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Experimental Population Dynamics of Amyloodiniumocellatum in the Spotted Seatrout, *Cynoscion nebulosus*, and the Red Snapper, *Lutjanus campechanus*

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by

Ignacio Masson

A Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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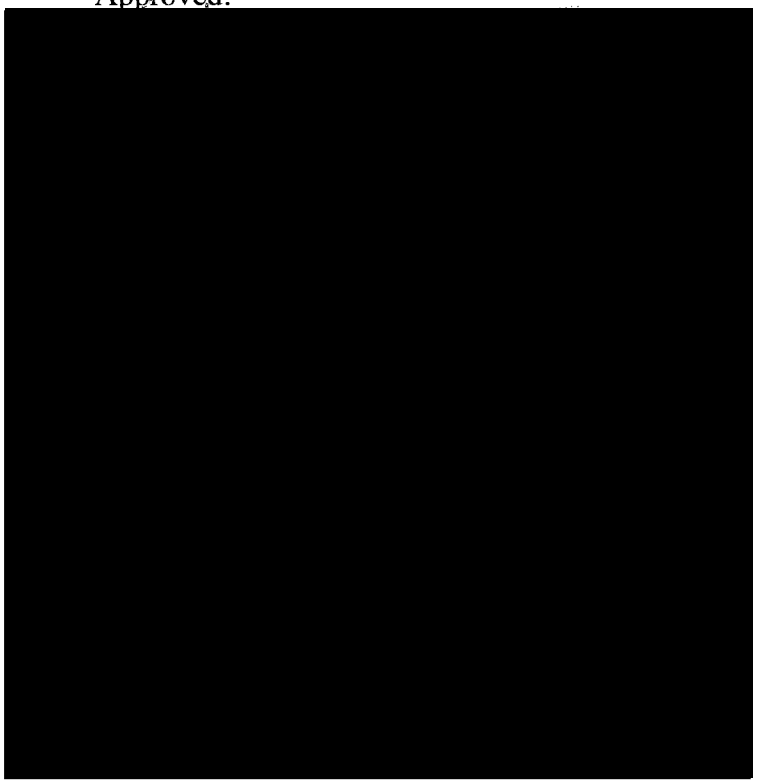

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ABSTRACT

EXPERIMENTAL POPULATION DYNAMICS OF *AMYLOODINIUM OCELLATUM* IN THE SPOTTED SEATROUT, *CYNOSCION NEBULOSUS,* AND THE RED SNAPPER, *LUTJANUS CAMPECHANUS*

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Amyloodinium ocellatum is a parasitic dinoflagellate that infects warm water marine bony fishes and causes high mortalities in aquaculture settings. It has three life history stages: the feeding trophont, the reproductive tomont, and the infective dinospore. This dissertation describes the characteristics of *A. ocellatum* infections in juvenile spotted seatrout, *Cynoscion nebulosus*, and red snapper, *Lutjanus campechanus*, studies the survival and fecundities of the stages in the life cycle of the parasite and presents a population model for *A. ocellatum.*

At 25°C and 33 ppt, the peak of trophont detachment occurred on day 2 post-infection in spotted seatrout and day 3 in red snapper. Trophont mean length at detachment was significantly larger in red snapper than in spotted seatrout (80 versus 72 μm, respectively), which translates to an increased mean dinospore production in red snapper (99 dinospores tomont^{-1} versus 82 in spotted seatrout). There was no difference either in the dinospore infection rate (0.36 and 0.34 dinospores d^{-1} for spotted seatrout and red snapper, respectively) or in the dinospore lethal dose (237,243 and 141,010 dinospores fish^{-1} for spotted seatrout and red snapper, respectively) between host species, but the mean trophont lethal load was higher in spotted seatrout (178,067 trophonts fish^{-1})

than in red snapper (123,160 trophonts $fish^{-1}$) suggesting that the red snapper is less tolerant of *A. ocellatum* infections than spotted seatrout.

The mean number of offspring per generation per individual for *A. ocellatum* (R_0) was 49.15 dinospores $tomont^{-1}$ with a generation time of 6.3 d. These rates were estimated using the mean tomont size for infections in spotted seatrout. Analysis of the population model revealed that changes in the mean number of dinospores $tomont^{-1}$ had the largest effect on *A. ocellatum* population growth, followed by the dinospore infection rate, the tomont sporulation rate and the dinospore mortality rate. A higher *A. ocellatum* population growth rate was observed in infections in red snapper than in spotted seatrout. These studies elucidate the dynamics of the life cycle of *A. ocellatum* and demonstrate the critical life cycle control points at which management strategies can be focused.

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TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF ILLUSTRATIONS

Figure

LIST OF ABBREVIATIONS

24-h dinospore infection rate.....β

48-h median dinospore lethal dose.....48-h LD_{50}

Amyloodinium ocellatum population growth rate.....λ

Confidence interval.....CI

Days.....d

Degrees Celsius.....°C

Dinospore fecundity .....m_D

Dinospore lifespan.....t_D

Dinospore mortality rate.....μ_D

Grams.....g

Hours.....h

Length.....L

Liters.....l

Mean number of total dinospores produced per tomont of the mean size.....*N*

Micrometers.....µm

Millimeters.....mm

Minutes .....min

Parts per million .....ppm

Parts per thousand.....ppt

Probability of wrongfully rejecting the null hypothesis.....p

Red snapper.....rs

Sample size.....n

Significance level.....α

Spotted seatrout .....st

Standard error of the mean.....SE

Sum of all stages' lifespan.....T

Tomont fecundity.....m_{To}

Tomont lifespan.....t_{To}

Tomont mortality rate .....μ_{To}

Tomont sporulation rate.....γ

Trophont detachment rate.....ϕ

Trophont fecundity.....m_{Tr}

Trophont lifespan.....t_{Tr}

Trophont mortality rate.....μ_{Tr}

Width.....W

CHAPTER I

INTRODUCTION

General Characteristics

Amyloodinium ocellatum is a parasitic dinoflagellate that infects fishes in tropical and subtropical marine waters (Brown and Hovasse 1946, Lawler 1977). It is considered one of the most troublesome pests of marine fish in captivity (Paperna 1984a, 1987) and the most important parasitic dinoflagellate affecting fish (Noga and Levy 2006). Its life cycle consists of three stages: a trophont, the parasitic feeding stage; a tomont, the dividing stage; and a dinospore, the free swimming infective stage (Brown 1934, Nigrelli 1936, Brown and Hovasse 1946, Paperna 1984a) (Fig. 1.1). Because *A. ocellatum* is a major pathogen in marine aquaculture, increasing our knowledge of its epidemiological characteristics could contribute to the control of the disease.

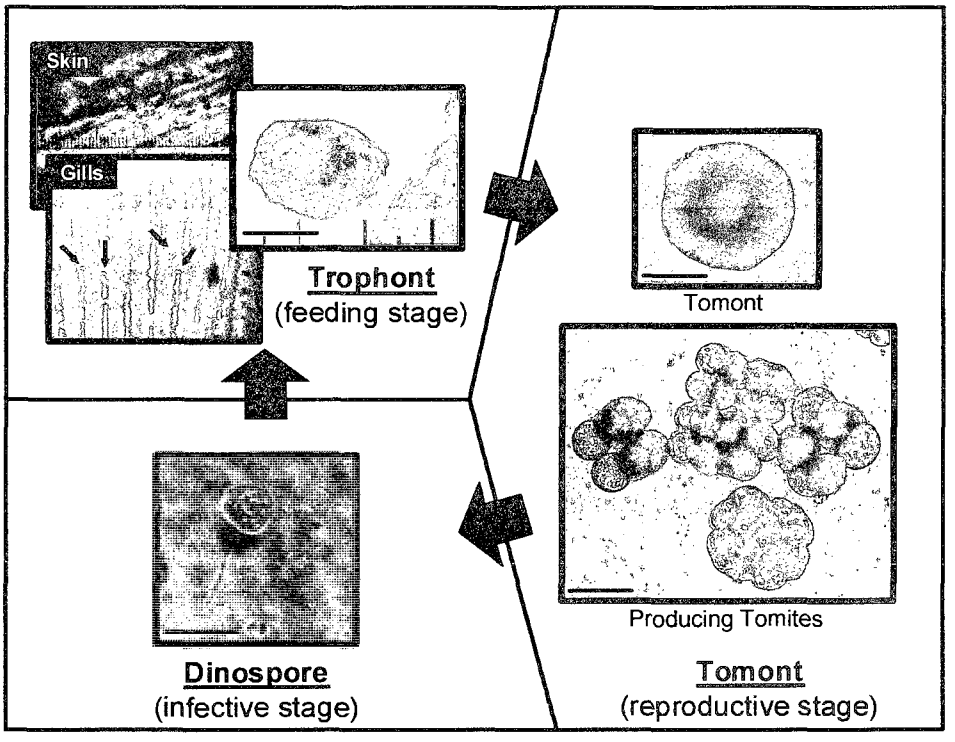

Figure 1.1. Micrographs of the stages in the life cycle of *Amyloodinium ocellatum*. Scale bar: 25 μm

Taxonomic Classification

In a botanical classification *Amyloodinium ocellatum* falls within the Phylum Dinoflagellida, Class Blastodiniphyceae, Order Blastodiniales, Family Oodiniaceae (Cachon and Cachon 1987). However, dinoflagellates also have been classified as zoological taxa because they share some characteristics with plants and animals (Noga and Levy 2006). According to the zoological classification, the organism belongs to the Phylum Sarcomastigophora, Subphylum Mastigophora (flagellates), Class Phytomastigophorea (phytoflagellates), Order Dinoflagellida (Levine et al. 1980). Among the plant (and algae) characteristics, many species of dinoflagellates present of cellulose walls, chloroplasts and photosynthetic pigments such as chlorophyll a and c_2, and starch as a storage product (Lee 1980). Among the animal characteristics are fibrous extrusomes such as mucocysts that are found in many dinoflagellate species. This and other structural and genetic details place dinoflagellates in close relationship with apicomplexans and ciliates, forming a group known as alveolates (Patterson 1999). However, it is now increasingly accepted that the International Code of Botanical Nomenclature should be used for classification of dinoflagellates (http://www.ucl.ac.uk/GeolSci/micropal/dinoflagellate.html, last accessed on 08/18/2009).

About 140 of the approximately 2,000 known species of dinoflagellates are parasites, mainly of invertebrates (Drebes 1984). *Amyloodinium* is one of five genera of dinoflagellates containing species parasitic in fish (Noga and Levy 2006). Parasitic dinoflagellates are a polyphyletic group formed by representatives of three botanical orders, Blastodiniales, Dinococcales (or Phytodiniales) and Syndiniales, that resemble one another due to convergent evolution (Cachon and Cachon 1987). Molecular studies

based on the sequence of the small subunit ribosomal RNA showed that *A. ocellatum* is closely related to *Pfiesteria piscicida*, the *Pfiesteria*-like dinoflagellate and cryptoperidiniopsoid sp., all belonging to the botanical Class Blastodiniphyceae (Litaker et al. 1999).

Five *A. ocellatum* isolates from different parts of the world including the Red Sea (Israel), the eastern Mediterranean Sea (Israel), the Adriatic Sea (Italy), the Gulf of Mexico (Florida), and one isolate from an unknown origin revealed insignificant variation in the sequence of the internal transcribed spacer region of the ribosomal DNA; thus, Levy et al. (2007) considered all isolates to be *A. ocellatum*. Nevertheless, because differences in environmental tolerances of some *Amyloodinium* isolates have been noted (e.g., Paperna 1984a versus Kuperman and Matey 1999), Levy et al. (2007) did not discount the possibility that further research would reveal variation at the subspecies level or lower.

Structure and Life Cycle

Trophonts are spherical or oval in shape and live attached to the gills or skin of fish. They are normally 20-120 μm in length just prior to detachment from the host (Brown and Hovasse 1946, Lom and Lawler 1973). Lawler (1980) reported trophonts of up to 350 μm on fish from the Mississippi Sound, but most were 150 μm or less. When they are small, trophonts have an oval or pear-shaped form becoming spherical as they increase in size. Trophonts have been described as colorless or unpigmented (Brown 1931, 1934, Brown and Hovasse 1946, Lawler 1977, 1980), and brownish or yellowish (Kupperman and Matey 1999, Cruz-Lacierda et al. 2004). Trophonts are surrounded by thecal plates covered by the outer cell membrane. The thecal plates are composed of cellulose and are perforated by the narrow discharge openings of mucocysts (Lom and

Lawler 1973). At the base of the trophont there is a gap in the thecal armor from which the peduncle arises. The peduncle bears an intense chromophilic ring and continues as a flattened attachment plate or disc. The attachment plate is ramified into numerous thin to filiform projections called rhizoids. Most of the rhizoids are deeply embedded in the cytoplasm of the epithelial cells (Brown 1934, Nigrelli 1936, Cheung et al. 1978, 1981). A tentacle-like movable projection called a stomatopode emerges from the cell interior and passes through the chromophilic ring in the peduncle and attachment plate and then extends laterally (Lom and Lawler 1973, Lom 1981). In some individuals the stomatopode can be up to about 30 μm long. The stomatopode has been thought to inject histolytic substances into the host's tissues and/or to be used for food ingestion (Cheung et al. 1981, Lom 1981).

The surface of the basal end of the trophont has two deep longitudinal invaginations, each with a flagellum. At least one of the invaginations is the rudiment of the sulcus with a flagellum and an opening into the cell. This area is called the phagocytic region and contains large digestive vacuoles that are often filled with ingested host cell cytoplasm. It extends essentially between the nucleus and the attached end of the trophont. The remainder of the cytoplasm has a spongious appearance and contains numerous starch grains and large digestive vacuoles with particulate food, but there is no trace of a chloroplast or other structure that could be interpreted as a rudimentary modified chloroplast (Brown and Hovasse 1946, Lom and Lawler 1973, Lom 1981). The nucleus is vesicular with a large endosome (Brown 1934). The presence of a red stigma or eyespot in all three life stages is characteristic of *A. ocellatum*, and from this feature it derives its trivial name (Brown 1934, Nigrelli 1936, Lawler 1980, Kuperman and Matey 1999). In the trophont stage, the eyespot is usually present in the peduncular region,

lateral in position. It is composed of a broad red and a thin black pigment bar, between which there is a clear refractile area. Occasionally, there may be two such eyespots (Nigrelli 1936).

Trophonts grow continuously and reach 100 μm in length within 4-5 d at 19-24°C (Paperna 1984a). Detachment of trophonts from the fish occurs gradually. It is dictated by the ability of the rhizoids to maintain the trophont attached to the host tissue and by the integrity of the tissue as it becomes necrotic or hyperplastic. This is also influenced by the velocity of the water flowing over the attached trophonts which creates friction over the integumental surface (skin and gills) and pulls the trophonts out from their attachment sites. After detachment, the rhizoids, peduncle and stomatopode are retracted within 2 to 5 min and a solid body wall is formed (Nigrelli 1936, Brown and Hovasse 1946, Cheung et al. 1981). Paperna (1984a) tentatively called this process encapsulation. A slightly depressed area at the zone where the attachment apparatus was retracted determines the plane of the first division of the tomont (Cheung et al. 1981). Tomonts start dividing within 3-6 h of trophont dislodgement at 23-27°C and 30 ppt (Paperna 1984a). The first division is longitudinal and succeeding ones occur at right angles to each other, giving rise to successive palmella stages of 2, 4, 8, 16, 32, 64 and 128 cells, referred to as tomites (Nigrelli 1936, Brown and Hovasse 1946). The sequence of divisions under optimal conditions (23-27°C, 30 ppt) is constant, with a division every 9-12 h and independent of trophont size at detachment (Paperna 1984a). In the last division each tomite divides to form two dinospores that rupture the membrane and swim away (Brown 1931, 1934, Nigrelli 1936, Brown and Hovasse 1946). The number of divisions before sporulation has been found to be related to the initial size of the trophonts. Larger trophonts undergo more divisions and produce more dinospores (Paperna 1984a).

Dinospores constitute the free-swimming, infective stage in the life cycle of *A. ocellatum*. They have a body approximately 12-15 μm long and 8-14 μm wide that contains two flagella, one situated in a well-marked girdle and the other one backwardly directed, longitudinal, and not contained in a well-marked sulcus (Brown 1931, 1934, Nigrelli 1936, Lawler 1980, Landsberg et al. 1994). Dinospore swimming is achieved by lashing movements of the longitudinal flagellum that propel the dinospore forward while the transverse flagellum causes it to rotate to the right (Nigrelli 1936). An eyespot is present in the neighborhood of the longitudinal flagellum. Landsberg et al. (1994) described the dinospore as anteriorly compressed, thinly armored, peridinioid and having an epitheca slightly shorter than the hypotheca. The "hamburger-shaped" body is circled by a deeply excavated cingulum that is displaced 1 to 1.5X. The sulcus is narrow distally and is about three times as wide proximally. The widest part is excavated and houses the peduncle and rhizoid-like complex that appears to protrude from under the plates in the right sulcal area. The peduncle would presumably extend to form the stomatopode after attachment. The longitudinal flagellum is located below the peduncle and is 20 to 25 μm long. The flagellum has a prominent longitudinal groove that separates two components, one of which sometimes extends approximately 5 μm beyond the other. The transverse flagellum is located in the cingulum, and fine hairs along its length attach it to the cell surface (Landsberg et al. 1994). Once dinospores attach to a fish, they transform into trophonts. This process was observed to take 5 to 20 min in cell culture (Noga 1987).

Effect of Temperature and Salinity on Tomont Division and Sporulation

Paperna (1984a) studied the effect of environmental factors on the life cycle of *A. ocellatum* and observed that temperatures from 18 to 30°C are optimum for tomont division and sporulation. However, at 23-27°C tomite reproduction was most efficient,

with complete sporulation requiring 2-3 d. At 29-30°C a decline in the number of divisions was evident, and, in some instances, final division and sporulation were delayed and terminated on day 4. At 18-20°C tomonts divided slowly (one division per 19-34 h) and there was a decline in division success (from 100 to 93-80% division success). At 18°C (and occasionally 20°C), however, tomonts had the greatest number of divisions before sporulation compared with those incubated at the higher temperatures. At 15°C there was a marked inhibition of the reproductive process, and few tomonts sporulated after 10-16 d of incubation with a yield of few dinospores. When returned to 20-25°C, even after 27 d at 15°C, recovery of the reproductive process and sporulation occurred, but at a reduced level. The process of division was totally inhibited at 8°C, but resumed when returned to 20°C. Similarly, Brown (1934) pointed out that cooling merely suspended divisions and the organism returned to an active condition when warmed. However, Paperna (1984a) observed that incubation at 8°C for 7-9 d resulted in a 2-3 d lag before divisions resumed, and only 16% of the tomonts resumed division and few sporulated. In addition, no division occurred in tomonts held at 8°C for 30 d and returned to 20°C. Temperatures approaching 35°C resulted in gradual death of tomites within the tomont, and in some tomonts, all tomites died. The few that sporulated produced non-motile dinospores.

Brown (1934) incubated tomonts in fresh water and noticed that only a small percentage divided and that the division process was very slow. In a set kept for 3 d in fresh water and then returned to sea water, half of the tomonts divided normally. Nigrelli (1936) observed that at 22°C the optimal salinity for development to the dinospore stage was from 18 to 39 ppt. Within this range, development into dinospores occurred by the end of day 2 or 3. At 55 ppt, division occurred slowly and no dinospore was formed. In

the majority of the tomonts at this salinity, development reached the 16-cell stage but only 2% developed to the 32-cell stage. However, when tomonts were transferred to a lower salinity as late as day 6, division continued to the 128-cell stage and dinospores were formed.

Paperna (1984a) concluded that tolerance to salinity was dependent on temperature. Tomonts demonstrated the widest tolerance to salinity at 24-25°C. Tolerance to salinity narrowed as temperatures deviated from 23-27°C. At 10 ppt, tomites within the tomont swelled and became pale and hydropic. Vacuoles appeared and eventually tomites burst. At 60 ppt, the tomites became dense and dark but did not collapse. Some division and sporulation occurred at salinities as low as 0.5 ppt and as high as 70 ppt. However, the few dinospores that were formed lost their motility upon hatching. At salinities above 80 ppt, tomonts did not divide and remained enclosed in a distinct capsule. Division and sporulation occurred in 25% or fewer of the tomonts when returned to the optimal salinity within 12 d.

Natural and Experimental Hosts

Amyloodinium ocellatum is a cosmopolitan parasite of warm water marine and brackish-water fishes and the most destructive dinoflagellate of fish in marine aquaria (Lawler 1977, Paperna 1984a, Bower et al. 1987). It has been found to infect 111 species of fish in 46 families in North America (Lawler 1979). In the first reported epidemics at the Aquarium of the Zoological Society of London (Brown 1931, 1934) and the New York Aquarium (Nigrelli 1936), most of the bony fish species in the infected tanks were susceptible to *A. ocellatum*. Lawler (1980) reported that 71 species in 39 families died of amyloodiniosis in aquaria. Among them were two individuals of the stingray *Dasyatis sabina*, the first record of an elasmobranch being susceptible to *A. ocellatum*. The author

also considered 16 species in 13 families as natural hosts for this parasite and stated that natural infections in Mississippi waters apparently were very light and did not cause major mortalities. This could be attributed to the normally low densities in the wild that would prevent repeated infections of the same individual host (Roberts-Thompson et al. 2006). The only reports of significant mortalities due to *A. ocellatum* in the wild are from Overstreet (1993) and Kuperman and Matey (1999). Overstreet (1993) reported significant fish mortality, mostly of spot *Leiostomus xanthurus*, caused at least in part by *A. ocellatum*. The low oxygen level in the water at that time could have acted as a stressor enhancing the severity of the infection. The episode occurred in Orange Beach Marina and Shotgun Canal, AL, USA, in 1984. Similarly, Kuperman and Matey (1999) observed an outbreak of amyloodiniosis and subsequent mortality of tilapia *Oreochromis mossambicus* and croaker *Bairdiella icistia* at the Salton Sea, CA, USA, in 1997-1998. These authors mentioned that low oxygen, high ammonia, a temperature of 40°C and a salinity of 46 ppt also could have contributed to the mortalities.

Immunity of Fish to *Amyloodinium ocellatum*

Brown (1934) and Nigrelli (1936) noted that some fish species apparently were more susceptible to *A. ocellatum* than others. Lawler (1977, 1980) observed that the fish species known to be more resistant also are known either for tolerating low oxygen levels or producing large amounts of mucus. Some of those species included the sheepshead minnow, *Cyprinodon variegatus*; the Gulf killifish, *Fundulus grandis*; the American eel, *Anguilla rostrata*; the Gulf toadfish, *Opsanus beta*; the sailfin molly, *Poecilia latipinna*; and the fat sleeper, *Dormitator maculatus*.

Mucus constitutes a part of the innate immunity of fish. It is secreted in copious amounts when the body surface is irritated, and it contains substances (e.g., lysozymes,

lectins, proteinases) that can prevent the attachment and/or establishment of parasites (Woo 1996). Landsberg et al. (1992) observed that the infectivity of *A. ocellatum* in cell culture was significantly reduced after exposure to mucus and serum from naïve blue tilapia, *O. aureus*, although mucus had considerably less inhibitory activity than serum, at least for that species. In addition, the skin, gills and spleen of rainbow trout *Oncorhynchus mykiss* and hybrid striped bass have proteins that are lethal to *A. ocellatum* (Noga et al. 2001). These proteins are endogenous, host-produced, with antibiotic activity. They are closely related to histones and are designated "histone-like proteins" (HLPs). Experimental evidence demonstrated that their activity is directed at the trophont stage since dinospores remained unaffected. When trophonts were exposed to HLPs they grew normally, but after forming tomonts they died (Noga et al. 2002). The mechanism responsible for the toxicity of HLPs to *A. ocellatum* is unknown, but trophont membrane lysis and/or interference with nucleic acids by binding are possibilities (Noga et al. 2001).

Studies examining the humoral response to *A. ocellatum* showed that serum of *O. aureus* immunized with antigens of live or sonicated dinospores produced a specific antibody response that was detectable by ELISA. The humoral response was no longer detectable after 8 weeks from the primary immunization with sonicated dinospores, but after a booster dose at week 9 the response increased and peaked at week 12. Over the next 6 weeks the antibody titer gradually declined, but the response was still positive at week 18 at the conclusion of the experiment (Smith et al. 1992). Likewise, the serum from *O. aureus* immunized intraperitoneally with dinospores diminished the motility and infectivity of dinospores and the growth of trophonts in cell culture (Smith et al. 1993). Another study showed that both hybrid striped bass immunized intraperitoneally with live dinospores and individuals of the same species that survived an *A. ocellatum* outbreak on

a fish farm developed substantial antibody responses. The study also showed that the mean anti-*A. ocellatum* serum titer declined over a 6-week period following successful treatment of the infection (Smith et al. 1994). In addition, Cobb (1997) observed that challenging 6-g tomato clownfish, *Amphiprion frenatus,* with 200,000 dinospores $fish^{-1}$ resulted in death while an exposure to 40,000 dinospores $fish^{-1}$ was not lethal and triggered immunity. However, the author pointed out that the number of tomonts recovered from the immunized fish after a 3-min dip in distilled water was significantly lower than the control (naïve fish) only when animals were challenged several times weekly with non-lethal doses of dinospores followed by freshwater dips and copper treatments before being exposed to lethal doses of dinospores. Cobb (1997) suggested that in intensive mariculture conditions, and if fish have not been previously exposed, the delay in the primary immune response would allow time for the parasite to reach lethal densities and to remain in sufficient numbers to kill fish introduced subsequently.

Treatments and Prevention

Copper sulfate ($CuSO_4$), a chemical used for treating a variety of external fish diseases, has been used commonly to control the outbreaks of *Amyloodinium ocellatum* in fish culture facilities (Noga and Levy 2006). After trophonts detach from the host tissues, a process perhaps enhanced by the copious secretion of mucus induced by copper, encapsulation and division proceeds normally to sporulation because the tomont cell wall limits entry of copper ions. At sporulation, sensitive dinospores are exposed to the cytotoxic effect of copper, thus interrupting the parasite's life cycle and preventing further spread of the disease (Dempster 1955, Lawler 1977, Paperna 1984b). Since it can take several days for tomonts to sporulate, depending on the environmental conditions (Paperna 1984a), copper levels need to be maintained until all tomonts hatch and release

their dinospores. Noga and Levy (2006) indicated that free copper ions must be maintained at 0.12-0.15 ppm for 10-14 d to control the epidemics.

Nitrofurazone (50 ppm), Furanace® (2.5 ppm) and acriflavin (6 ppm) were observed to halt tomont division or kill this stage over a 4 to 17 d treatment period, but parasite development often resumed after completion of the treatment (Noga and Levy 2006). Aureomycin, chlortetracycline, tetracycline and potassium permanganate, among other substances, were ineffective in controlling *A. ocellatum* (Lawler 1977).

Montgomery-Brock et al. (2000) observed that striped mullet, *Mugil cephalus,* fry mortalities due to amyloodiniosis declined dramatically within 3 d following a treatment with 25 ppm hydrogen peroxide for 30 min. After the treatment, fish began to feed and recovered their characteristic schooling swimming behavior. A similar study with the Pacific threadfin, *Polydactylus sexfilis,* showed that single treatments with hydrogen peroxide at 75 or 150 ppm for 30 min eliminated trophonts without causing loss of fish (Montgomery-Brock et al. 2001). Likewise, Cruz-Lacierda et al. (2004) observed that either 1 h immersion in fresh water or 1 h immersion in 200 ppm hydrogen peroxide prior to return to clean sea water was equally effective in removing 100% of the trophonts in milkfish, *Chanos chanos*, and mangrove snapper, *Lutjanus argentimaculatus*. However, overexposure or the use of an excessive concentration of hydrogen peroxide could be lethal to the fish, so care should be taken when using this chemical. In addition, although hydrogen peroxide reduced the mortalities of fish due to amyloodiniosis, it is unknown if tomonts remain viable after the treatment (Montgomery-Brock et al. 2001).

Because *A. ocellatum* is firmly attached to the host gill epithelium and surrounded by a protective mucus coating, the attached stage may become refractory to chemical bath treatments (Kingsford 1975). Based on this premise, Lewis et al. (1988) studied the use

of the antimalarial drug chloroquine diphosphate as a systemic approach for treating amyloodiniosis in red drum, *Sciaenops ocellatus*, and concluded that oral administration at 50 mg drug kg^{-1} body weight was effective, protecting the fish for 7 d. Most of the drug disappeared from plasma and mucus coat within 30 d. *In vitro* culture of the dinospores in Ott's medium (Ott 1965) showed they were active for as long as 96 h at 20°C. However, loss of activity was observed when exposed to 2.5 ppm chloroquine for 24 h. Noga and Levy (2006) indicated that chloroquine is very safe and effective, controlling an *A. ocellatum* experimental infestation of clown anemonefish, *Amphiprion ocellaris,* after a 10-d exposure to a single bath treatment of 5-10 ppm. Chloroquine has no effect on tomont division, but kills dinospores immediately upon their excystment. The concentration is not toxic to fish, but is highly toxic to algae and invertebrates.

Benzalkonium chloride applied at 1 ppm followed by another application at the same concentration 2 d later was effective in controlling *A. ocellatum* without being toxic to fish. However, concentrations above 2 ppm are notably toxic to fish. A possible advantage of using benzalkonium would be that in contrast to copper treatments which bear the risk of the buildup of precipitated copper to levels toxic to fish, it does not precipitate or accumulate (Johnson 1984).

The sodium salt of lasalocid (3,N- methylglucamine), a water soluble ionophorous antibiotic, proved to be effective against trophonts and tomonts by interfering with their ionic balance. *In vitro*, trophont infection was significantly reduced at 0.1 ppm and eliminated at 1.0 ppm, with motile dinospore excystment from tomonts reduced or prevented at levels as low as 0.001 ppm. This treatment was not toxic to 0.5-1.0-g red drum (*S. ocellatus*) fry. However, the authors of the study recommended further work on

the drug's toxicity to other species, tissue residue levels, possible effluent levels, and a full-scale field trial (Oestmann and Lewis 1996).

Nauplii of *Artemia salina* were observed to feed on *A. ocellatum* dinospores, and stocking of nauplii in tanks with infected fish suggests an alternative treatment for amyloodiniosis in aquaculture systems. Red drum from tanks stocked with nauplii of *A. salina* had 65% fewer trophonts in their gills than those from tanks with the same initial number of dinospores but not stocked with nauplii (10.75 vs. 3.75 trophonts filament^{-1}). *In vitro* studies showed that dinospores were completely eliminated by 20 h in the presence of 1 nauplius for every 2,500 dinospores and by 8 h in the presence of one nauplius for every 1,250 dinospores. Dinospores were afterwards seen in the intestinal tracts and fecal casts of the nauplii (Oestmann et al. 1995).

Water treatment with ultraviolet radiation or diatomaceous earth filtration also may help to control the spread of amyloodiniosis, but these methods have not been proven completely effective (Bower 1987). Another factor to consider is that dinospores are capable of traveling in aerosol droplets and were observed to infect naïve fish at a distance of at least 4 m from their origin under certain experimental conditions (Roberts-Thompson 2006). Thus, minimizing the airflow between tanks could help to prevent the spread of the disease.

Brock et al. (1999) and Montgomery-Brock and Brock (2001) performed studies to evaluate the maintenance of low light conditions as a means to inhibit the life cycle of *A. ocellatum* in Pacific threadfin (*P. sexfilis*) culture tanks. The rationale for using darkness to combat amyloodiniosis was based on the belief that a significant reduction in light would slow down or stop the proliferation of macroalgae on the bottom of the tanks, reducing the favorable substrate for *A. ocellatum*. Investigators also speculated that the

low light conditions would be unfavorable to the dinospores. When infected fish were placed in a covered tank in which the light level was reduced to near darkness for 2 weeks, trophont counts on the gills declined below a detectable level compared to those fish kept in a tank exposed to direct sunlight. When the covered tank was uncovered and exposed to light for a month, trophonts were detected at a low level. Conversely, when the uncovered tank was covered for 25 d, trophonts were not detected in gill biopsies. These results suggest that the maintenance of Pacific threadfin under limited light conditions may be an effective tool for prevention and control of amyloodiniosis.

Information from Pacific threadfin culture facilities in Hawaii, indicates that once fish start dying from amyloodiniosis, the losses will be high even if the fish are treated with hydrogen peroxide and/or transferred to a clean raceway (Brock et al. 1999). As a consequence, treatment or management procedures would be more effective if initiated prior to the onset of clinical disease (morbidity and mortality). When the signs of the disease are recognized, the fish are often so weakened they do not survive the treatments employed (Lawler 1977). In spite of this, Brock et al. (1999) developed a protocol for monitoring of subclinical *A. ocellatum* infections and tested it in a commercial Pacific threadfin farm. The technique consisted of doing gill biopsies of 5 fish per tank weekly. This procedure was found to be effective as a means to monitor subclinical infections. Pacific threadfin were asymptomatic and feeding well in tank populations when the average trophont count/field remained at 5 or less. There was elevated risk of an outbreak of amyloodiniosis when the average trophont count/field score rose to 20 or higher. Average trophont count/field scores of >75 were associated with clinical signs of amyloodiniosis in the Pacific threadfin (Brock et al. 1999).

Use of Mathematical Models in Epidemiology

The application of mathematics to the study of infectious disease began over 200 years ago, but it was not until the beginning of last century when Hamer and Ross formulated specific theories about the transmission of infectious diseases (measles and malaria, respectively) in simple but precise mathematical statements and investigated the properties of the resulting models (Anderson and May 1991). The models are developed by compartmentalizing components of a system and generating mathematical expressions for each one (Anderson and May 1979, May and Anderson 1979, Ebert 1999). Previous research with aquatic pathogens included the estimation of the epidemiological parameters of white spot syndrome disease in *Litopenaeus vannamei* and *L. setiferus* (Soto and Lotz 2001) and the development of a model of white spot syndrome epidemics in *L. vannamei* (Lotz and Soto 2002). Similar studies were done for Taura syndrome (Lotz et al. 2003) and necrotizing hepatopancreatitis (Vincent et al. 2004) infecting *L. vannamei*. In all cases, the parameters were estimated experimentally under specified conditions and used in the mathematical models. These models described the changes in the disease stages of the hosts (e.g., susceptible, acutely infected, dead infected) rather than the number of viral or bacterial organisms. To model the change in numbers of a pathogen, the pathogen number is the variable and the host disease state is ignored.

Population Model for *Amyloodinium ocellatum*

Amyloodinium ocellatum population growth can be modeled based on the life cycle stages of the parasite. Many organisms have survival and reproductive rates that are dependent on the stage rather than the age of the organism. In these cases, it is suitable to use a stage-structured life cycle approach to perform population projections (Ebert 1999). Each of the three life stages of *A. ocellatum* forms a separate compartment of the model.

Transition coefficients are the rates of transformation from one stage to the next, and regulate the number of individuals in each stage over time. Once the mathematical model is developed, the transition coefficients can be estimated experimentally, and the resulting estimates can be used in the model to study the population growth of the parasite and to examine the effect of the changes in those transition coefficients on the population growth. This last application can be useful to pinpoint which vital rate has the largest effect on the population growth and may aid in the development of management strategies to control or prevent outbreaks (Benton and Grant 1999, Ebert 1999).

Although the stages in the life cycle of *A. ocellatum* are the same for any species it parasitizes, the values of the transition coefficients likely depend on the host. Some fish species are more susceptible to *A. ocellatum* than others (Brown 1934, Nigrelli 1936, Lawler 1977, 1980), but there is a lack of studies that accurately calculate infection rates that account for the proportion of dinospores attached per unit of time and the effect of the dinospore dose and exposure time. Likewise, there is no study in which the dinospore lethal dose and trophont lethal loads have been estimated and compared among host species.

This dissertation was conducted using primarily the spotted seatrout, *Cynoscion nebulosus,* as the model host. The spotted seatrout is the focus of a stock enhancement research program at University of Southern Mississippi's Gulf Coast Research Laboratory. The program is a collaborative project with the Mississippi Department of Marine Resources and has operated since 2004. Almost 60,000 fish have been tagged and released from 2006 to October 2009; thus, a consistent supply of experimental animals was available. Laboratory-reared red snapper, *Lutjanus campechanus*, also became available in moderate quantities while this study was in progress so this species was

incorporated into to provide a comparative study on how *A. ocellatum* transition coefficients change among different host species.

Objectives

1. To develop a dynamic model of the life cycle of *A. ocellatum* infecting spotted seatrout in a closed system,
2. To determine the probability of death of the trophont, tomont and dinospore stages per day,
3. To determine the probability that a tomont sporulates per day, the time to sporulation and the number of dinospores produced by tomonts of different size per day,
4. To determine the probability that a trophont detaches from its host per day and the size achieved by trophonts at detachment,
5. To determine the dinospore lethal dose and trophont lethal load for spotted seatrout,
6. To determine the probability that a dinospore infects a spotted seatrout per day, and
7. To perform a comparative study of the same characteristics with red snapper.

CHAPTER II

DETACHMENT OF *AMYLOODINIUM OCELLATUM* TROPHONTS FROM SPOTTED SEATROUT, *CYNOSCION NEBULOSUS*, AND RED SNAPPER, *LUTJANUS CAMPECHANUS*: A COMPARATIVE STUDY

Introduction

Spotted seatrout and red snapper are among the most discussed candidates for culture in the Gulf of Mexico region and are well known for being susceptible to *A. ocellatum* infections (Blaylock and Whelan 2004). However, different host species may have different susceptibilities to *A. ocellatum* (Brown 1934, Nigrelli 1936, Lawler 1977, 1980). Indeed, our own incidental observations during outbreak episodes have suggested that infections in red snapper reach lethal levels faster than in spotted seatrout. Because the size of the trophonts is directly related to the time spent attached to the host and the size of the trophont determines the number of dinospores produced, the intensity of an outbreak can be influenced by, among other factors (e.g., trophont lethal load, host capability of tolerating low dissolved oxygen levels), the rate and size at which trophonts detach from the host.

My goal in this experiment was to evaluate spotted seatrout, *Cynoscion nebulosus*, and red snapper, *Lutjanus campechanus*, as hosts for *A. ocellatum*. I compared the growth and detachment of the trophont stage from juvenile spotted seatrout and red snapper and estimated the rates of trophont detachment, the length, volume and biomass distribution of trophonts at detachment, and the trophont growth rate, for each of the two host species.

Methods

The culture of *A. ocellatum* was started by introducing a naturally infected wild striped mullet, *Mugil cephalus,* to a tank containing juvenile lab-reared spotted seatrout, *Cynoscion nebulosus*, wild-caught striped mullet, Atlantic croaker, *Micropogonias undulatus*, and spot, *Leiostomus xanthurus*. The infection was maintained in a 570-l tank filled with approximately 200 l of sea water. The sea water was prepared with dechlorinated and aged tap water and Crystal Sea® Marinemix salt (Marine Enterprises International Inc., Baltimore, MD). Continuous aeration was supplied by air stones connected to a regenerative blower.

As fish died, they were removed from the tank, dissected and inspected to confirm infection with *A. ocellatum*. Infected gills were excised and returned to the tank. Fish of the species previously mentioned, but most commonly Atlantic croaker and lab-reared spotted seatrout, were continuously added to the *A. ocellatum*-source tank to sustain the infection. Salinity in the tank was maintained at 33±2 ppt. Dechlorinated tap water was added periodically to account for evaporation. No mechanical filtration was used. Biofiltration was accomplished using submerged blue, bonded filter pads (Aquatic Ecosystems, Apopka, FL). Temperature was maintained at 25±2°C by controlling the air temperature with a thermostatically controlled heating and cooling unit. Temperature, salinity, alkalinity, pH, and ammonia and nitrite concentrations were monitored daily to ensure parameters were within the desired ranges (23–27 °C, 180–240 ppm $CaCO_3$, 31–35 ppt, pH 7.5–8.5, 0–0.25 ppm and 0–1 ppm, respectively). Illumination in the room was provided by sets of standard fluorescent tubes on a 14 h light / 10 h dark photoperiod. Infected and uninfected fish were fed twice daily to satiation with

commercial flakes (Aquatic Ecosystems, Apopka, FL, USA) or pellets (Skretting, Stavanger, Norway).

Dinospores for the experiments were obtained by introducing naïve hatchery-reared juvenile spotted seatrout to the previously described tank where the infection was maintained. Upon display of signs indicating heavy infection (e.g., gasping at the water surface, aimless swimming, and listlessness), fish were euthanized by pithing. The gills were excised and placed in a Petri dish with artificial sea water (25°C, 33 ppt) until most of the trophonts detached. The dislodgement of the trophonts from the gills was promoted by gently shaking the Petri dish, washing the gills with a stream of sea water and/or moving the gills with a pair of forceps. The water with the detached trophonts was poured through a 125 μm filter and collected in a tube. The tube was set aside for 20-30 min to allow settling and transformation of trophonts into tomonts. The water overlay was removed by vacuum aspiration. The tomonts, settled at the bottom of the tube, were washed by re-suspending them in sea water. This process was repeated three times with a 10-15 min interval between washes to allow settling of the tomonts. The tomonts were then transferred from the bottom of the tube to a 6-well culture plate (Corning Life Sciences, Corning, NY, USA) and incubated for 72 h at 25°C, the approximate time at which maximum dinospore excystment has been reported (Oestmann and Lewis 1995). After 72 h of incubation, swimming dinospores were pipetted from the water column into a container of a known volume to assess their number for use in the challenges. The number of dinospores obtained was assessed volumetrically with a Sedgwick-Rafter counting cell using a method similar to the one described by Dickerson et al. (1981) for counting tomites of *Ichthyophthirius multifiliis*, except that 0.5 ml of the dinospore suspension was combined with 0.5 ml of formalin to kill the dinospores to allow for

counting. The number of dinospores in the 0.5-ml sample was then used to estimate the total number of dinospores collected and establish the volume needed to obtain the dinospore doses used for the challenges. Assuming the dinospores were randomly distributed in the sample (due to the mixing and stirring done with the micropipette), the SE of the 20,000 dinospore dose was estimated using the Poisson distribution, with SE being the square root of the mean (20,000) divided by the square root of the number of observations (3) done to calculate the dose.

Trophont detachment was studied using 26 juvenile hatchery-reared spotted seatrout (53.3±1.5 mm, mean total length±SE) and 11 hatchery-reared juvenile red snapper (46.0±2.5 mm, mean total length±SE) which were individually exposed to 20,000±82 dinospores $fish^{-1}$ (mean dose±SE) for 3 h in 3-l aquaria filled with 2.5 l of water at 25°C and 33 ppt.

After being exposed to dinospores, each fish was moved to a 475-ml plastic cup (Fisher Scientific Company L.L.C., Pittsburg, PA) filled with 75% the cup's volume of clean water of the same physicochemical characteristics as the water used for the exposure (25°C, 33 ppt). Every 24 h, each fish was moved to a new cup with clean artificial sea water and the number and size of the tomonts in the old cup was determined. This operation was repeated daily for 6 d, and on day 6 each fish was euthanized and dissected to determine if trophonts were still attached (Fig. 2.1). All measurements and counts are presented as mean±SE, with an alpha = 0.05.

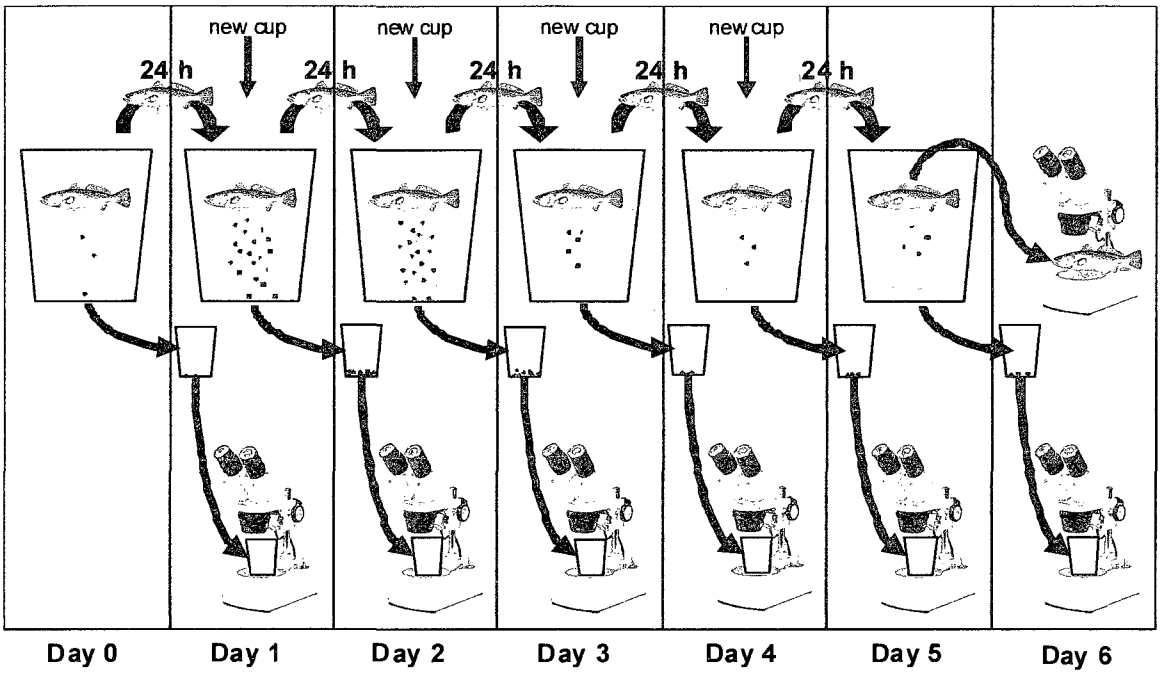

Figure 2.1. Experimental procedure used to determine the number and size of trophonts detached over time from spotted seatrout and red snapper. After a 3-h challenge with 20,000 dinospores $fish^{-1}$, fish were moved to individual cups filled with clean sea water. Every 24 h, fish were moved to a "new" cup and the "old" cup was examined to account for the number and length of the detached trophonts. On day 6 fish were dissected and examined to count trophonts still attached.

To assess the number and size of the trophonts detached from the host, cups were left undisturbed for 15-30 min after fish removal to allow tomonts to settle. Tomonts were counted with the aid of a grid and a dissecting microscope. A random sample of ten tomonts per cup was measured daily using an ocular micrometer resulting in 260 observations d^{-1} for spotted seatrout and 110 observations d^{-1} for red snapper. Because tomonts are ovoid in shape, the formula for the ovoid sphere volume, $0.75\ \pi\ [0.25\ (L+W)]^3$, with L and W representing the tomont length and width, was used to calculate a tomont volume. Furthermore, as tomonts present isometry in length and width (Paperna 1984a), the length of each tomont was measured and used to estimate its width using W=L-0.075, based on Paperna's (1984a) regression equations for L and W. The mean

volume of a trophont at detachment was estimated as the sum of the product of the mean volume at detachment at day x and the number of trophonts that detached on that day for each tested fish (with x ranging from day 1 to day 6). ANOVA and Tukey HSD tests were used to compare the trophont mean volumes at detachment among days and between spotted seatrout and red snapper. The trophont growth rates for each host species were estimated from the best fit exponential curves for the change in trophont volume over time, with time zero being the dinospore stage for which its volume was calculated in the same manner as for trophonts. The data were fitted by least squares to the expression $y=a.e^{b.x}$, with e^b being the growth rate. Systat version 12.0 (Systat 2007) was used to perform the curve fitting analysis. To determine if there were statistical differences in trophont growth rates between spotted seatrout and red snapper, I used the $\log_{10}$ transformed data and linear regression analysis to test for differences in slopes. The trophont mean time to detachment for each host species was estimated using survival analysis. S-PLUS® version 6.2 (S-Plus 2003) was used for the survival analysis.

I estimated and compared the biomass of detached trophonts each day for each host species. The trophont biomass was estimated as the product of the number of tomonts detached per fish per day and the mean volume of the trophonts detached from each fish each day. ANOVA and Tukey HSD tests were performed to determine if there were significant differences among the biomass of trophonts detached each day within and between host species.

To estimate the trophont detachment rate for spotted seatrout and red snapper, I plotted the cumulative percentage of trophonts remaining attached to each host species over time (d) and calculated the best fist exponential curve for the expression $y=a.e^{b.x}$, with $1-e^b$ being the probability that a trophont is released from a fish per day or the

trophont detachment rate (ϕ). Systat version 12.0 (Systat 2007) was used to perform the curve fitting analysis. To determine if there were statistical differences in trophont detachment rates between spotted seatrout and red snapper, I $\log_{10}$ transformed the data and used linear regression analysis to test for differences in slopes.

Results

Figure 2.3 shows the trophont volume at detachment over time based on 260 observations d^{-1} for spotted seatrout and 110 observations d^{-1} for red snapper. The overall trophont mean volume at detachment was 95,427±5,757 μm^3 for spotted seatrout and 132,108±6,828 μm^3 for red snapper. There were significant differences between the two mean values ($p < 0.05$). Trophonts achieved the maximum mean volume on day 3 for both spotted seatrout (158,419±5,906 μm^3) and red snapper (189,429±6,248 μm^3), with the largest trophont found on day 4 for both spotted seatrout (303,223 μm^3) and red snapper (289,910 μm^3). In both host species, trophonts were found in the cups and on the fish on day 6; however, evidence suggests that reinfection occurred and the small, newly formed trophonts caused the trophont mean volume to decrease beginning on day 4 (Fig. 2.2, refer to Discussion for details).

The number of trophonts on the fish at day 6 was 1,768±427 trophonts $fish^{-1}$ for spotted seatrout and 831±178 trophonts $fish^{-1}$ for red snapper. The mean volume of a trophont attached to spotted seatrout on day 6 was 60,099±3,841 μm^3; the mean volume of a trophont attached to red snapper was 62,103±4,941 μm^3.

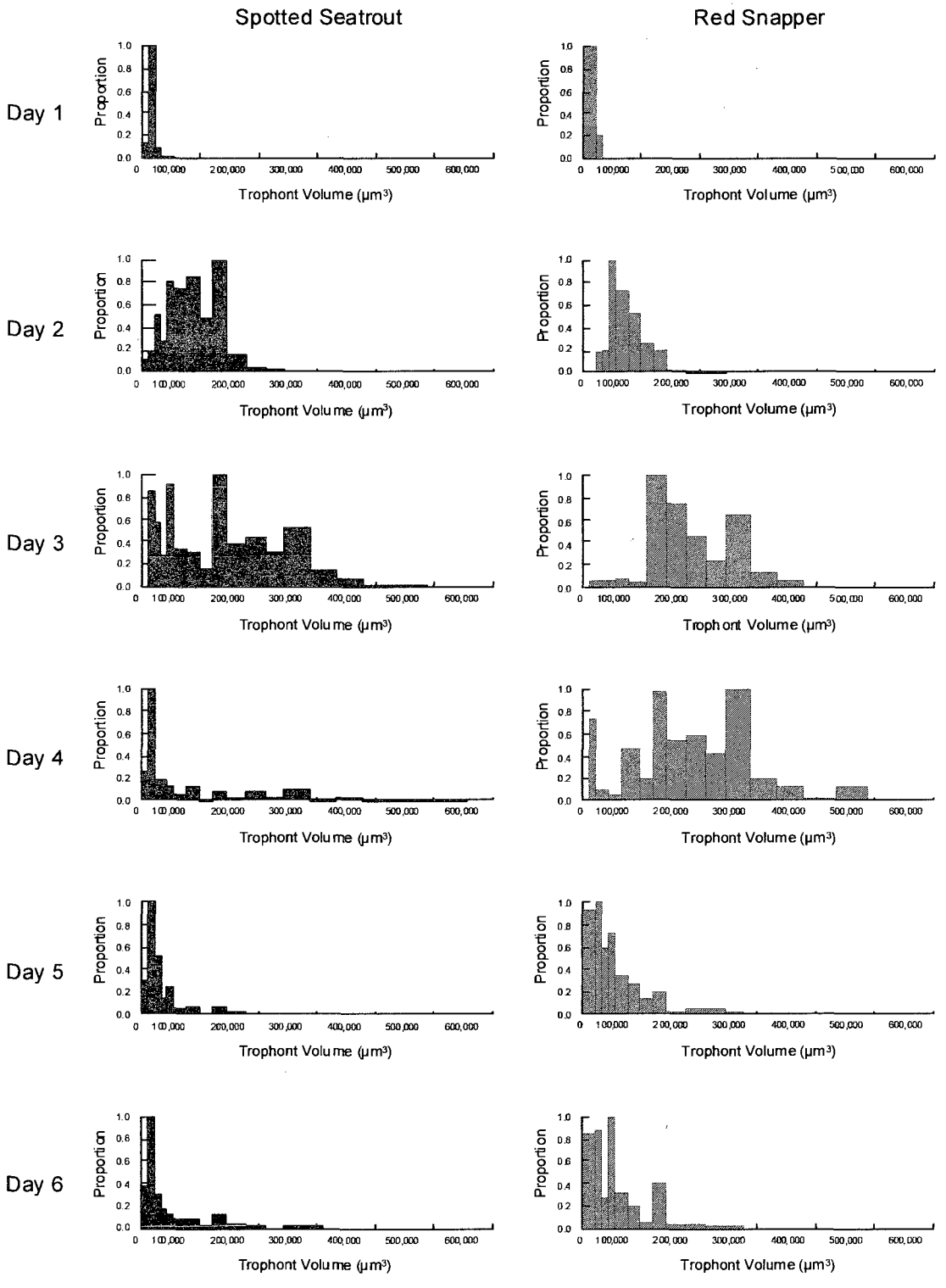

Figure 2.2. Proportion of trophonts of different volumes detached from day 1 to day 6 from spotted seatrout (solid bars) and red snapper (hatched bars). Note that from days 4 to 6 in spotted seatrout and 5 to 6 in red snapper, most of the trophonts detached were comparable in volume to the ones detached during days 1 and 2 for both species.

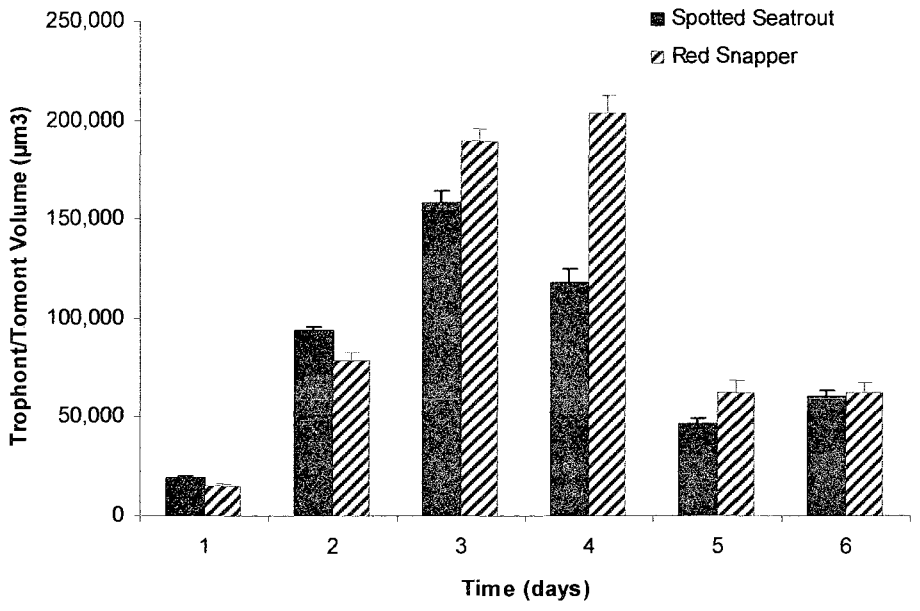

Figure 2.3. Mean trophont volume at detachment from spotted seatrout (solid bars) and red snapper (hatched bars) over time. Error bars show the positive SE.

The trophont growth rate was estimated using the best fit exponential curve $y=a.e^{b.x}$ for the relationship trophont volume versus time, with e^b being the trophont growth rate (Systat 2007). However, I used data from days 0 to 3 only, with the mean dinospore volume as the starting point for day 0 (680±74 μm^3 $dinospore^{-1}$) (n=21). Day 4-6 observations were excluded because reinfection by newly produced dinospores resulted in a decrease in trophont mean volume, as indicated before. The trophont growth rate in volume was 2.12 $d^{-1}$for spotted seatrout and 2.71 d^{-1} for red snapper.

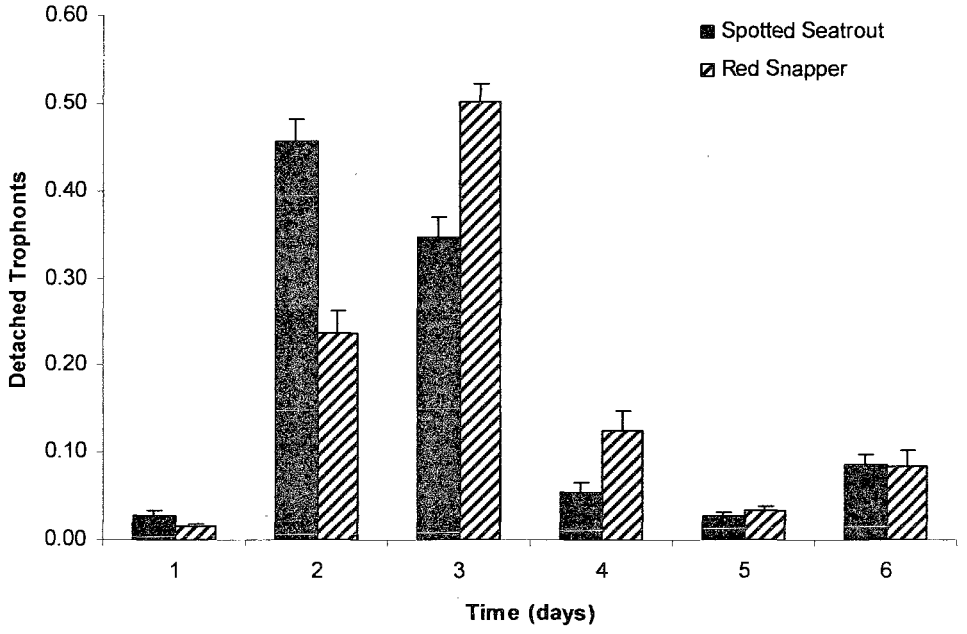

Figure 2.4. Mean proportion of trophonts detached from spotted seatrout (solid bars) and red snapper (hatched bars) over time. Error bars represent the positive SE.

The daily trophont detachment from spotted seatrout and red snapper is shown in Fig. 2.4. Only 0.03±0.01 and 0.02±0.00 of the total trophonts collected during the 6-d experiment detached from spotted seatrout and red snapper on day 1, respectively. These proportions were not significantly different. The proportion of trophonts that detached from spotted seatrout on day 2 (0.46±0.02) was significantly larger than that which detached from red snapper (0.24±0.03) ($p < 0.05$). However, on days 3 and 4, the proportion of trophonts that detached from spotted seatrout was significantly smaller than the proportion that detached from red snapper for the same day (0.35±0.02 and 0.50±0.02 for spotted seatrout and red snapper, respectively, on day 3; 0.05±0.01 and 0.13±0.02 for spotted seatrout and red snapper, respectively, on day 4) ($p < 0.05$). On days 5 and 6, only a small proportion of trophonts detached: 0.03±0.00 for both spotted seatrout and red snapper on day 5; 0.09±0.01 and 0.08±0.02 for spotted seatrout and red snapper,

respectively, on day 6. Those proportions were not significantly different between host species. Trophont detachment peaked on day 2 for spotted seatrout (0.46±0.02), whereas peak detachment was on day 3 (0.50±0.02) for red snapper. Survival analysis detected significant differences in the trophont detachment cumulative distributions between host species ($p < 0.05$). The trophont mean time to detachment was 3.45±0.003 d in spotted seatrout and 3.53±0.005 d in red snapper. The 95% confidence intervals for the mean values in each host species did not overlap, which suggests significant differences in trophont mean time to detachment between them (95% CI were 3.44 - 3.46 d and 3.52 – 3.54 d for spotted seatrout and red snapper, respectively). The daily biomass of trophonts detached from spotted seatrout and red snapper over time (Fig. 2.5) also followed a similar pattern as the one shown in Fig. 2.4 for the proportion of trophonts detached over time.

The cumulative proportion of trophonts attached to spotted seatrout and red snapper was plotted and fit to the exponential curve $y=a.e^{b.x}$ (Fig. 2.6). The trophont detachment rates calculated from these curves were 0.5262±0.0121 trophonts d^{-1} for spotted seatrout and 0.4811±0.0182 trophonts d^{-1} for red snapper. Regression analysis of the $\log_{10}$ transformed data showed no significant differences in trophont detachment rates between host species ($p < 0.05$).

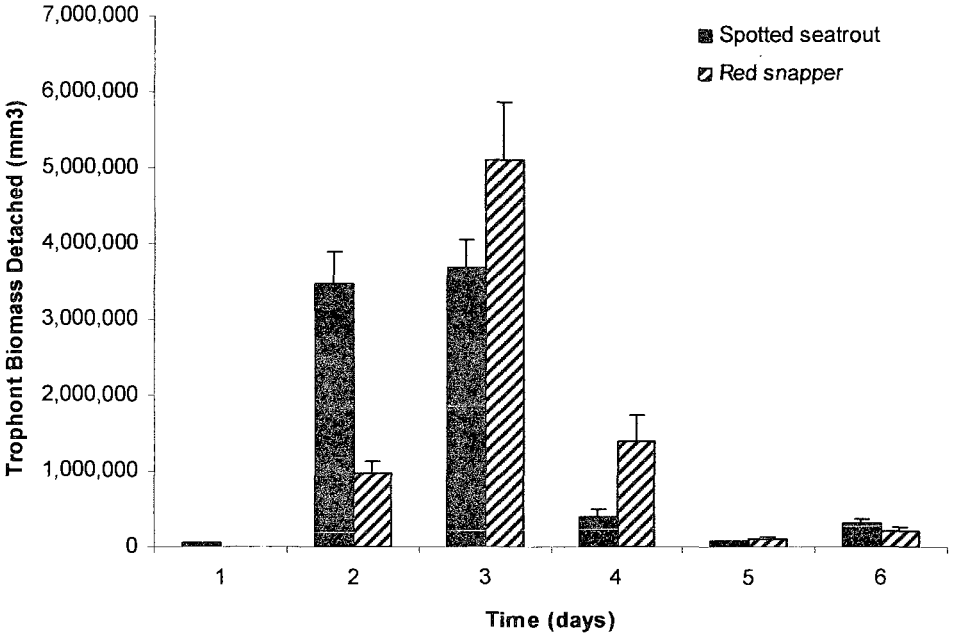

Figure 2.5. Mean trophont biomass detached from spotted seatrout (solid bars) and red snapper (hatched bars) over time. Error bars represent the positive SE.

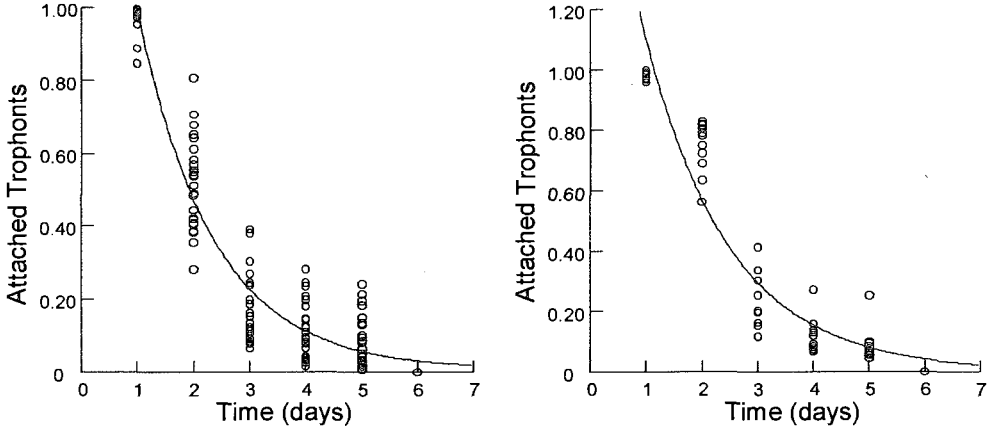

Figure 2.6. Observed data (circles) and best fit exponential curves used to estimate the trophont detachment rate (ø) for spotted seatrout (left) and red snapper (right).

Discussion

Several authors have described the processes that lead to trophont release from the host. Nigrelli (1936) commented that as trophonts attain larger sizes, the rhizoids can no longer maintain attachment and trophonts drop off. In addition, the integrity of the host tissue which constitutes the substrate to which rhizoids are anchored is lost. Similarly, Paperna (1984a) indicated that the detachment of trophonts occurs as a consequence of either an increase in size of the parasite (amplifying the friction with the water) or a decline in attachment efficiency as the host tissue becomes necrotic or hyperplastic (Lom and Lawler 1973). While this study cannot address the physical processes that result in trophont release, it showed that smaller trophonts indeed were less likely than large ones to be lost. Less than 0.02 of trophonts detached during the first day post-infection when trophonts were approximately five times smaller than the ones that detached during the second day in both host species. Similarly, Paperna (1984a) observed no significant trophont detachment during the first day post-infection in post-larvae of *S. aurata* at 19-24°C, with the trophonts that detached on day 1 being 5.6 times smaller than the ones that detached on day 2.

The overall mean daily trophont detachment rates in red snapper and spotted seatrout were not significantly different; however, there were differences in the pattern of trophont detachment between species. Trophont detachment peaked on day 2 in spotted seatrout, while it peaked on day 3 in red snapper (Fig. 2.4). The differences in daily trophont detachment between spotted seatrout and red snapper could be in part related to the latter species being a less active swimmer than the former (red snapper commonly remained stationary when held in static tanks or aquaria while spotted seatrout moved frequently, personal observation). Because water flows less rapidly over the tegument of

a stationary fish, less friction is produced thereby reducing trophont detachment. Thus, red snapper would be expected to lose trophonts more slowly than spotted seatrout.

Only on day 2 post-infection were trophonts significantly larger on spotted seatrout than on red snapper. As previously noted, a higher proportion of trophonts detached from spotted seatrout than from red snapper on day 2. However, by day 3, the size of the trophonts was larger in red snapper (189,429±6,248 μm^3) than in spotted seatrout (158,419±5,906 μm^3) and so was the proportion of trophonts that detached (0.50±0.2 versus 0.35±0.02 for red snapper and spotted seatrout, respectively), with this tendency continuing for days 4 and 5. Thus, the results of this study support the idea that the size of the trophont is a primary factor in determining when a trophont detaches.

Trophonts that detached beyond day 4 (inclusively for spotted seatrout) should have been larger in mean volume than trophonts that detached on the previous days; however, the mean volume of trophonts decreased beyond day 4. This suggests that reinfection occurred and that the influx of younger, smaller trophonts "masked" the growth of trophonts from the original infection, which would have been decreasing in number (Fig. 2.2). Paperna (1984a) observed trophonts 20-25 μm long that detached from *S. aurata* during the first day post-infection which failed to divide and sporulated within 24 h (yielding 2 dinospores $tomont^{-1}$). Similarly, the re-infection seen in our experiment probably was due to small trophonts falling off the fish, encapsulating and hatching within the 24-h period each fish stayed in the cup. The newly hatched dinospores would have been re-infecting the fish and adding to the total number of trophonts collected in the subsequent examinations of the cups.

The characteristics of the skin and mucus of spotted seatrout and red snapper could have caused the parasite to grow and detach differentially in each host species. In

contrast to the innate immunity that the hosts' tegument and mucus provide to reduce the establishment and survival of invading parasites (Woo 1996), adaptive immunity seems not to play an important role in reducing survival of *A. ocellatum* initially because the parasite can reach lethal levels much faster than the antibody response can develop (Cobb 1997). Fish used in our experiments were lab-reared and never exposed to the parasite, thus ensuring no immunological memory for a quick adaptive response. Differences in the innate immune responses of spotted seatrout and red snapper are not known, but such responses could have played a role in the differences observed.

Considering that there is no significant difference in infection rates between the species (Chapter III), I attribute the observation that infections in red snapper reach lethal levels faster than in spotted seatrout to a higher trophont growth rate, a larger trophont mean size at detachment and a tendency for trophonts to remain attached longer in red snapper than spotted seatrout. It is important to note that the harm caused by trophonts to the host is a function of their number and volume which can be expressed as the trophont biomass. Although the general pattern of trophont biomass detachment over time (Fig. 2.5) was similar to that of the proportion of trophonts detached over time (Fig. 2.4), the differences in the detachment patterns between host species with respect to the biomass were more pronounced than with respect to the proportion of detached trophonts. For day 2, the trophonts that detached from spotted seatrout were not only more numerous but also larger than the ones that detached from red snapper, whereas for days 3 and 4 trophonts were more numerous and larger in red snapper than in spotted seatrout. A higher biomass of trophonts remaining attached to red snapper than to spotted seatrout beyond the second day of infection would cause more harm to the former species than to

the latter one which further supports the incidental observations that *A. ocellatum* reaches a lethal level faster in red snapper than in spotted seatrout.

Knowing the pattern of trophont detachment in a defined host and under specified conditions is important for understanding the population dynamics of *A. ocellatum* and also for assessment of the infection rate. To estimate the infection rate in a host species it is necessary to quantify how quickly trophonts detach so that the appropriate period of time between infection with dinospores and the counting of the attached trophonts can be established. Bower et al. (1987) reported infection rates between 0.36 and 0.51 for hybrid striped bass, *Morone chrysops x Morone saxatilis,* exposed to 1,000 dinospores $fish^{-1}$ (of different age groups) for 30 min and then returned to clean sea water for 2 d before counting the number of detached trophonts following a 6-min freshwater bath. In a similar way, Cobb et al. (1998b) observed a mean infection rate of 0.20 after exposing tomato clownfish, *Amphiprion frenatus,* to 40,000 dinospores $fish^{-1}$ for 30 min, moving the fish to clean sea water for 3 d and counting the detached trophonts from each fish following a 3-min dip in distilled water. Admittedly, Bower et al. (1987) and Cobb (1998b) were not studying the population growth of *A. ocellatum*. However, in both cases, due to the time allowed between infection with dinospores and trophont counting, the reported infection rates likely would have been underestimated. The prolonged time the fish were held before the number of trophonts on them was assessed (2 and 3 d, respectively) would have allowed a proportion of the trophonts to grow, detach from the host and be missed in the assessment of the infection rate. Indeed, our study showed that at least 0.26 trophonts detached by the second day post infection and that at least 0.76 detached by the third day (combining the data from spotted seatrout and red snapper). Some other factors that also could have introduced bias into the infection rates reported

by Bower et al. (1987) and Cobb et al. (1998b) were that no time unit was provided (e.g., percentage of dinospores attached per day or hour), exposures to dinospores were only for 30 min followed by holding the fish for 2- 3 d, and the re-infection with dinospores produced by young trophonts dropping off, metamorphosing into tomonts and producing more dinospores was neither considered nor corrected for in their results. Also the effectiveness of a 3-6 min freshwater dip may be limited for inducing the detachment of all trophonts.

In contrast, the detachment patterns detailed in this study were considered in Chapter III to design the experiments aimed at estimating the dinospore infection rates of spotted seatrout and red snapper. The infection rates were obtained by exposing a fish to dinospores for 24 h, euthanizing the fish at the end of those 24 h, and immediately counting the trophonts attached (refer to Chapter III for more details). The number of trophonts that could have detached during the 24 h period the fish were exposed to dinospores (although minimal as shown here) was corrected for in the computation of the infection rate using data from the trophont detachment study presented in this chapter.

The trophont detachment rate and pattern of trophont detachment derived in this portion of the study will be used in Chapter V to construct a population model for *A. ocellatum* infecting spotted seatrout and red snapper. The population model will allow determination of the parameters that have the most influence on the parasite's population growth. This approach, which will be detailed in the following chapters, can help identify critical life cycle control points at which control measures can be directed.

CHAPTER III

SUSCEPTIBILITY AND TOLERANCE OF SPOTTED SEATROUT, *CYNOSCION NEBULOSUS*, AND RED SNAPPER, *LUTJANUS CAMPECHANUS*, TO *AMYLOODINIUM OCELLATUM* INFECTIONS

Introduction

Some host species are more resistant than others to infections with *A. ocellatum*. Lawler (1977, 1980) observed that among the more resistant species were fish that either tolerate low oxygen or produce large amounts of mucus. Resistant species include the sheepshead minnow, *Cyprinodon variegatus*; the Gulf killifish, *Fundulus grandis*; the American eel, *Anguilla rostrata*; the Gulf toadfish, *Opsanus beta*; the sailfin molly, *Poecilia latipinna*; and the fat sleeper, *Dormitator maculatus*. Noga and Levy (2006) reported that clown anemonefish, *Amphiprion ocellaris*, were more susceptible than striped bass, *Morone saxatilis*, which were, in turn, more susceptible than Mozambique tilapia, *Oreochromis mossambicus*, but the authors did not provide details on how differences in susceptibility were quantified.

Susceptibility can be defined as a measure of the host's ability to become infected by a parasite or a disease-causing organism. Hosts that are more readily infected would be classified as more susceptible than those that are less readily infected. Tolerance, on the other hand, refers to a host's capacity to sustain a parasite load. Hosts that carry a higher parasite load would be considered more tolerant than those that carry lower loads. Spotted seatrout, *Cynoscion nebulosus*, and red snapper, *Lutjanus campechanus*, are both susceptible (i.e., they get infected) to *A. ocellatum*, and during *A. ocellatum* outbreak episodes, I have noticed that infections appear to reach lethal levels faster in red snapper than in spotted seatrout (personal observation). Therefore, I undertook a comparative

study of the susceptibility and tolerance of the two species to *A. ocellatum*. For both species, I estimated the dinospore infectivity rate (the probability that a dinospore infects a fish per day), the dinospore lethal dose (the number of dinospores needed to kill a fish) and the trophont lethal load (the number of trophonts following death after exposure to the dinospore lethal dose).Different dinospore doses were used to determine if there was a relationship between infection rate and challenge dose. In addition, after determining that spotted seatrout tolerated a larger trophont load than red snapper, I evaluated the gill surface area to body weight ratio in both species to determine if that ratio could explain differences in tolerance.

Methods

Dinospore Collection and Quantification

A stock of *A. ocellatum* was continuously maintained at 25°C and 33 ppt in live fish (primarily spotted seatrout but occasionally Atlantic croaker, *Micropogonias undulatus*) (Chapter II). The sea water used for all experiments was prepared by dissolving Artificial Crystal Sea® Marinemix salt (Marine Enterprises International Inc., Baltimore, MD) in dechlorinated and aged tap water to achieve a salinity of 33 ppt at 25°C. To obtain dinospores for challenges, uninfected fish were introduced into the stock tank. When fish showed behavioral signs of infection (erratic swimming, gulping, anorexia), they were removed, euthanized, and their gills excised and placed in an 80-ml Petri dish filled with 40 ml of sea water (25°C, 33 ppt). The body of the fish was placed in a 500-ml beaker with 300 ml of sea water (25°C, 33 ppt). After 30-60 min, the gills and body were removed from their respective containers and the remaining water with detached trophonts was filtered through a 125-μm mesh sieve to remove large debris. The filtrate was collected in a 500-ml beaker which was left undisturbed for 15 min to allow

the trophonts to settle. The supernatant was removed by siphoning and the remaining 50-100 ml containing the settled trophonts was split and transferred in approximately equal parts to two 50-ml test tubes. The tubes were left undisturbed for 15 min to allow the trophonts to settle. The supernatant was removed by siphoning, leaving a 1-cm water layer containing the settled trophonts. Trophonts were re-suspended in clean sea water (25°C, 33 ppt) by refilling the test tubes to the 40-ml mark. This washing was performed three times. After the last washing, trophonts were transferred to a six-well culture plate with 16-ml wells (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and the wells were filled with clean sea water (25°C, 33ppt) to a final volume of 10 ml $well^{-1}$.

After 72 h of incubation at 25°C, swimming dinospores were pipetted from the culture plate to a 300-ml beaker and sea water was added to achieve a final volume of 200 ml. The total number of dinospores was estimated using a Sedgwick-Rafter counting cell (S-R cell). The S-R cell was filled with 0.5 ml of the dinospore suspension, previously mixed and stirred using the micropipette, and 0.5 ml of 10 % buffered formalin to kill dinospores and allow counting. The mean number of dinospores from three 0.5-ml samples of the dinospore suspension was multiplied by 2 to obtain the number of dinospores per ml in the original dinospore suspension which then was used to calculate the volume of the dinospore suspension needed for each dinospore dose used in the challenges. Assuming the dinospores were randomly distributed in the sample (due to the mixing and stirring), the SE of each dinospore dose was calculated using the Poisson distribution, with SE being the square root of the mean divided by the square root of the number of observations (3) done to determine the dinospore dose.

Estimation of the Dinospore Infection Probabilities

Laboratory-reared spotted seatrout juveniles (13.50±0.23 cm in total length and 22.83±1.322 g, mean±SE) were individually challenged with 5,000±41, 20,000±82, 100,000±183 and 200,000±258 dinospores fish^{-1} (n=25 individual fish dose^{-1}) in 3-l aquaria filled with 2.5 l of sea water. Twenty-five laboratory-reared juvenile red snapper (5.84±0.15 cm in total length and 3.31±0.28 g, mean±SE) were exposed individually to 20,000 dinospores. A single dose was used due to the limited availability of laboratory-reared red snapper. The temperature and salinity in the exposure aquaria were maintained at 25°C and 33 ppt, respectively. Continuous aeration was supplied by air stones connected to a regenerative blower (one air stone per aquarium).

After 24 h of exposure to dinospores, fish were dissected and the attached trophonts counted. Gills were removed by clipping each gill arch dorsally and ventrally and placing in an 80-ml Petri dish with distilled water for 45-60 min to allow trophonts to detach. Further dislodgement was obtained by gently rubbing the gill surface with a dissecting needle until no more attached trophonts were observed. The contents of the Petri dish then were poured into a 300-ml beaker and the beaker filled with sea water to a 200-ml final volume. Trophonts were counted using the S-R cell as previously described for counting dinospores.

The number of trophonts attached to the body of the fish was estimated in the same manner. After removing all gill arches, the body was placed in a plastic 300-ml container filled with enough distilled water to cover the body for 45-60 min until examined. At examination, the body surface was gently brushed with a round, size 3, red sable paint brush (Bluementhal Craft, Carlstadt, NJ) and rinsed with a stream of distilled

water to enhance trophont release. The contents of the container were then poured into a 300-ml beaker and counted using the method previously described.

The infection rate is the probability that a dinospore infects a fish per day and is estimated as the proportion of dinospores that result in trophonts. ANOVA of the arcsin root transformed proportions was used to test for significant differences in infection probabilities between spotted seatrout and red snapper ($\alpha \leq 0.05$).

In addition, because the number of resulting trophonts produced by infection with dinospores was counted for the gills and the body separately, the mean proportions of trophonts in the gills and in the body were calculated. ANOVA of the arcsin root transformed proportions was performed to determine if there were statistical differences between host species at the 20,000 dinospores fish^{-1} dose (the only dose used for red snapper) ($\alpha \leq 0.05$).

Determining the 48-h Median Dinospore Lethal Doses

One hundred and twenty lab-reared spotted seatrout (12.06±0.18 cm in total length and 16.36±0.82 g, mean±SE) were placed individually in 120 3-l aquaria filled with 2.5 l of sea water (25°C, 33 ppt) with continuous aeration and challenged with 100,000±183, 150,000±224, 200,000±258 or 250,000±289 live dinospores (mean dose±SE) (30 fish per dosage) for 48 h. The dinospore doses and exposure time were chosen based on preliminary observations which showed that all juvenile spotted seatrout exposed to 200,000±258 and 500,000±408 dinospores fish^{-1} (mean dose±SE) died within 48 h. This indicates that these dinospore doses are above the median lethal dinospore dose and that a 48-h exposure was sufficient to determine if a dose was lethal. In addition, a 48-h maximum time of exposure considerably reduces trophonts loss and reinfection, compared with longer exposure times (Chapter II).

For red snapper, only 28 animals (10.15±0.37 cm in total length and 17.15±2.04 g, mean±SE) were available for exposure (seven for each dosage level). Because of the limited availability of red snapper, 300,000±316 (mean dose±SE) dinospores was used as the upper dosage level instead of the 250,000 used for seatrout to increase the likelihood of detecting a difference in mortality compared with that of the 200,000 dosage level. The mortality rate was computed at each dinospore dose and probit analysis was used to estimate the 48-h LD_{50}.

Determining the Trophont Lethal Loads at the 48-h LD_{50}

To determine the trophont lethal loads, I counted the number of trophonts on the fish that died from the infection during the 48-h LD_{50} study using the techniques described above. In addition, to account for any trophonts that may have detached and metamorphosed into tomonts, I counted the number of tomonts settled in the aquarium. This number was obtained after removing the airstone from the aquarium and allowing the water to settle 5 min, after which the water was siphoned down to leave a 1-2-cm layer containing the settled tomonts. The settled tomonts were then transferred to a container of known volume and the S-R cell was used to count tomonts. The trophont lethal load was calculated as the sum of the number of trophonts attached to the fish and the number of tomonts recovered from the aquarium. Linear regression analysis was used to obtain the best fit lines and equations from which the trophont lethal loads at the 48-h LD_{50}s for spotted seatrout and red snapper were estimated. ANCOVA was used to test for significant differences in trophont lethal loads at the 48-h LD_{50} between spotted seatrout and red snapper ($\alpha \leq 0.05$). The covariate was dose.

Estimation of the Gill Surface Area to Body Weight Ratio

Five spotted seatrout (27.50±1.31 g, mean weight±SE), 5 red snapper (47.98±1.87 g, mean weight±SE) and another group of 5 spotted seatrout (74.90±5.86 g, mean weight±SE) were euthanized and weighed. Their gill arches (one side per fish) were dissected, laid flat on a glass slide with the inner side facing up and photographed at a resolution of 3072 x 2304 megapixels with a digital camera. Using Adobe Illustrator CS4 (Adobe Systems 2008), I outlined the area corresponding to the filaments in the digital picture and used a digital grid application provided by this software. I calculated the area by counting the number of whole squares and partial squares that fell within the outlined gill area. Then I summed the number of whole squares and half the number of partial squares and multiplied that value by the area of a square. The gill surface area to body weight ratio was then computed by summing the calculated area for each gill and multiplying the resulting value by 4 (to account for the other side of the gill and the other side of the fish). Mann–Whitney U tests were performed to determine if there were statistical differences among the three fish groups (each group was composed of 5 fish).

Results

The overall mean dinospore infection rate for spotted seatrout was 0.36 dinospores d^{-1} (0.33-0.38 dinospores d^{-1}, 95% CI). There was no significant difference in mean infection rates among doses ($p < 0.05$) in spotted seatrout (Fig. 3.1). For red snapper, the overall mean dinospore infection rate (based on 20,000 dinospores $fish^{-1}$ as the only challenge dose) was 0.34 dinospores d^{-1} (0.31-0.37 dinospores d^{-1}, 95% CI) (Fig. 3.2). No significant difference was detected in overall mean infection rates between spotted seatrout and red snapper or between the mean infection rates in each species at the 20,000 dinospores $fish^{-1}$ d^{-1} dose.

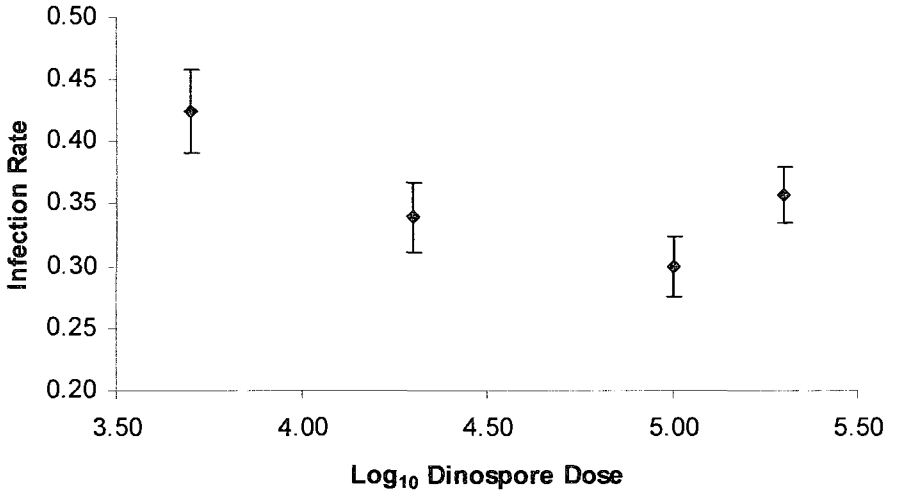

Figure 3.1. Mean dinospore infection rates in spotted seatrout exposed to 5,000, 20,000, 100,000 and 200,000 dinospores fish^{-1} d^{-1} (n=25 fish dose^{-1} d^{-1}). Error bars show the SE. No significant differences in mean infection rates were detected among doses ($p > 0.05$).

I counted trophonts attached to the gills and the body separately; therefore, I calculated the proportion of the total number of trophonts in the gills and on the body. Combining all dinospore doses for spotted seatrout, the proportion of trophonts in the gills was 0.44±0.02 (mean±SE). At the 20,000 dinospore fish^{-1} dose, the proportion of trophonts in spotted seatrout gills was 0.43±0.03 (mean±SE). For red snapper, the proportion of trophonts in the gills was 0.32±0.02 (mean±SE). ANOVA of the arcsin root transformed proportions showed a significant difference in the mean proportion of trophonts in the gills between spotted seatrout and red snapper at the 20,000 dinospores fish^{-1} dose.

The 48-h median dinospore lethal doses (48-h LD_{50}s) were calculated from the probit distributions shown in Fig. 3.3. The 48-h LD_{50}s were 237,243 dinospores fish^{-1} (195,980-350,306 dinospores fish^{-1}, 95% CI) for spotted seatrout and 141,010 dinospores fish^{-1} (85,892-210,230 dinospores fish^{-1}, 95% CI) for red snapper. Logistic regression analysis detected no significant difference in the 48-h LD_{50}s between spotted seatrout and

red snapper at $\alpha \leq 0.05$ (p=0.055). There was no statistically significant difference in mean weight between the spotted seatrout and red snapper used in this experiment.

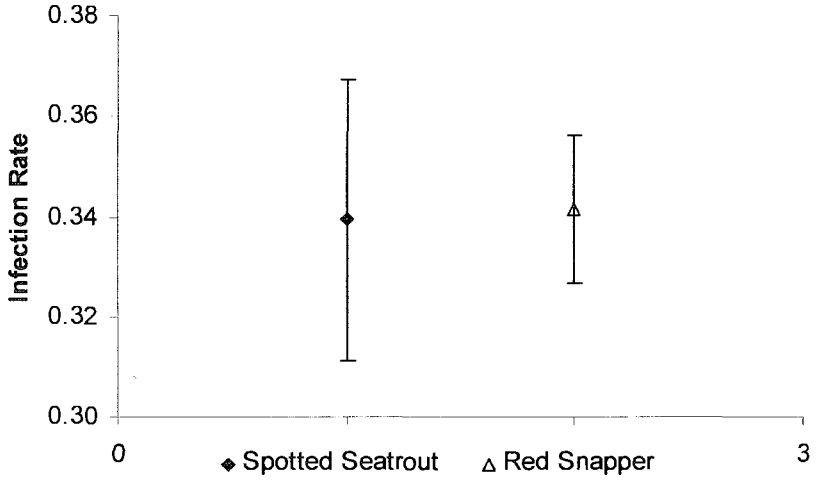

Figure 3.2. Comparison of mean infection rates between spotted seatrout (solid rhombus) and red snapper (hollow triangle) challenged with 20,000 dinospores $fish^{-1}$ d^{-1} (n=25 observations $species^{-1}$). Error bars show the SE. No significant difference in mean infection rates between host species ($p > 0.05$).

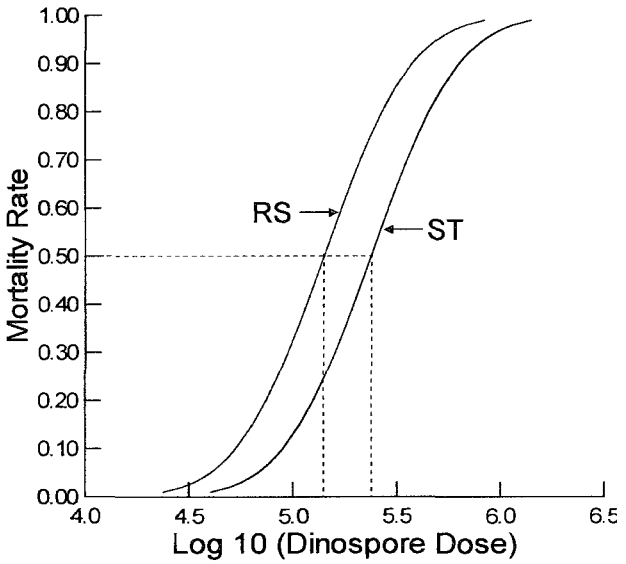

Figure 3.3. Host mortality rates at various dinospore doses and probit distributions used to estimate the 48-h LD_{50} for spotted seatrout (ST) and red snapper (RS). The resulting 48-h LD_{50}s for spotted seatrout and red snapper were 237,243 (n=120) and 141,010 dinospores $fish^{-1}$(n=28), respectively. No significant difference in 48-h LD_{50}s between host species (p=0.055).

Figure 3.4 shows the number of trophonts recovered from the spotted seatrout and the red snapper that succumbed to the infection and the best fit regression lines used to estimate the trophont lethal loads at 48-h LD_{50} for each species. The trophont lethal loads at 48-h LD_{50} were 178,067 trophonts $fish^{-1}$ (75,026-281,107 trophonts $fish^{-1}$, 95% CI) for spotted seatrout and 123,160 trophonts $fish^{-1}$ (2,439-243,880 trophonts $fish^{-1}$, 95% CI) for red snapper. ANCOVA detected significant differences in trophont lethal loads at the 48-h LD_{50} between species ($p < 0.05$).

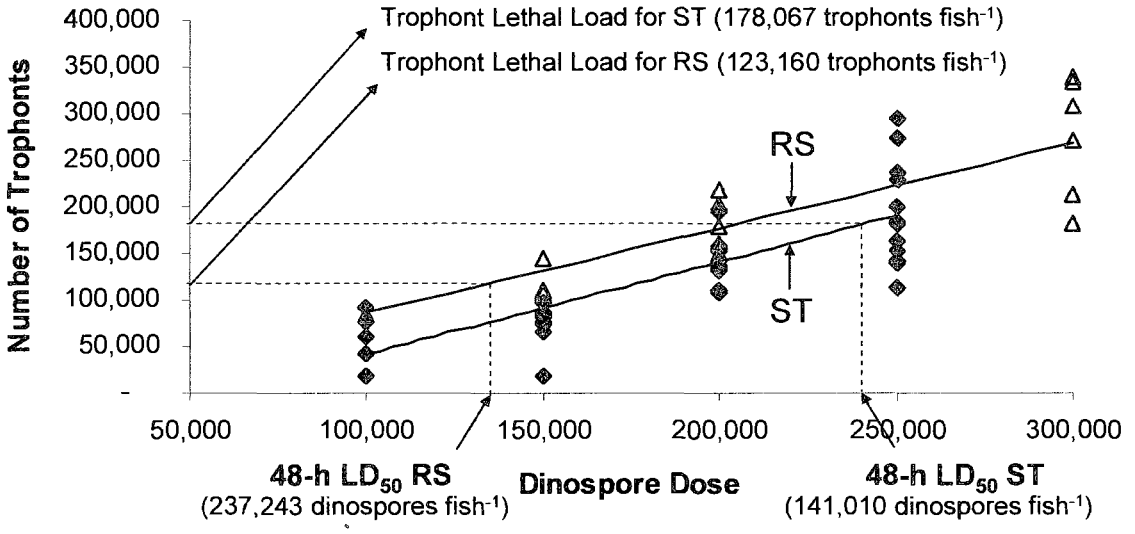

Figure 3.4. Number of trophonts recovered from the spotted seatrout (ST) (solid rhombi) and the red snapper (RS) (hollow triangles) that died at the dinospore doses tested and best fit regression lines used to estimate the trophont lethal loads at the 48-h LD_{50}s. The resulting trophonts lethal loads at the 48-h LD_{50}s for ST (237,243 dinospores $fish^{-1}$) and RS (141,010 dinospores $fish^{-1}$) were 178,067 (n=40) and 123,160 trophonts $fish^{-1}$ (n=17) for spotted seatrout and red snapper, respectively. Significant differences in trophont lethal loads at the 48-h LD_{50}s between host species ($p < 0.05$).

The mean gill surface area to body weight ratio for the first group of spotted seatrouts (27.50±1.31 g, mean weight±SE) was 0.48±0.01 (mean±SE). The mean gill surface area to body weight ratio for the second group of spotted seatrout (74.90±5.86 g, mean weight±SE) was 0.34±0.02 (mean±SE). The mean gill surface area to body weight ratio for the group of red snappers (47.98±1.87 g, mean weight±SE) was 0.55±0.02 (mean±SE). The group of red snapper had a significantly larger ratio than either of the two groups of spotted seatrout ($p<0.05$). In addition, red snapper gills have a more feathery appearance than gills of spotted seatrout, with longer and looser secondary lamellae. Secondary lamellae in spotted seatrout are shorter and more compact (Figure 3.5).

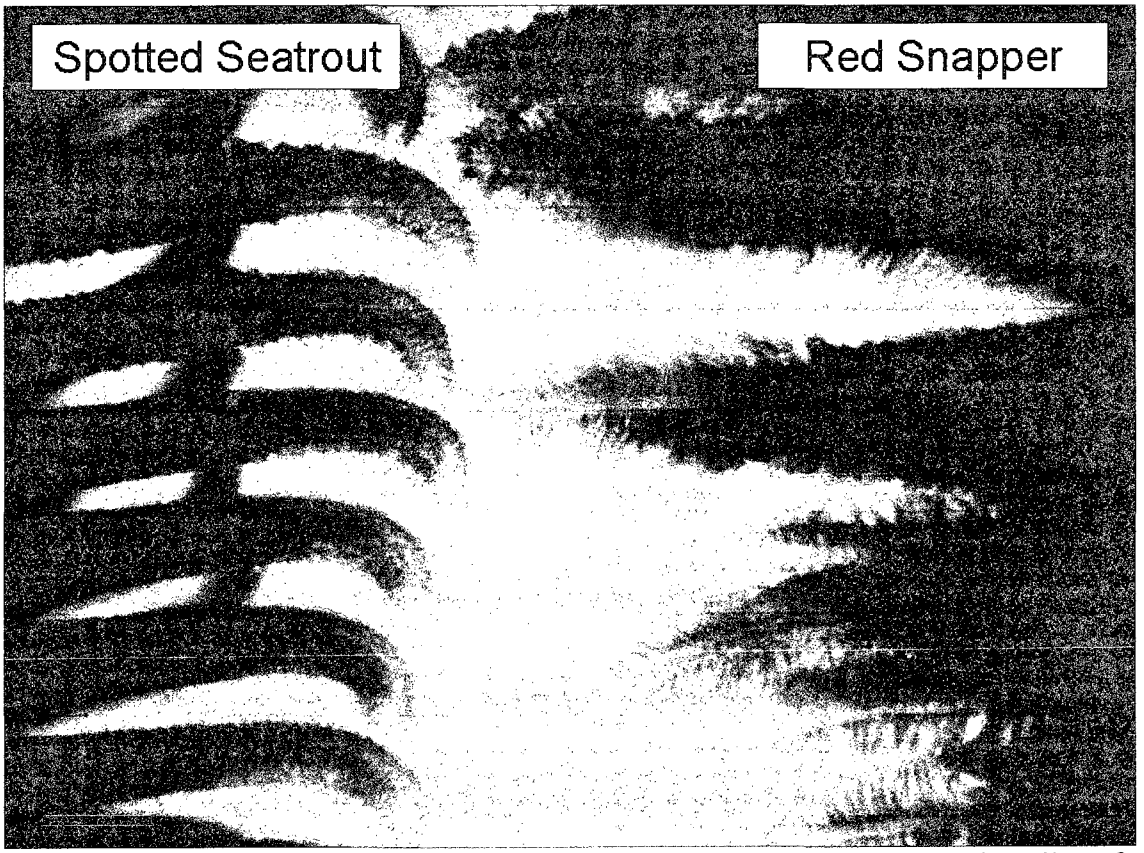

Figure 3.5. Micrograph showing the gill's primary filaments and secondary lamellae of spotted seatrout and red snapper. Scale bar: 200 μm.

Discussion

The mean infection rates at the four dinospore doses used for spotted seatrout were not significantly different from each other, which indicates that the dinospore infection rate is independent of the dinospore dose, at least at the tested levels. However, this assumption could not be tested in red snapper because only one dinospore dose was used for the challenge in this species.

While spotted seatrout and red snapper have the same mean infection rates (i.e., are equally susceptible), the higher trophont lethal load in spotted seatrout indicates that this species is more tolerant of infections than red snapper. Because there was no significant difference in mean weight between the spotted seatrout and the red snapper used in this study, it is unlikely that the differences in trophont lethal loads could be attributed to variation in fish size. Because spotted seatrout and red snapper have different body shapes (i.e., spotted seatrout are more torpedo-shaped and dorso-ventrally shallow than red snapper), weight instead of length was used to quantify size differences. The difference in trophont lethal loads (i.e., tolerance) means that more time is required for an infection to reach a lethal level in a culture system stocked with spotted seatrout than with red snapper, possibly giving the producer a greater chance of administering a successful treatment to control the outbreak.

The difference in gill surface area to body weight ratio (with red snapper having a higher ratio) and the difference in gill structure between the two host species do not explain why the red snapper is less tolerant of infections than the spotted seatrout. If red snapper had a smaller gill surface area to body weight ratio than spotted seatrout then (although the loads were different) the densities of trophonts on the gills might be equal. However, trophont lethal density in gills of red snapper were lower than in gills of

spotted seatrout, and the seatrout was still more tolerant of infection than red snapper. In addition, even though the gill surface area to body weight ratio was lower in spotted seatrout than in red snapper, spotted seatrout had a higher proportion of trophonts in the gills than on the body than red snapper and still was more tolerant to trophont loads than red snapper.

Although this study does not address the mechanisms that could explain this difference in tolerance to *A. ocellatum* between spotted seatrout and red snapper, it is known that factors such as the characteristics of the mucus, the epithelium and its extracellular fluid may play a role in preventing the attachment of parasites (Woo 1996), and variations in these characteristics between spotted seatrout and red snapper may explain the differences in the severity of infections. Likewise, the capacity of withstanding low dissolved oxygen concentrations also may have a role in enhancing the tolerance to high trophont loads because trophonts on the gills interfere with gas exchange (Lawler 1977, 1980). However, I am not aware of studies evaluating oxygen requirements in these species to test this hypothesis.

Fish used in this project had never been exposed to *A. ocellatum*; therefore, a lack of immune memory was ensured. Nevertheless, a differential capacity for developing acquired immunity also could explain differences in tolerance. However, Cobb (1997) has shown that for tomato clownfish, *Amphiprion frenatus*, it takes several (1-week apart) exposures to non-lethal doses of dinospores to trigger immunity. This author even pointed out that in intensive mariculture conditions, and if fish have not been previously exposed to *A. ocellatum*, the delay in the primary immune response would allow time for the parasite to reach lethal densities and remain in sufficient numbers to kill fish introduced subsequently. For that reason, it is unlikely that the fish used in this study

could have developed acquired immunity to *A. ocellatum* in less than the 48-h exposure timeframe used in this experiment.

Other workers have reported *A. ocellatum* infection rates for other host species, but these rates are not directly comparable to the ones presented in this study. Bower et al. (1987) reported infection rates between 0.36 and 0.51 for hybrid striped bass, *Morone chrysops x Morone saxatilis*, exposed to 1,000 dinospores $fish^{-1}$ for 30 min and then returned to clean sea water for 2 d. Similarly, Cobb et al. (1998b) observed a mean infection rate of 0.20 after exposing tomato clownfish to 40,000 dinospores $fish^{-1}$ for 30 min and maintaining them in clean sea water for 3d. In my study, the spotted seatrout and the red snapper were exposed to dinospores for 24 h (the entire time for which the dinospore infection rate was calculated). This method provided a dinospore infection rate on a per day basis suited for use in the population model of *A. ocellatum* presented in Chapter V. In addition, because I estimated the dinospore infection rate over a 24-h period, not enough time was allowed for reinfection (Chapter II).

The infection rates presented in this chapter were calculated with the intent to use them in a population model of *A. ocellatum* that would allow the estimation and comparison of the population growth rate of the parasite on spotted seatrout and red snapper. Therefore, the methods and measurements used by Bower et al. (1987) and Cobb et al. (1998b) do not adequately account for the processes measured in my study. The data presented in this chapter provide a quantitative baseline measurement of the infection process for this parasite and provide infection rates for spotted seatrout and red snapper that can be used to make inferences about the population dynamics of the parasite infecting these host species.

CHAPTER IV

SURVIVAL AND FECUNDITY OF *AMYLOODINIUM OCELLATUM*

Introduction

The population dynamics of any organism are driven by the fecundity and lifespan of the various components in its life cycle. Life tables are the basis for modeling the population growth of an organism and they constitute various ways of viewing survival and reproductive rates of individuals grouped according to some common trait (e.g, life history stage, age, size). Because *A. ocellatum* has a life cycle with definite, discrete stages (like *Ichthyophthirius multifiliis*, for example, which has a trophont, a cyst and a theront stage) and because the survival and reproductive rates are dependent on the stage at which the organism is rather than on its age or size, it is suitable to use a stage-structured life table analysis (in contrast to human populations, for example, which are age-structured) for the study of the population dynamics of *A. ocellatum*. Life table analysis uses lifespan and fecundity to provide an estimate of the net reproductive rate (R_0) (Ebert 1999). Therefore, in this study, I used a laboratory challenge model to estimate the lifespans and fecundities of the trophont, tomont and dinospore stages of *A. ocellatum* in order to construct a life table which will be used in Chapter V to construct a population model that could be used to identify the vital rates that have the largest effect on the parasite's population growth.

Methods

Trophonts used for the experiments were obtained by adding naïve, lab-reared spotted seatrout to the tank where the infection was maintained (25°C and 33 ppt) (see Chapter II). Spotted seatrout showing behavioral signs of heavy infection were euthanized, dissected and processed to collect the trophonts. Gills were excised and

placed in Petri dishes with sea water at 25°C and 33 ppt. After a few minutes, the detached trophonts were transferred individually to a 96-well culture plate (Corning Life Sciences, Corning, NY, USA) (i.e., 1 trophont per well) using a glass pipette with a capillary tip. Wells were previously filled to half their volume (0.16 ml) with sea water (25°C, 33 ppt). The length of each trophont was measured. The culture plate was incubated in the dark at 25°C (VWR®1535 General Purpose Incubator, OR, USA). Culture plates were examined once daily in the morning to assess the survival of the trophonts, tomonts and dinospores, the number of tomont divisions, the time to sporulation and the number of dinospores produced by tomonts. Three trials were done successively such that 288 trophonts were monitored for development through the successive stages. Three trophonts were discarded due to error; therefore, the sample for this study consisted of a maximum of 285 trophonts. The time unit used in all cases is day.

Trophont Lifespan

The mean lifespan of the trophont (t_{Tr}) is inversely related to the probability that a trophont dies per day (μ_{Tr}) and the probability that a trophont detaches from spotted seatrout per day (ϕ). Either death or metamorphosis to a tomont ends the trophont stage.

To estimate the probability that a trophont dies per day (μ_{Tr}), I calculated the ratio of the number of trophonts that did not divide to the total number of incubated trophonts and multiplied the resulting value by the probability that a trophont detaches from spotted seatrout per day (ϕ), estimated in a separate study (Chapter II).

t_{Tr} was then computed as $t_{Tr} = \dfrac{1}{[1-(1-\mu_{Tr})(1-\phi)]}$ (5.1)

Tomont Lifespan

The tomont lifespan (t_{To}) is inversely related to the probability that a tomont dies per day (μ_{To}) and the probability that a tomont sporulates per day (γ). Either death or sporulation ends the tomont stage.

To calculate the probability that a tomont dies per day (μ_{To}), I counted the trophonts that transformed into tomonts and began dividing. For the tomonts that did not sporulate, the time at which they stopped dividing was recorded. These data were used to plot the number of tomonts that remained alive over time. Data were fit to the exponential function $y=a.e^{b.x}$, with $1-e^{b}$ being μ_{To}. Systat version 12.0 (Systat 2007) was used to perform the curve fitting.

The probability that a tomont sporulates per day (γ) was obtained by plotting the cumulative percentage of unsporulated tomonts over time. The data were fit to the exponential function $y=a.e^{b.x}$, with $1-e^{b}$ being γ. Systat version 12.0 (Systat 2007) was used to perform the curve fitting.

t_{To} was computed as $t_{To} = \dfrac{1}{[1-(1-\mu_{To})(1-\gamma)]}$ (5.2)

Tomont Fecundity

The tomont is the only reproductive stage in the life cycle of *A. ocellatum*, thus it is the only stage at which fecundity is greater than zero. Tomont fecundity (m_{To}) is the number of dinospores produced by a tomont per time unit. It is a function of the number of dinospores produced by a tomont of the mean size and the mean time it takes a tomont to sporulate. These parameters were estimated by maintaining tomonts in the 96-well culture plates and examining them once daily until sporulation. Sporulation may be protracted, hence sporulation was tracked until all tomites sporulated or decomposed. The

number of tomites just before sporulation was used to determine the number of dinospores produced per tomont (i.e., each tomite produces two dinospores). I examined the relationship between fecundity and tomont size. Linear regression was used to obtain a functional relationship between tomont length and the number of dinospores produced. Also, I used linear regression to describe the functional relationship between the mean time to tomont sporulation and tomont size.

For the life table, the fecundity of tomonts (m_{To}) was computed as the ratio of the mean number of dinospores produced per tomont of the mean size to the mean time to tomont sporulation, with the resulting value representing the number of dinospores produced per tomont per time unit.

Dinospore Lifespan

The dinospore lifespan (t_D) is inversely related to the probability that a dinospore dies per day (μ_D) and the probability that a dinospore infects a host per day (β). Either death or metamorphosis to a trophont ends the dinospore stage.

The probability that a dinospore dies per day (μ_D) was estimated by observing newly sporulated dinospores until dinospore motility ceased. However, only data from tomonts releasing all dinospores within a 24-h period (i.e., between two consecutive observations) were used to construct the dinospore survival curve from which the probability of dinospore death per day was calculated. This ensured that all dinospores in each well had a maximum age difference of 24 h.

Dinospore survival over time was fit to the exponential function $y=a.e^{b.x}$, with $1-e^{b}$ being the probability that a dinospore dies per day (μ_D). Systat version 12.0 (Systat 2007) was used to perform the curve fitting.

The dinospore infection rate (β) for spotted seatrout was estimated in a separate study [β_{st}=0.36 dinospores d^{-1} (0.33 – 0.38, 95% CI), Chapter III].

t_D was computed as $t_D = \frac{1}{[1-(1-\mu_D)(1-\beta)]}$ (5.3)

Results

Trophont Lifespan

Only two out of the 285 (0.007) incubated trophonts failed to divide and sporulate. The probability that a trophont dies per day (μ_{Tr}) was then calculated multiplying the ratio of dead trophonts to the total number of incubated trophonts by the trophont detachment rate (ϕ) (ϕ= 0.53 trophonts d^{-1} (0.50-0.55, 95% CI) as determined in Chapter II). The resulting value for μ_{Tr} was 0.0037 trophonts d^{-1} (0.0035-0.0038, 95% CI). Solving equation 5.1 using the mean values and 95% CI limits for μ_{Tr} and ϕ results in t_{Tr}=1.89 d (1.81-1.99, 95% CI).

Tomont Lifespan

Figure 4.1 shows the number of live tomonts over time and the best fit exponential curve $y=a.e^{b.x}$ used to estimate the probability that a tomont dies per day (μ_{To}), with $\mu_{To}=1-e^b$. The estimated value for μ_{To} was 0.010 tomonts d^{-1} (0.004-0.016, 95% CI).

The mean sporulation rate of the tomonts (γ) was calculated from the best fit exponential curve for the expression $y=a.e^{b.x}$ where $\gamma=1-e^b$ (Fig. 4.2). These data were obtained by observing 268 tomonts (of the 285 originally incubated trophonts, 2 died and 15 did not sporulate, leaving a total of 268 tomonts for this part of the study) of various sizes ranging from 32 to 112 μm for sporulation over time. The estimated value for γ was

0.35 tomonts d^{-1} (0.25 - 0.45, 95% CI). Solving equation 5.2 using the mean values and 95% CI limits for μ_{To} and γ results in t_{To}=2.78 d (2.20-4.01, 95% CI).

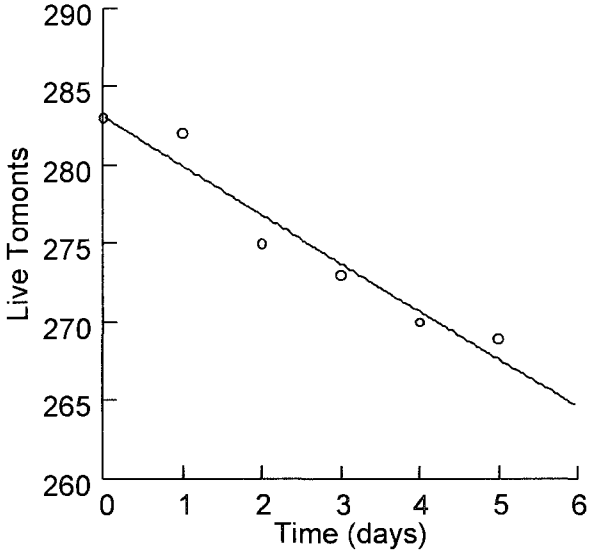

Figure 4.1. Number of tomonts remaining alive over time. Observed data (circles) and best fit exponential curve used to estimate the death rate of the tomonts (μ_{To}).

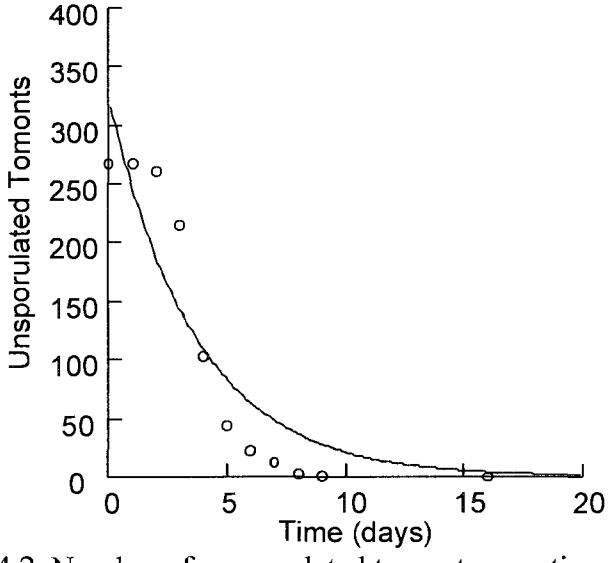

Figure 4.2. Number of unsporulated tomonts over time. Observed data and best fit exponential curve used to estimate the sporulation rate of the tomonts (γ).

Tomont Fecundity

Figure 4.3 shows the number of dinospores produced by each one of 268 tomonts of different lengths and the best fit regression line. Previously I estimated the mean

length of tomonts from spotted seatrout to be 71.55 µm (68.89–74.21, 95% CI) (Chapter II). From the regression equation, I estimated that tomonts of mean length produced 81.90 dinospores tomont^{-1} (66.10-97.31, 95% CI).

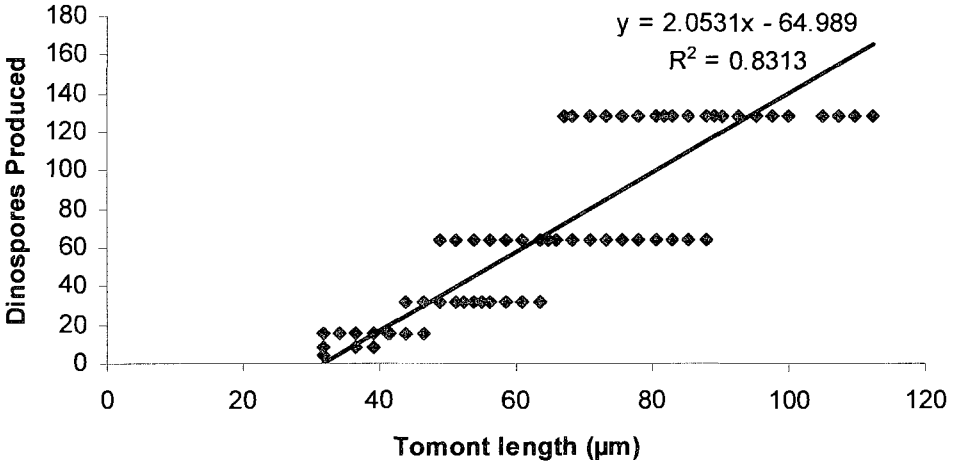

Figure 4.3. Number of dinospores produced by 283 tomonts of different lengths and regression line used to estimate the mean number of dinospores produced by tomonts of the mean length.

Figure 4.4 shows the time to sporulation for the various lengths of tomonts, and the best fit regression line. The mean time to sporulation for tomonts of the mean length (71.55 µm) was 4.63 d (3.51–5.75 d, 95% CI).

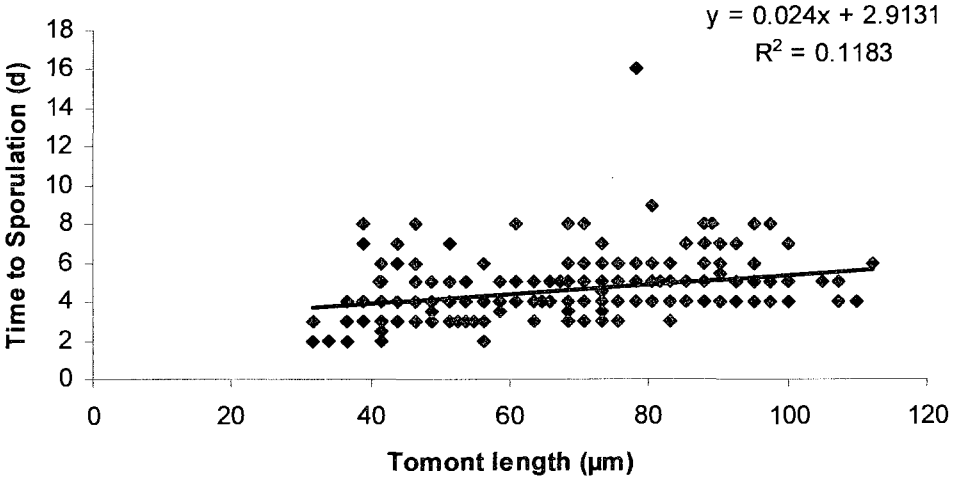

Figure 4.4. Time to sporulation of 283 tomonts of different lengths and regression line used to estimate the mean time to sporulation for tomonts of the mean length.

The resulting tomont fecundity (m_{To}) calculated as the ratio of the mean number of dinospores produced by a tomont of the mean length to the mean time to sporulation was 17.69 dinospores tomont^{-1} d^{-1} (16.92–18.83, 95% CI).

Dinospore Lifespan

Dinospore survival over time is shown in Fig. 4.5. The probability that a dinospore dies per day (μ_D) was estimated using the best fit exponential curve $y=a.e^{b.x}$, where $\mu_D=1-e^b$. The estimated value for μ_D was 0.38 dinospores d^{-1} (0.34-0.41, 95% CI). In a separate study it was estimated the probability that a dinospore infects spotted seatrout per day (β) to be 0.36 dinospores d^{-1} (0.33 – 0.38, 95% CI) (Chapter III).

Replacing the mean values and 95% CI limits of μ_D and β in equation 5.3 resulted in t_D=1.67 d (1.57 – 1.79 d, 95% CI).

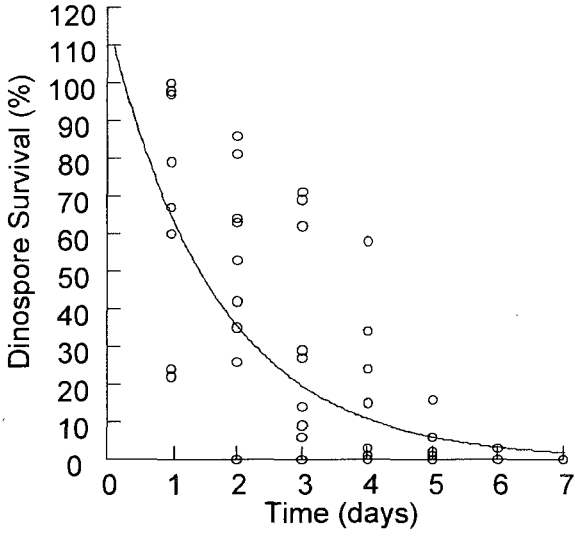

Figure 4.5. Survival of dinospores produced by 20 tomonts individually incubated. Observed data and best fit exponential curve used to estimate the death rate of the dinospores (μ_D).

Life Table for Amyloodinium ocellatum

Each stage's lifespan and daily fecundity were used to construct a life table (Table 4.1). The sum of the products of the lifespan and fecundity of each stage was 49.15 dinospores tomont^{-1} (37.23-75.47, 95% CI). This value represents the net reproductive rate per generation (R_0). By adding the lifespan for each of the stages in the life cycle of *A. ocellatum*, I also calculated the generation time (T), which had a mean value of 6.3 d (5.6-7.8 d, 95% CI).

Table 4.1. Life table for *A. ocellatum*

Stage	Lifespan (t_x) mean (95% CI)	Fecundity (m_x) mean (95% CI)	Product ($t_x.m_x$) mean (95% CI)
Trophont	$t_{Tr} = 1/ [1-(1-\mu_{Tr})(1-\phi)] = 1.89$ d (1.81-1.99)	$m_{Tr} = 0$	$t_{Tr}.m_{Tr} = 0$
Tomont	$t_{To} = 1/[1-(1-\mu_{To})(1-\gamma)] = 2.78$ d (2.20-4.01)	m_{To} = dinosp tomont^{-1} d^{-1} = 17.69 dinospores tomont^{-1} d^{-1} (16.92-18.83)	$t_{To}.m_{To} = 49.15$ dinospores tomont^{-1} (37.23-75.47)
Dinospore	$t_D = 1/[1-(1-\mu_D)(1-\beta)] = 1.67$ d (1.57-1.79)	$m_D = 0$	$t_D.m_D = 0$
			Σ = mean number of offspring per generation per individual (R_0) = 49.15 dinospores tomont^{-1} (37.23-75.47)

Discussion

The results from this study explain quantitatively how epidemics of *A. ocellatum* suddenly occur and quickly explode. The low mortality rates of the trophont and tomont stages (i.e., 0.004 and 0.010 per day, respectively) at 25°C and 33ppt indicate that most of those stages will successfully develop into the next life history stage. Although the temperature and salinity values used for the study were chosen based on Paperna's (1984a) optimal ranges for tomont survival and sporulation, variation in trophont and

tomont mortality would be expected as conditions deviate from the optima (Bower et al. 1987).

If left unchecked, the reproductive capacity of tomonts can result in devastating numbers of dinospores. Although tomonts have the potential to produce 256 dinospores, this occurs only rarely (Nigrelli 1936, Brown and Hovasse 1946, Paperna 1984a). The maximum number of dinospores produced by tomonts observed in my study was 128. Paperna (1984a) observed that the number of dinospores produced by tomonts was determined by the initial size of the tomont but that it was the temperature and salinity that determined whether tomonts divided to their maximum potential. He pointed out that tomonts yielded the greatest number of cell divisions at the lower limit of the optimum temperature range (18°C) and that only 5% of the largest tomonts he studied (91-100 μm in length) produced 256 dinospores. The temperature at which this study was performed (25°C) may explain why the large tomonts (> 91 μm) produced 128 dinospores but failed to produce 256 dinospores.

Another factor making *A. ocellatum* such a difficult problem in fish culture is that trophonts of various sizes released at the same time develop and sporulate at different rates, thus providing a continuous source of dinospores over a prolonged period of time. My results showed that most tomonts sporulated in 2 to 9 d; however, one tomont was observed to sporulate as late as day 16.

Other authors have speculated that once hosts are removed, all dinospores should emerge within 2-3 d of encapsulation at 23-27° C (Paperna 1984a) and die after another 7 to 9 d at 24-26°C (Bower 1987). Bower (1987) hypothesized that a fish holding/culture system should be free of the parasite about 2 weeks after the last infected fish has been removed. My study shows that the tomont lifespan (t_{To}) is 2.78 d (2.20-4.01, 95% CI)

with a single most extreme case of a tomont that sporulated after 16 d of trophont detachment. Dinospores, in contrast, lived a maximum of 8 d under the studied conditions. The sum of the lifespans (T) estimated in this study indicates that it takes 5.6-7.8 d (95% CI range) for *A. ocellatum* to complete its life cycle at 25°C and 33 ppt when a host is present. However, combining the most extreme case of the time it takes a tomont to sporulate and the maximum time for dinospore survival, I observed, it could take 24 d from trophont detachment until the death of the last dinospore. As a consequence, if a fish holding system can not be decontaminated and needs to be reused after an outbreak, it is thus recommended that fish not be reintroduced until at least 4 weeks after the removal of the last fish (assuming optimum conditions).

However, in aquaculture settings it is usually impractical to remove fish from a contaminated system. Therefore, aquaculturists must treat intact systems. Although there are some techniques that can physically remove dinospores from a culture system, copper sulfate is the most commonly used treatment. When trophonts are exposed to copper sulfate, they detach from the host, encapsulate and become tomonts. Tomonts are not affected by copper sulfate. It is only the dinospore stage which is sensitive to copper ions; therefore, copper must remain in the system at an adequate level until the last tomont sporulates (Dempster 1955, Lawler 1977, Paperna 1984b). Noga and Levy (2006) recommended copper sulfate at therapeutic concentrations (i.e., 0.12-0.15 ppm) for 10-14 d to control epidemics. My results suggest that 14 d is insufficient to control the infection. Rather, the standard *in situ* copper sulfate treatment at a concentration of at least 0.15 ppm is recommended for perhaps as long as 4 weeks to account for the most extreme cases of survival and reproduction such as the tomont that took 24 d to sporulate.

CHAPTER V

POPULATION MODEL FOR *AMYLOODINIUM OCELLATUM* INFECTING A SINGLE HOST SPECIES IN A CLOSED SYSTEM

Introduction

The application of mathematics to the study of infectious disease began over 200 years ago, but it was not until the beginning of the last century when Hamer and Ross formulated specific theories about the transmission of infectious diseases (measles and malaria, respectively) in simple but precise mathematical statements and investigated the properties of the resulting models (Anderson and May 1991). The mathematical models are developed by compartmentalizing components of a system and generating mathematical expressions for each one (Anderson and May 1979, May and Anderson 1979, Ebert 1999). Previous research with aquatic pathogens includes the estimation of the epidemiological parameters of white spot syndrome disease in *Litopenaeus vannamei* and *L. setiferus* (Soto and Lotz 2001) and the development of a model of white spot syndrome epidemics in *L. vannamei* (Lotz and Soto 2002). A similar study also was done for Taura syndrome virus and the necrotizing hepatopancreatitis bacterium infecting *L. vannamei* (Lotz et al. 2003 and Vincent et al. 2004, respectively). In all cases, the parameters were estimated experimentally under specified conditions and used in the mathematical models. These models described the changes in the disease stages of the hosts (e.g., susceptible, acutely infected, dead infected) rather than the number of viral or bacterial organisms. To model the change in numbers of a pathogen, one must consider the pathogen number as the variable and ignore the host disease state. The population dynamics of *A. ocellatum* can be modeled using the life history stages of the pathogen as compartments in the model. These "stage-structured" models are suitable for species that

have survival and reproductive rates (i.e., vital rates) dependent on the stage rather than the age or size of the organism (Ebert 1999). Each of the three life history stages (the trophont, the tomont and the dinospore) of *A. ocellatum* forms a separate compartment in the model (Fig. 5.1). The transitions among stages are given by the vital rate probabilities for each of the stages. These probabilities determine the changes in number of individuals in each stage over time and were estimated experimentally as shown in previous chapters.

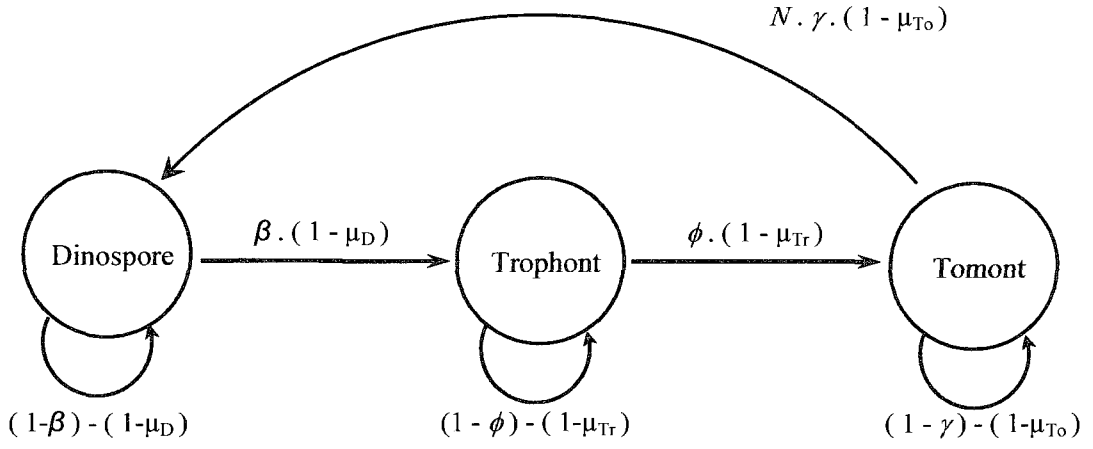

Figure 5.1. Life cycle graph for an *Amyloodinium ocellatum* population showing the transitions between and within stages and the reproduction rate; all arcs take one time period to complete. β is the probability that a dinospore infects a fish d^{-1}; μ_D is the probability that a dinospore dies d^{-1}; ϕ is the probability that a trophont detaches from a fish d^{-1}; μ_{Tr} is the probability that a trophont dies d^{-1}; γ is probability that a tomont sporulates d^{-1}; μ_{To} is probability that a tomont dies d^{-1}; and N is the mean number of dinospores produced per tomont.

The *A. ocellatum* life cycle graph (Fig. 5.1) with its compartments representing the life history stages and the arcs representing the transitions among stages can be converted into a matrix population model to make population projections under defined conditions (the conditions that were used for the experimental estimation of the probabilities associated with the model). A matrix population model can be analyzed for how sensitive the parasite's population growth rate is to perturbations in specific vital rates. Such analysis is called perturbation analysis. It includes sensitivity and elasticity

analyses. Sensitivity analysis estimates the impact of an absolute change in a vital rate on population growth, and elasticity analysis estimates the effect of a proportional change in the vital rate on population growth (Benton and Grant 1999, Ebert 1999). This approach provides insight into which parameters are under the most intense selection pressure and which parameters might be focused on for management. One example used by Benton and Grant (1999) to describe the type of conclusions arrived at after performing perturbation analysis was about the biocontrol of cattle tick, *Boophilus microplus*, an important disease vector in cattle. The elasticity analysis of a matrix model suggested that reducing the tick fecundity and off-host survival would aid control whereas neither factor was implicated in simulation models. Another example given by these authors was about the nodding thistle, *Carduus nutants*, an invasive weed in New Zealand. Elasticity analysis indicated that its "Achilles' heel" is seed and seedling mortality. Therefore, integrated management strategies involving adding weevils (to increase seed mortality) and increase grazing (to increase seedling mortality) were suggested to increase production.

This study presents a stage-structured matrix population model for *A. ocellatum*. I used the model to perform simulations of the population growth of *A. ocellatum* infecting two host species separately, spotted seatrout, *Cynoscion nebulosus,* and red snapper, *Lutjanus campechanus.* The vital rates estimated in previous studies (Chapters II-IV) are incorporated into the model. The effect of perturbation of each vital rate on *A. ocellatum* population growth was studied to identify the vital rates that have the greatest effect on the parasite's population growth.

Methods

The population model for *A. ocellatum* was analyzed using Mathcad 13 (Mathsoft 2005). A graphic representation of the triphasic life cycle of *A. ocellatum* is presented in Fig. 5.1. Each node represents one of the three life cycle stages. The arcs indicate the transitions among stages. The expressions and coefficients that appear on the arcs constitute the probabilities of transition from one stage to the other as well as the fecundities of each stage during one unit of time. The probability that a dinospore infects a fish per day (the infection rate) is represented by β, μ_D is the probability that a dinospore dies per day (the mortality rate of dinospores), ϕ is the probability that a trophont detaches from a fish per day (the detachment rate of trophonts), μ_{Tr} is the probability that a trophont dies per day (the mortality rate of trophonts), γ is the probability that a tomont sporulates per day (the sporulation rate of tomonts), μ_{To} is the probability that a tomont dies per day (the mortality rate of tomonts) and N is the mean number of dinospores produced per tomont.

The parameters (vital rates) for *A. ocellatum* infecting spotted seatrout were estimated experimentally at 25°C and 33 ppt as described in Chapters II-IV. To perform the perturbation analysis, the *A. ocellatum* life cycle graph (Fig. 5.1) was transformed into its equivalent matrix form (M). In the life cycle graph, transitions are from the trophont stage to the tomont stage, from the tomont stage to the dinospore stage, and from the dinospore stage back to the trophont stage. Also, there are transitions represented by self loops (the probabilities of remaining in that stage). In a matrix, transitions always are from the class represented by a column to the class represented by a row, so the life cycle graph (Fig. 5.1) was translated from a node to a node into a column to a row in the matrix.

$$M = \begin{bmatrix} (1-\beta).(1-\mu_D) & 0 & N.\gamma.(1-\mu_{Tr}) \\ \beta.(1-\mu_D) & (1-\phi).(1-\mu_{Tr}) & 0 \\ 0 & \phi.(1-\mu_{Tr}) & (1-\gamma).(1-\mu_{To}) \end{bmatrix} \quad (6.1)$$

The transition matrix M along with the estimates of the vital rates were used to calculate the population growth rate (maximum eigenvalue), the sensitivities (change in the population growth rate with change in a parameter), and elasticities (percent change in growth rate per percent change in a parameter) for each of the parameters involved in the *A. ocellatum* population model.

The *A. ocellatum* asymptotic rate of population growth or simply the population growth rate (λ) is given by the dominant eigenvalue of the transition matrix M. The stable stage-structure distribution function (i.e., the function describing the proportions of each stage when the population is growing exponentially at a constant rate, λ) and the reproductive value function (i.e., the relative contribution to future population growth an individual in a certain stage is expected to make) are given by the corresponding right and left eigenvectors, **w** and **v**. The sensitivity, s_{ij}, of λ to changes in the matrix element, a_{ij}, of M is given by the partial differential δ (Benton and Grant 1999). The symbol "<>" in the equation below represents the scalar product of the vectors.

$$s_{ij} = \frac{\delta.\lambda}{\delta.a_{ij}} = \frac{v_j.w_j}{< w, v >} \quad (6.2)$$

Although the sensitivities measure how λ changes with an absolute change in each a_{ij}, they can be scaled to test the effect of proportional changes of each a_{ij} on λ. These new variables known as elasticities (e_{ij}) represent the proportional contributions of each element in the transition matrix to λ (Benton and Grant 1999).

$$e_{ij} = \frac{a_{ij}}{\lambda}\frac{\delta.\lambda}{\delta.a_{ij}} = \frac{\delta.\log\lambda}{\delta.\log a_{ij}} \quad (6.3)$$

The effect of a 50% change in each vital rate on λ was determined in spotted seatrout and red snapper and the vital rate threshold values (point where λ=1) for the *A. ocellatum* population increase were estimated by performing iterations of the vital rate values for the transition matrix M. Mathcad 13 (Mathsoft 2005) was used for the calculations.

Table 5.1. Summary of the mean values (95% CI) of the vital rates estimates for *Amyloodinium ocellatum* infecting spotted seatrout and red snapper. Refer to Fig. 5.1 for the definition of symbols

Vital Rate	Vital rate mean estimates (95% CI) (Chapters II-IV)	
	Spotted Seatrout	Red Snapper
N	81.90 (66.10 – 97.31)	98.90 (82.16 – 115.65)
β	0.3552 (0.3279 – 3825)	0.3414 (0.3122 – 0.3707)
γ	0.3534 (0.2465 – 0.4457)	
μ_D	0.3787 (0.3443 – 0.4120)	
μ_{To}	0.0100 (0.0040 – 0.0159)	
μ_{Tr}	0.0037 (0.0035 – 0.0038)	0.0034 (0.0031 – 0.0036)
ϕ	0.5262 (0.5014 – 0.5498)	0.4811 (0.4435 – 0.5162)

Results

Table 5.1 contains the vital rate estimates that were used in the model (Chapters II-IV). The vital rate estimates for *A. ocellatum* infections in spotted seatrout and red snapper were entered in the transition matrix (M). The resulting transition matrices for the life cycle of *A. ocellatum* infecting spotted seatrout (M_{st}) and red snapper (M_{rs}) were

$$M_{st} = \begin{bmatrix} 0.4006 & 0 & 28.6540 \\ 0.2207 & 0.4720 & 0 \\ 0 & 0.5243 & 0.6401 \end{bmatrix} \quad M_{rs} = \begin{bmatrix} 0.4092 & 0 & 34.6017 \\ 0.2121 & 0.5171 & 0 \\ 0 & 0.4795 & 0.6401 \end{bmatrix} \quad (6.4, 6.5)$$

The *A. ocellatum* population growth for spotted seatrout (λ_{st}) was 1.9988 d^{-1}, whereas for red snapper (λ_{rs}) it was 2.0461 d^{-1}. The stable stage structure functions for the

A. ocellatum life cycle in spotted seatrout (w_{st}) and red snapper (w_{rs}) are shown below, with the top to bottom values in the matrices representing the proportional contributions of the dinospore, trophont and tomont stages, respectively

$$w_{st} = \begin{bmatrix} 1.0000 \\ 0.1445 \\ 0.0558 \end{bmatrix} \qquad w_{rs} = \begin{bmatrix} 1.0000 \\ 0.1387 \\ 0.0473 \end{bmatrix} \qquad (6.6, 6.7)$$

Likewise, the reproductive value functions for the *A. ocellatum* life cycle in spotted seatrout (v_{st}) and red snapper (v_{rs}) are as follows:

$$v_{st} = \begin{bmatrix} 1.0000 \\ 7.2419 \\ 21.0900 \end{bmatrix} \qquad v_{rs} = \begin{bmatrix} 1.0000 \\ 7.7173 \\ 24.6102 \end{bmatrix} \qquad (6.8, 6.9)$$

The following are the sensitivities for the *A. ocellatum* life cycle in spotted seatrout (S_{st}) and red snapper (S_{rs}):

$$S_{st} = \begin{bmatrix} 0.3103 & 0 & 0.0173 \\ 2.2469 & 0.3248 & 0 \\ 0 & 0.9458 & 0.3650 \end{bmatrix} \qquad S_{rs} = \begin{bmatrix} 0.3091 & 0 & 0.0146 \\ 2.3857 & 0.3310 & 0 \\ 0 & 1.0554 & 0.3599 \end{bmatrix} \qquad (6.10, 6.11)$$

The resulting elasticities for the *A. ocellatum* life cycle in spotted seatrout (E_{st}) and red snapper (E_{rs}) were:

$$E_{st} = \begin{bmatrix} 0.0622 & 0 & 0.2481 \\ 0.2481 & 0.0767 & 0 \\ 0 & 0.2481 & 0.1169 \end{bmatrix} \qquad E_{rs} = \begin{bmatrix} 0.0618 & 0 & 0.2473 \\ 0.2473 & 0.0836 & 0 \\ 0 & 0.2473 & 0.1126 \end{bmatrix} \qquad (6.12, 6.13)$$

The maximum elasticity values corresponded to the transitions from the dinospore to the trophont stage, from the trophont stage to the tomont stage and from the tomont stage back to the dinospore stage in both host species. The values were 0.2481 and 0.2473 in spotted seatrout and red snapper, respectively. This means that a 1% change in each matrix component ($\beta (1 - \mu_D)$, $\phi (1 - \mu_{Tr})$, or $N \gamma (1 - \mu_{To})$) will increase λ by

0.2481% and 0.2473%, in spotted seatrout and red snapper, respectively. The elasticity values for the three transitions are all equal (in their respective host species) because each transition within a loop (i.e., a circuit of arcs returning to the starting compartment and encountering no compartment more than once) has the same elasticity (Benton and Grant 1999).

Because each matrix component involved more than one vital rate, I analyzed the effect of a 50% change in each vital rate on the *A. ocellatum* population growth rate in spotted seatrout (λ_{st}) and red snapper (λ_{rs}) (Table 5.2). In both species, the greatest percent increase in λ for a 50% change in a vital rate was given by N, β, γ and μ_D (in decreasing order). In addition, equal changes in μ_{To} and μ_{Tr} had little effect (< 0.2%) on λ.

Table 5.2. Effect of a 50% change in a vital rate on the *A. ocellatum* population rate for infections is spotted seatrout and red snapper. Refer to Fig. 5.1 for the definition of symbols

Vital Rate	Percent increase in the *A. ocellatum* population growth rate for spotted seatrout (λ_{st}) and red snapper (λ_{rs}) due to a 50% change in a vital rate.	
	Spotted Seatrout	Red Snapper
N	15.34%	15.30%
β	13.61%	13.68%
γ	11.91%	12.00%
μ_D	10.32%	10.28%
μ_{To}	0.19%	0.18%
μ_{Tr}	0.06%	0.05%
ϕ	Not computed due to interference with N	

Further analysis of the vital rates showing the threshold values for *A. ocellatum* population increase (λ=1) in spotted seatrout and red snapper is presented in Table 5.3.

Table 5.3. Vital rate threshold values for *A. ocellatum* population increase (λ=1) and fold difference with their actual values (from Chapters II-IV) for infections in spotted seatrout and red snapper. Refer to Fig. 5.1 for the definition of symbols

	Vital rate threshold values for *A. ocellatum* population increase (λ=1)					
	Spotted Seatrout			Red Snapper		
Vital Rate	Threshold value	Actual value	Fold difference	Threshold value	Actual value	Fold difference
N	2.814	81.90	29.1	2.885	98.90	34.3
β	0.0078	0.3552	45.5	0.0064	0.3414	53.3
γ	0.0003	0.3534	1178.0	0.0002	0.3534	1767.0
μ_D	0.9652	0.3787	2.5	0.97	0.3787	2.6
μ_{To}	0.911	0.0100	91.1	0.9237	0.0100	92.4
μ_{Tr}	0.9371	0.0037	253.3	0.9416	0.0034	276.9
ϕ	Not computed due to interference with N					

When comparing these values with the actual vital rates estimated in Chapters II-IV (Table 5.1) I noticed that a 2.5 and a 2.6 fold increase in μ_D (for spotted seatrout and red snapper, respectively) brings this vital rate value below the threshold limit for an epidemic to occur. Instead, N would need to be reduced 29.1 and 34.3 times (for spotted seatrout and red snapper, respectively) to end up below its threshold value; β would need to be decreased 45.5 and 53.3 times its actual value and γ would be to be reduced 1,178.0 and 1,767.0 times its actual value to prevent an epidemic (for spotted seatrout and red snapper, respectively). Regarding μ_{To} and μ_{Tr}, their magnitudes would need to be managed to increase 91.1 and 92.4 times (for spotted seatrout and red snapper, respectively) and 253.3 and 276.9 times (for spotted seatrout and red snapper, respectively) for the former and latter vital rate, respectively. The effect of a change in the trophont detachment rate (ϕ) was not computed because changes in this vital rate would consequently affect the mean number of dinospores produced per tomont (N) (refer to discussion for more details).

Discussion

The *A. ocellatum* matrix population model elucidates the relative contribution of the various vital rates on the population growth rate (λ). The number of dinospores produced per tomont has the largest effect on λ, followed by the dinospore infection rate (β), the tomont sporulation rate (γ) and the dinospore mortality rate (μ_D). This indicates that control efforts should focus primarily at those vital rates to have a greater effect on the parasite's population growth. A focus on reducing N should result in the greatest reduction in λ; therefore, one might increase the flow of water through the culture system. The water flow causes friction over the fish tegument (where the trophonts attached). The greater the surface area of a trophont, the higher the probability that the trophont is removed by the water flow. If water flows faster, trophonts may detach at smaller sizes and produce fewer dinospores at tomont sporulation (Paperna 1984a). The infection rate (β) could be reduced through vaccination (Smith et al. 1993, Cobb 1997, Cobb et al. 1998) or selection of less susceptible culture species (Lawler 1977, 1980). Reduced light intensity has been observed to reduce dinospore activity and perhaps infectivity (Brock et al. 1999, Montgomery-Brock and Brock 2001). Similarly, γcould be reduced by acclimating the cultured fish to temperatures or salinities outside the optimum range for tomont reproduction (18-30°C and 10-60ppt, Paperna 1984a). Finally, μ_D could be increased by treating the water with chemicals such as copper sulfate which at proper doses would kill dinospores without affecting fish (Dempster 1955, Lawler 1977, Paperna 1984b, Noga and Levy 2006), by filtering the water to exclude dinospores (e.g., diatomaceous earth filtration) (Bower 1987), or by using nauplii of *Artemia salina* which have been found to feed on dinospores as a biological control agent (Oestmann et al. 1995), among others. Moreover, because a comparison of the vital rate threshold values

for *A. ocellatum* population increase (Table 5.3) with the actual values estimated in previous studies (Chapters II-IV) (Table 5.1) shows that increasing μ_D 2.5 times its actual value would produce a reduction in the parasite's population size (versus 27 to 1,178-fold increase for the other vital rates), this would be the vital rate to focus on from a management perspective. For the producer, the decision of which treatment to use to affect this parameter will be based on cost and effectiveness. Copper sulfate at 0.12-0.15 ppm for at least 4 weeks seems to be a cost-effective method for killing the infective dinospores and controlling an outbreak (Noga and Levy 2006, Chapter IV).

The model suggests that a reduction in trophont detachment rate (ϕ) should cause a decrease in λ. However, because the model does not account for the fact that if trophonts remain attached longer they will grow larger and produce more dinospores per tomont (and cause an increase in λ), ϕ was not considered in the analysis.

The lower λ in spotted seatrout (1.9988 d^{-1}) compared with red snapper (2.0461 d^{-1}), when computed using the actual vital rate estimates obtained from my studies (Chapters II-IV), may explain the incidental observations that suggest that infections in red snapper seem to reach lethal levels faster than in spotted seatrout. Another factor not considered in the proposed model that may have an effect on the parasite's population growth is the host density. The experiments performed to estimate the vital rates for infections in spotted seatrout and red snapper were carried out with individual fish. However, in aquaculture systems where fish are usually reared in numerous quantities, crowding stress could make hosts more susceptible to infections (Snieszko 1974, Yin et al. 1995). Thus, the model presented here represents an approximation to what may be found in culture conditions. Deviations from the estimated values are possible. Investigation of the effects of deviations from the estimated values and conditions could

provide a deeper understanding of the population dynamics of this important parasite and lead to improved strategies for its control.

CHAPTER VI

SUMMARY

The parasitic dinoflagellate *Amyloodinium ocellatum* is considered one of the most devastating parasites affecting warm water marine bony fishes in captivity. Although its life cycle, which consists of three life history stages - the trophont, the tomont, and the dinospore - has been studied extensively, there is a lack of information regarding the vital rates regulating transitions among the stages. This information can be useful in modeling the dynamics of the life cycle of *A. ocellatum*, which can elucidate the course of an outbreak under specified conditions. Likewise, despite the fact that host susceptibility to *A. ocellatum* varies among species, there is no study estimating and comparing the factors that ultimately determine the susceptibility of different host species.

The specific objectives of this dissertation were to: 1) develop a dynamic model of the life cycle of *A. ocellatum* infecting spotted seatrout in a closed system, 2) determine the probability of death of the trophont, tomont and dinospore stages on a per day basis, 3) determine the probability that a tomont sporulates per day, the time to sporulation and the number of dinospores produced by tomonts of different sizes, 4) determine the probability that a trophont detaches from its host per day and the size achieved by trophonts at detachment, 5) determine the dinospore lethal dose and load for spotted seatrout, 6) determine the probability that a dinospore infects a spotted seatrout per day, and 7) perform a comparative study with red snapper.

Amyloodinium ocellatum was maintained *in vivo* by periodically adding susceptible fish, typically spotted seatrout, to a tank containing the infection. Trophonts collected from juvenile spotted seatrout were used to perform the *in vitro* experiments to

determine the fecundity and survival of each of the *A. ocellatum* life history stages. Juvenile spotted seatrout and red snapper were challenged with different doses of dinospores to estimate and compare the detachment rate and size of the trophont stage, the dinospore infection rate and the trophont lethal load. All these data were used to develop a stage-structured deterministic model to study the population growth of *A. ocellatum* in spotted seatrout and red snapper. All experiments were performed at 25°C and 33 ppt, which are within the optimal temperature and salinity ranges for *A. ocellatum* development and reproduction.

Although the trophont detachment rates were not significantly different between host species, the trophont detachment patterns were different. Only 0.03±0.01 and 0.02±0.00 (mean±SE) of the total trophonts collected during the 6-d experiment detached from spotted seatrout and red snapper on day 1, respectively (no significant differences between mean values). On day 2, the proportion of trophonts that detached from spotted seatrout (0.46±0.02) was significantly larger than the proportion that detached from red (0.24±0.03) snapper ($p < 0.05$). However, on days 3 and 4, the proportion of trophonts that detached from spotted seatrout was significantly smaller than the proportion that detached from red snapper (0.35±0.02 and 0.50±0.02 for spotted seatrout and red snapper, respectively, on day 3; 0.05±0.01 and 0.13±0.02 for spotted seatrout and red snapper, respectively, on day 4) ($p<0.05$). On days 5 and 6, only a small proportion of trophonts detached (0.03±0.00 and 0.03±0.00 for spotted seatrout and red snapper, respectively, on day 5; 0.09±0.01 and 0.08±0.02 for spotted seatrout and red snapper, respectively, on day 6). Trophont detachment peaked on day 2 for spotted seatrout (0.46±0.02), and on day 3 for red snapper (0.50±0.02). Survival analysis detected significant differences in the trophont detachment cumulative distributions between host

species ($p<0.05$). The trophont mean time to detachment was 3.45 d (3.44-3.46) in spotted seatrout and 3.53 d (3.52–3.54) in red snapper [mean (95% CI)]. In addition, the mean length of the trophonts detached from red snapper [80 μm (77-83)] was significantly larger than the mean size of the trophonts detached from spotted seatrout [72 μm (69-74)]. This translates into a larger number of dinospores produced by tomonts at sporulation on red snapper [99 dinospores tomont^{-1} (82-116)] than on spotted seatrout [82 dinospores tomont^{-1} (66-97)].

There were no significant differences in dinospore infection rates and dinospore lethal doses between spotted seatrout and red snapper. Mean 24-h dinospore infection rates were 0.36 dinospores d^{-1} (0.33-0.38) for spotted seatrout and 0.34 dinospores d^{-1} (0.31-0.37) for red snapper. Mean 48-h median dinospore lethal doses were 237,243 dinospore fish^{-1} (195,980-350,306) for spotted seatrout and 141,010 dinospore fish^{-1} (85,892-210,230) for red snapper. However, spotted seatrout could withstand higher trophont loads than red snapper. Mean trophont lethal loads were 178,067 trophonts fish^{-1} (75,026-281,107) for spotted seatrout and 123,160 trophonts fish^{-1} (2,439-243,880) for red snapper . Thus, spotted seatrout can be considered more tolerant to infections.

The trophont mortality rates were 0.0037 trophonts d^{-1} (0.0035-0.0038) in spotted seatrout and 0.0034 trophonts d^{-1} (0.0031-0.0036) in red snapper (no significant differences between mean values). The mortality rates for the off-host stages were 0.010 tomonts d^{-1} (0.004-0.016) and 0.38 dinospores d^{-1} (0.34-0.41). The lifespan of the trophont stage was 1.89 d (1.81-1.99) for spotted seatrout and 2.07 d (1.93-2.25) for red snapper. The lifespan of the tomont stage (non-host dependent) was 2.78 d (2.20-4.01). The lifespan of the dinospore stage was 1.67 d (1.57-1.79) in spotted seatrout and 1.69 d (1.59-1.82) in red snapper (no significant differences between species).

The fecundity of the tomonts, the only reproductive stage, was 17.69 dinospores tomont^{-1} d^{-1} (16.92-18.83) in spotted seatrout and 20.48 dinospores tomont^{-1} d^{-1} (20.11-23.41) in red snapper, reflecting the bigger size of trophonts at detachment in red snapper. The net reproductive rate per *A. ocellatum* generation was 49.15 dinospores tomont^{-1} (37.23-75.47) with a generation time of 6.3 d (5.6-7.8) in infections on spotted seatrout, and 56.90 dinospores tomont^{-1} (44.25-93.81) with a generation time of 6.4 d (5.6-7.8) in infections on red snapper. Fifty percent of the dinospores live 3.5 d under host-deprived conditions and none lives beyond day 8.

Finally, the *A. ocellatum* population model helps understand the effect of the different vital rates in the life cycle of this parasite and their relative effects on its population growth (λ). The mean number of dinospores produced per tomont (N) had the largest effect on λ, followed by the dinospore infection rate (β), the tomont sporulation rate (γ) and the dinospore mortality rate (μ_D). This would indicate that efforts to control the parasite's population growth should be focused primarily at affecting these vital rates. In particular, an increase in μ_D, which is only 2.5 times smaller than its actual value (compared to the other vital rates which have threshold values for ceasing the parasite's population growth that are from 29 to 1,767 times higher than their actual values), would have a profound effect on the parasite's population growth.. To increase μ_D (i.e., kill the dinospores), the infected system can be treated with chemicals such as copper sulfate that at proper doses kill dinospores without affecting fish, the water can be filtered to exclude dinospores (e.g., diatomaceous earth filtration), or nauplii of *Artemia salina*, which have been found to feed on dinospores, can be used as a biological control agent,. Copper sulfate at 0.12-0.15 ppm, however, seems to be a proven, cost-effective method for killing the infective dinospores and controlling an outbreak.

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