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LOCAL ADAPTATION AND COSTS OF PARASITISM FOR WHITE SANDS PUPFISH (CYPRINODON TULAROSA) BY GYRODACTYLUS TULAROSAE

(THESIS)

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LOCAL ADAPTATION AND COSTS OF PARASITISM FOR WHITE SANDS PUPFISH (*CYPRINODON TULAROSA*) BY *GYRODACTYLUS TULAROSAE*

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Title

Local Adaptation and Costs of Parasitism for White Sands Pupfish (*Cyprinodon tularosa*) by *Gyrodactylus tularosae*

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

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Signature

ABSTRACT

Vinje, Jason L., M.S., Department of Biological Sciences, College of Science and Mathematics, North Dakota State University, May 2007. Local Adaptation and Costs of Parasitism for White Sands Pupfish (*Cyprinodon tularosa*) by *Gyrodactylus tularosae*. Major Professor: Dr. Craig A. Stockwell.

Assessing local adaptation and costs of parasitism is a vital step in the management of threatened and endangered species, especially where management protocols call for translocations. In this study, local adaptation and costs of parasitism were assessed for White Sands pupfish (*Cyprinodon tularosa*) parasitized by the monogenean ectoparasite *Gyrodactylus tularosae*.

Field surveys showed gyrodactylids to co-occur with both native (Salt Creek and Malpais Spring) and introduced populations (Lost River and Mound Spring) of White Sands pupfish. Varying levels of *G. tularosa*e prevalence and intensity were found in the Salt Creek, Lost River, and Mound Spring pupfish populations. Gyrodactylids also co-occurred with pupfish at Malpais Spring, but whether this population is unique is not known.

To assess parasite local adaptation, a laboratory experiment was conducted using *G. tularosae* to infect Salt Creek and Malpais Spring strains of White Sands pupfish. There was no significant difference in parasite prevalence and intensity between the two strains of pupfish. Infections on all fish followed a pattern of rapid parasite increase, followed by a rapid decrease. Similarly, costs associated with *G. tularosae* infection were evaluated in the laboratory. *G. tularosae* from Salt Creek fish were used to infect fish from Lost River. There was no significant difference in survival, growth (standard length and mass gained), or fat content between treatment (infected) and control (uninfected) groups.

Thus, no evidence was found for local adaptation or costs of parasitism associated

with *G. tularosae* infection of White Sands pupfish; however, field data show that *G. tularosae* is able to occupy extreme environments varying considerably in salinity, temperature, and flow.

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CHAPTER 1. INTRODUCTION

Host-parasite Interactions

Host-parasite interactions and their influence on population dynamics have important implications for conservation biology (Scott 1988; Daszak et al. 2000). Specifically, parasites may play crucial roles in regulating host populations (Anderson and May 1978; Shaw and Dobson 1995), mediating competition (Price 1980; Clayton and Moore 1997; Prenter et al. 2004), altering host behavior (Moore 1984; Lafferty and Morris 1996; Thomas et al. 2005), and influencing host physical condition (Jokela et al. 1999; Ranzani-Paiva and Silva-Souza 2004; Bradley and Altizer 2005).

By their ecological definition, parasites decrease the survival or reproduction of their hosts (Anderson and May 1978) and are, therefore, presumed to be costly; however, costs are often assumed but often not directly assessed (Collyer 2000; Collyer & Stockwell 2004). Additionally, parasites may have subtle, yet costly, effects on host competitive ability (Bedhomme et al. 2005). Assessing the costs associated with parasitism is especially relevant today, where management practices sometimes call for the translocation of threatened species (Corn and Nettles 2001; Stockwell and Leberg 2002). Specifically, translocations may result in the spread of exotic parasites, leading to novel host-parasite associations (Scott 1988; Leberg and Vrijenhoek 1994).

The concern with novel host-parasite associations is the potential for increased virulence of an introduced parasite on its new host (Esch and Fernandez 1993). An increase in virulence is thought to be the result of parasites being maladapted to their hosts (Toft and Karter 1990; Ewald 1995); however, Sasal et al. (2000) found no evidence of increased virulence of the digenean *Labratrema minimus* infecting naïve common gobies

(*Pomatoschistus microps*). Similarly, Ebert and Hamilton (1996) cited many studies in which novel host-parasite associations resulted in decreased transmissibility and virulence.

Virulence ultimately depends on the nature of the interaction between host and parasite (Schjørring and Koella 2003). These interactions lead to the parasite being locally adapted (Ebert 1994; Lively and Dybdahl 2000; Osnas and Lively 2004), not locally adapted (Strauss 1997; Sasal et al. 2000; Uller and Olsson 2004), or locally maladapted (Kaltz et al. 1999; Oppliger et al. 1999).

The extent of local adaptation is influenced by spatial scale, genetic aspects of resistance and pathogenicity, environmental stochasticity, and life histories of both host and pathogen (Thrall et al. 2002). It is generally believed that the ability of parasites to become locally adapted is a result of their numbers, short generation times, and higher rates of mutation (Cory and Myers 2004). Gandon and Michalakis (2002) found that higher mutation and migration rates led to local adaptation, but shorter generation times did not always lead to local adaptation (when genetic variability is limiting). Similarly, Lively (1999) found that parasites can have slower generation times than their hosts and still be locally adapted because parasites track host genotypes independently of generation time.

Overall, parasite local adaptation is increased with shorter generation time when migration and mutation are not limiting; however, when migration and mutation are limiting, shorter generation time results in decreased genetic variance, and thus local adaptation is not likely to occur (Gandon and Michalakis 2002). In contrast to parasite migration rates, Oppliger et al. (1999) speculated that hosts having higher migration rates than parasites would lead to parasite local maladaptation.

Situations involving local adaptation are of particular interest for species that have

been widely translocated; such is the case for many cyprinodontids. The White Sands pupfish (*Cyprinodon tularosa*) and its monogenean ectoparasite *Gyrodactylus tularosae* provide an excellent system for assessing parasite-host interactions. Specifically, its length of isolation, genetically distinct populations, and threatened status make White Sands pupfish an ideal host to study local adaptation and the potential costs imposed by *G. tularosae*. Further, Moen and Stockwell (2006) recently reported that *G. tularosae* was locally adapted to White Sands pupfish, as *G. tularosae* preferred White Sands pupfish over the sheepshead minnow (*Cyprinodon variegatus*), a closely related congener.

Background on Gyrodactylids

Worms of the genus *Gyrodactylus* are viviparous monogenean ectoparasites found on teleost fish (Cable et al. 2002a), aquatic tetrapods (Harris and Tinsley 1987), and cephalopod mollusks (Llewellyn 1984). Gyrodactylids lack a specific transmission stage and thus are not dependent upon intermediate hosts (Cable et al. 2002a). Each worm contains several generations of embryos developing inside one another (Cable and Harris 2002) (Figure 1.1). The young are born fully grown, attaching directly to the host alongside their parents where they feed on host mucus and epithelial cells (Cable et al. 2002b). Transmission of worms occurs via contact with living or dead hosts. Worms may also attach to the substratum or drift in the water column until they come in contact with a host (Bakke et al.1992b).

Gyrodactylids are generally very host specific, but due to their life cycle and colonization ability, if a worm does switch hosts, it has a high probability for

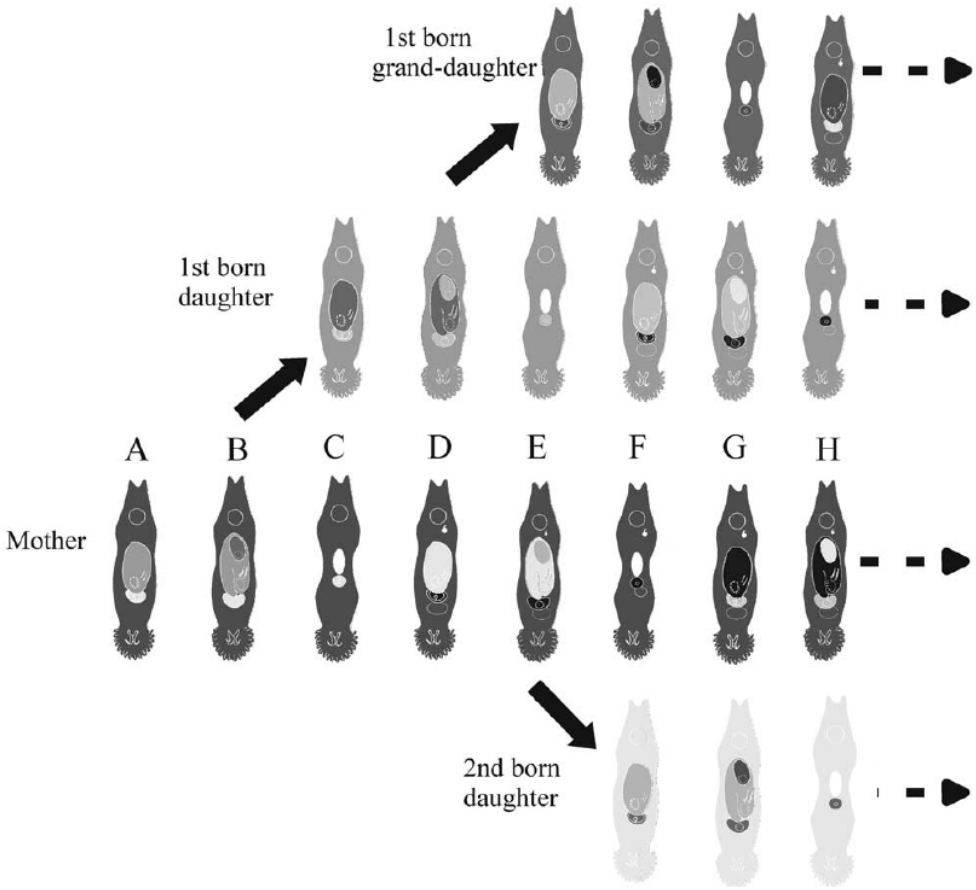

Figure 1.1. *Gyrodactylus* sp. reproductive stages. Letters A-H represent the life cycle stages of a newborn fluke. The first-born daughter, at stage B, develops asexually while its mother is an embryo. The second-born daughter, at stage E, develops parthenogenetically from an oocyte. All subsequent offspring develop either parthenogenetically or are sexually reproduced after the reproductive system is fully mature (Cable and Harris 2002). Figure redrawn with permission from Cable and Harris (2002).

speciation because of restricted gene flow (Zięta ra and Lumme 2002).

Gyrodactylids are of special interest because they have been reported to be pathogenic (Bakke et al. 1992b; Leberg and Vrijenhoek 1994; Soleng et al. 1998; Hedrick et al. 2001). For instance, Atlantic salmon are highly susceptible to infection by *Gyrodactylus salaris*, generally resulting in high levels of mortality (Bakke et al. 1992b; Soleng et al. 1998). Although some species of *Gyrodactylus* can be pathogenic, many studies showed no apparent costs (MacKenzie 1970; Cone and Odense 1984; Bakke et al. 1991; Bakke et al. 1992a; Jansen and Bakke 1995; Bakke et al. 1996; Buchmann and Uldal 1997; Soleng and Bakke 2001; Sterud et al. 2002).

It is perhaps a combination of their ubiquity and potential negative impacts to fish populations that have led to numerous studies being conducted on various species of *Gyrodactylus* and their associated fish hosts (Table 1.1). The majority of the papers included in Table 1.1 address host specificity and infection dynamics, followed by a fairly equal number focused on systematics, host response, fluke biology, and fluke physiology. Although they are grouped into categories, many studies included in Table 1.1 address multiple issues.

While fish vary in their response to *Gyrodactylus* infections (Bakke et al. 1992b), an example of the possible outcome of infestation by a species of *Gyrodactylus* is illustrated by Cable et al. (2002a) in their description of *Gyrodactylus turnbulli* and its pathogenicity toward guppies. Infected fish are first characterized by their erratic swimming behavior and flattened dorsal fin. In the latter stages of the infection, the host fins become contracted, the fin rays fuse together, and the fish dies.

Table 1.1. Literature review of *Gyrodactylus* species and hosts.

Subject	Host(s)	Focus	Source
TAXONOMY/SYSTEMATICS			
G. unicopula	Plaice (*Pleuronectes platessa*)	Fluke description, with notes on fluke ecology	MacKenzie (1970)
G. salmonis, G. nerkae n. sp., *G. colemanensis, G. avalonia,* and *G. brevis.*	Cutthroat trout (*Salmo clarki*), Atlantic salmon (*Salmo salar*), golden trout (*Salmo aquabonita*), rainbow trout (*Onchorhyncus mykiss*), brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*), coho salmon (*Oncorhyncus kisutch*), sockeye salmon (*Oncorhyncus nerka*), and splake (*S. fontinalis x S. namaycush*)	Fluke descriptions	Cone et al. (1983)
G. asiaticus, G. birmani, G. brachymystacis, G. derjavini, G. lavareti, G. lenoki, G. magnus, G. salaris, G. taimeni, G. thymalli, and *G. truttae*	Salmonids and Thymallids	Fluke descriptions, with notes on specificity and site attachment	Ergens (1983)
G. fryi	Musky (*Esox masquinongy*)	Fluke description	Cone and Dechtiar (1984)
G. longidactylus	Lozano's goby (*Pomatoschistus lozanoi*)	Fluke description	Geets et al. (1998)
G. salaris and *G. thymalli*	Atlantic salmon (*Salmo salar*) and grayling (*Thymallus thymallus*)	Differentiating fluke species based on specificity, pathogenicity and genetics	Sterud et al. (2002)
24 *Gyrodactylus* species	Cyprinids, Salmonids, Percids, Esocids, Gasterosteids, Gobitids	Speciation and phylogeny	Ziętra and Lumme (2002)

Table 1.1. (Continued)

Subject	Host(s)	Focus	Source
G. quadratidigitus	Leopard-spotted goby (*Thorogobius ephippiatus*)	Fluke description	Longshaw et al. (2003)
HOST RESPONSE/COSTS			
G. adspersi, G. avalonia, G. bullatarudis, G. spp., and *G. salmonis*	Cunner (*Tautogolabrus adspersus*), three-spined stickleback (*Gasterosteus aculeatus*), guppy (*Poecilia reticulata*), goldfish (*Carassius auratus*), and rainbow trout (*Oncorhyncus mykiss*)	Pathology (host tissue damage)	Cone and Odense (1984)
G. derjavini	Rainbow trout (*Oncorhyncus mykiss*)	Host mucous cell density and fluke population increase on testosterone treated fish	Buchmann (1997)
G. derjavini	Rainbow trout (*Oncorhyncus mykiss*)	Host response and mucous cell density	Lindenstrøm and Buchmann (2000)
G. turnbulli	Gila topminnow (*Poeciliopsis o. occidentalis*)	Parasite induced mortality and specificity	Hedrick et al. (2001)
G. derjavini	Rainbow trout (*Oncorhyncus mykiss*)	Parasite influence on cortisol production	Stoltze and Buchmann (2001)
G. derjavini (in association with *Flavobacterium psychrophilum*)	Rainbow trout (*Oncorhyncus mykiss*)	Host mortality and infection levels	Busch et al. (2003)
G. turnbulli	Guppy (*Poecilia reticulata*)	Host feeding response and specificity	Van Oosterhout et al. (2003)

Table 1.1. (Continued)

Subject	Host(s)	Focus	Source
G. salaris and *G. derjavini*	Rainbow trout (*Oncorhyncus mykiss*), Atlantic salmon (*Salmo salar*) and carp (*Cyprinus carpio*)	Host immune reponse and specificity	Buchmann et al. (2004)
SPECIFICITY/INFECTION AND TRANSMISSION DYNAMICS			
G. bullatarudis	Guppy (*Poecilia reticulata*)	Challenge infections	Scott and Robinson (1984)
G. stellatus	English sole (*Parophrys vetulus*)	Prevalence and intensity in wild and population growth in lab	Kamiso and Olson (1986)
G. colemanensis and *G. salmonis*	Rainbow trout (*Oncorhyncus mykiss*), brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*)	Specificity, site attachment, seasonal incidence, and pathology	Cone and Cusack (1988)
G. turnbulli	Guppy (*Poecilia reticulata*)	Attachment site specificity	Harris (1988)
G. colemanensis	Rainbow trout (*Oncorhyncus mykiss*)	Parasite attachment location and dispersal	Cone and Cusack (1989)
G. salaris	Rainbow trout (*Oncorhyncus mykiss*)	Specificity (fluke survival and infection dynamics)	Bakke et al. (1991)
G. salaris	Brook trout (*Salvelinus fontinalis*)	Specificity	Bakke et al. (1992a)
Various *Gyrodactylid* species	Various	Specificity and fluke dispersal	Bakke et al. (1992b)
G. salaris	Atlantic salmon (*Salmo salar*)	Fluke seasonal incidence	Mo (1992)

Table 1.1. (Continued)

Subject	Host(s)	Focus	Source
G. salaris	Brown trout (*Salmo trutta*)	Specificity	Jansen and Bakke (1995)
G. salaris	Arctic charr (*Salvelinus alpinus*)	Specificity	Bakke et al. (1996)
G. derjavini	Rainbow trout (*Oncorhyncus mykiss*), brown trout (*Salmo trutta*), and Atlantic salmon (*Salmo salar*)	Specificity and parasite site selection	Buchmann and Uldal (1997)
G. derjavini	Rainbow trout (*Oncorhynchus mykiss*)	Fluke microhabitat selection and host mucous cell density	Buchmann and Bresciani (1998)
G. salaris	Atlantic salmon (*Salmo salar*)	Fluke reproductive success	Cable et al. (2000)
G. salaris	Arctic char (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*), and brown trout (*Salmo trutta*)	Host susceptibility following immunosuppression	Harris et al. (2000)
G. salaris	Grayling (*Thymallus thymallus*)	Specificity	Soleng and Bakke (2001)
Various *Gyrodactylus* species	Various	Host specificty dynamics	Bakke et al. (2002)
G. turnbulli	Guppy (*Poecilia reticulata*)	Transmission and parasite behavior	Cable et al. (2002a)
G. perforatus	Arrow goby (*Clevelandia ios*)	Fluke prevalence and intensity in wild populations	Walberg et al. (2003)

Table 1.1. (Continued)

Subject	Host(s)	Focus	Source
FUNCTIONAL MORPHOLOGY AND PHYSIOLOGY			
G. elegans	White crappie (*Pomoxis annularis*), stickleback (*Gasterosteus williamsoni microcephalus*), and large mouth bass (*Micropterus salmoides*)	Control/treatment and transmission of flukes	Guberlet et al. (1927)
G. elegans	Golden shiner (*Notemigonus crysoleucas*)	Control/treatment of flukes	Lewis and Lewis (1963)
G. spp.	*Clarias batrachus*	Control/treatment of flukes	Amatyakul (1972)
G. alexanderi	Freshwater sticklebacks (*Gasterosteus aculeatus leiurus*) and marine sticklebacks (*Gasterosteus a. trachurus* and *G. a. semi-armatus*)	Fluke reproduction, mortality and effect on host	Lester and Adams (1974)
G. salaris	Atlantic salmon (*Salmo salar*)	Fluke salinity tolerance	Soleng and Bakke (1997)
G. salaris	Atlantic salmon (*Salmo salar*)	Killing of flukes by host immune response	Harris et al. (1998)
G. salaris	Atlantic salmon (*Salmo salar*)	Salinity and parasite dispersal	Soleng et al. (1998)
Various *Gyrodactylus* species	Various	Fluke developmental biology	Cable and Harris (2002)
G. gasterostei	Three-spined stickleback (*Gasterosteus aculeatus*)	Fluke survival, feeding and embryo development	Cable et al. (2002b)
G. salaris (variant)	Rainbow trout (*Oncorhyncus mykiss*) and Atlantic salmon (*Salmo salar*)	Fluke infection biology, morphology and genetics	Lindenstrøm et al. (2003)

Table 1.1. (Continued)

Subject	Host(s)	Focus	Source
G. arcuatus, G. derjavini, G. gasterostei, G. salaris, and *G. truttae*	Not available	Fluke functional morphology	Shinn et al. (2003)
G. rysavyi	Nile catfish (*Clarias gariepinus*)	Fluke swimming behavior	El-Naggar et al. (2004)
OTHER			
G. anguillae and *G. nipponensis*	Australian eels (*Anguilla reinhardtii* and *A. australis*), American eel (*A. rostrata*), European eel (*A. anguilla*), and Asian eel (*A. japonica*)	Parasite global distribution and genetic variation	Hayward et al. (2001)
Various *Gyrodactylus* species	Various	Parasite-host interactions	Buchmann and Lindenstrøm (2002)
Various *Gyrodactylus* species	Various	*Gyrodactylus* species and principal hosts	Harris et al. (2004)

Background on White Sands Pupfish

The White Sands pupfish is endemic to the Tularosa Basin of New Mexico and is listed as threatened in the state of New Mexico. White Sands pupfish occur at Malpais Spring, Mound Spring, and Salt Creek, all located on the White Sands Missile Range, and Lost River, located on Holloman Air Force Base. The populations at Salt Creek and Malpais Spring are native, while the populations at Mound Spring and Lost River were introduced from Salt Creek between 1967 and 1973 (Stockwell et al. 1998; Pittenger and Springer 1999) (Figure 1.2). Stockwell et al. (1998) reported that the native Salt Creek and Malpais Spring populations have diverged at both microsatellite and allozyme markers. This degree of divergence rivals divergence between other recognized species of pupfish. This led Stockwell et al. (1998) to recommend that the Malpais Spring and Salt Creek populations be recognized as evolutionary significant units (ESUs) of White Sands pupfish. This designation effectively elevates the conservation status of each population.

White Sands pupfish are host to a number of parasites. Parasites are a concern because recent work has shown that both white grubs and heterophyid parasites are costly for White Sands pupfish in terms of various life history characteristics and morphology (Harstad 2003; Collyer and Stockwell 2004; Rogowski and Stockwell 2006); however, parasitism varies among habitats due to environmental variation in salinity (Rogowski and Stockwell 2006). Differences in salinity limit the distribution of snails and complex life cycle parasites that infect White Sands pupfish (Rogowski and Stockwell 2006). Physid snails and associated white grub parasites occur at Malpais Spring and Mound Spring, where salinity levels are approximately 3.5 ppt (Collyer and Stockwell 2004). A recently discovered springsnail, *Juturnia tularosae*, and its associated heterophyid parasite occur

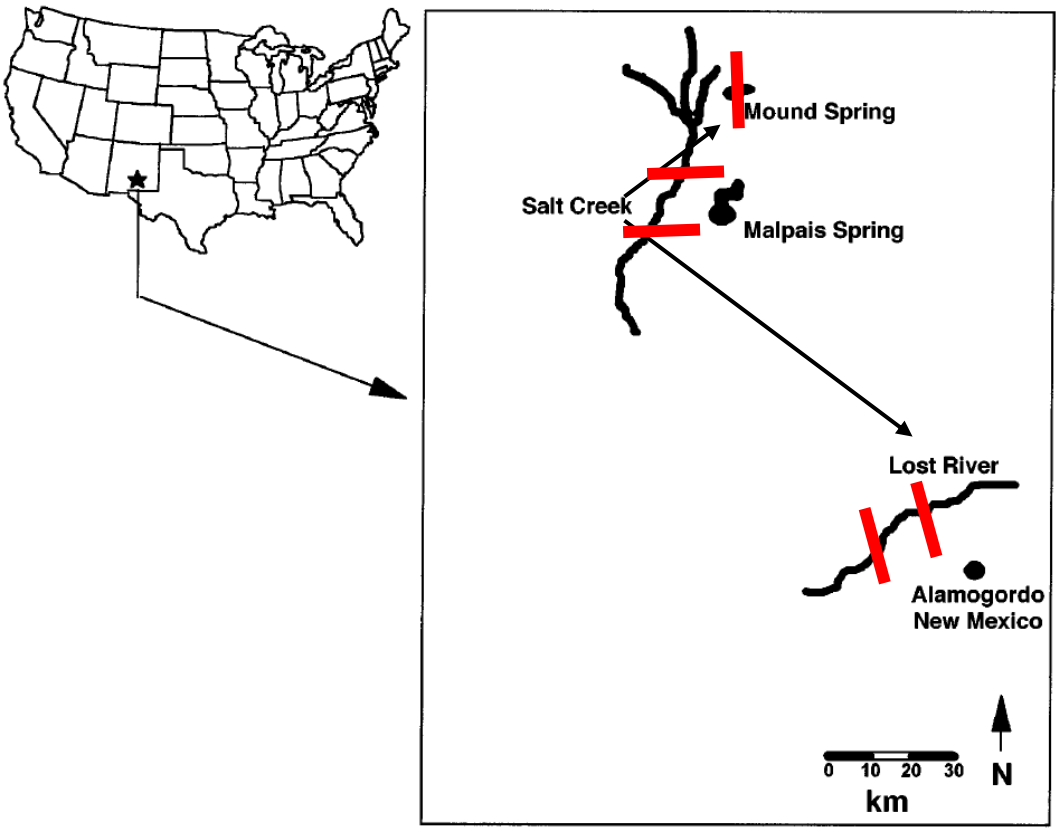

Figure 1.2. White Sands pupfish distribution within the Tularosa Basin, New Mexico. Mound Spring and Lost River populations were introduced from the native Salt Creek population (from Stockwell et al. 1998). Solid bars represent barriers to fish migration.

only at Salt Creek (Hershler et al. 2002; Rogowski and Stockwell 2006), where salinity can reach levels greater than 88 ppt. At this habitat, the springsnail is limited in distribution by salinity (Rogowski and Stockwell 2006). No snails and associated complex life cycle parasites occur at Lost River.

In addition to complex life cycle parasites, direct life cycle, gyrodactylid monogenean parasites occur at Salt Creek, Malpais Spring and Mound Spring, but until this current work, appeared to be absent from Lost River. *Gyrodactylus tularosae* was recently described by Kritsky and Stockwell (2005) based on worms from Salt Creek pupfish, but whether the Malpais Spring population of gyrodactylids is distinct has not been evaluated. Within this thesis, *G. tularosae* will refer to cases that involve worms from any of the Salt Creek ESU populations (Salt Creek, Lost River, and Mound Spring). The term gyrodactylid will also be used in a more generic context and for cases where un-diagnosed gyrodactylids from Malpais Spring are discussed.

Little is known about the spatial distribution of gyrodactylids within White Sands pupfish habitats. This is of interest because pupfish habitats vary considerably in salinity. Further, it is not known if these parasites have become locally adapted in relation to their hosts. Earlier work showed *G. tularosae* to prefer White Sands pupfish (Salt Creek ESU) over sheepshead minnow (Moen and Stockwell 2006). These two pupfish species diverged approximately 1.6-1.9 million years ago (Echelle et al. 2005); however, it is not known if *G. tularosae* is locally adapted to its specific strain (Salt Creek and Malpais Spring) of pupfish. This could have important management implications in terms of deciding where to establish refuge populations. Further, the costs of these parasites to White Sands pupfish have not been evaluated.

White Sands Pupfish Habitat Descriptions

The four pupfish habitats can be characterized as streams (Salt Creek and Lost River) or brackish springs (Mound Spring and Malpais Spring). Each location can be divided into sections based on barriers to fish movement (Rogowski 2004) (Figure 1.2). Additionally, salinity and temperature values within and among these habitats vary considerably (Stockwell and Mulvey 1998; Rogowski 2004).

Salt Creek can be divided into upper, middle, and lower sections. A waterfall separates the upper and middle sections, while a system of culverts separates the middle and lower sections; however, during flood events, stream flow is high enough to allow fish migration from the lower to the middle section (personal observation). Salinity and temperature levels in Salt Creek increase from the upper area to the lower area and are subject to significant variation depending on the time of year (Rogowski 2004). Salinity in Salt Creek is generally high, ranging from 7.4 to > 88 ppt (Rogowski 2004), but it rapidly decreases to as low as 1.5 ppt during floods (Craig Stockwell, personal observation). Temperature in Salt Creek ranges from 2.73 to 36.92°C (Rogowski 2004).

Lost River can be divided into upper, middle, and lower sections. A system of culverts running under Range Road 9 separates the upper and middle sections. The middle section runs from Range Road 9 to the end of a large playa which is typically dry; the river re-emerges downstream and runs until it terminates into the gypsum dunes. During wet years, Lost River extends into White Sands National Monument. On average, salinity and temperature are higher in Lost River compared to Salt Creek (Stockwell and Mulvey 1998). Salinity in Lost River ranges from 13.5 to > 88 ppt, with temperature ranges from 2.74 to 38.13°C (Rogowski 2004). Additionally, in contrast to Salt Creek, salinity values decrease

downstream.

The Malpais Spring complex can be separated into upper, middle, and lower sections as described by Rogowski (2004). The upper section is a small area composed of a springhead and subsequent outflow which travels for a short distance to a small waterfall. From there, the middle section consists of a small channel and wetland complex located approximately 50-60 meters south of the springhead. The lower section is a wetland and playa system located approximately 2 km south of the springhead. This is in the area of the "Malpais Spring ponds" that were discussed by Miller and Echelle (1975) in their original description of White Sands pupfish. Temperature and salinity generally increase with distance from the springhead, as do fluctuations in these values (Stockwell and Mulvey 1998). Salinity in the upper sections is generally about 3 to 3.5 ppt, whereas the lower sections may have higher levels of salinity ranging from about 5 to 21.5 ppt (Stockwell and Mulvey 1998). Temperatures in the Malpais Spring complex range from 3.2 to 29°C in the lower portion and from 13.20 to 17.6°C in the upper portion (Stockwell and Mulvey 1998).

Mound Spring is a system of two ponds separated into upper and lower sections by an overflow drainage pipe. Upper Mound Spring is shallower (maximum depth of approximately 2.5 m) and less vegetated than Lower Mound Spring (maximum depth of approximately 4 m) (Rogowski 2004). The temperature and salinity in both ponds are similar, with minimal fluctuations in salinity (Stockwell and Mulvey 1998). Salinity ranges from 1.5 to 4 ppt in Upper Mound and from 2 to 4 ppt in Lower Mound (Stockwell and Mulvey 1998). Temperatures in Upper Mound range from 9.4 to 26.30°C (Stockwell and Mulvey 1998) while Lower Mound ranges from 4.69 to 30.81°C (Rogowski 2004).

Objectives

The purpose of this research is to address the following: 1) Evaluate the spatial distribution of gyrodactylids within White Sands pupfish habitats. 2) Have historic translocations influenced the parasite-host relationship for Salt Creek ESU? 3) Has local adaptation occurred in *G. tularosae* populations associated with the Malpais Spring and Salt Creek pupfish populations? 4) What are the costs of parasitism associated with *G. tularosae*?

CHAPTER 2. *GYRODACTYLUS* FIELD SURVEY

Introduction

Environmental conditions can have a significant impact on parasite-host interactions (Esch et al. 1975; Lafferty and Kuris 1999; Lenihan et al. 1999; Gilbert and Granath 2003; Rogowski and Stockwell 2006). In aquatic ecosystems, the physiological condition, survival, and reproduction of both parasite and host can be influenced by temperature, salinity, and dissolved oxygen (Lenihan et al. 1999). This is especially true in desert aquatic habitats, such as streams which can experience substantial spatial and temporal variation in salinity, temperature, and flow (Miller 1981; Meffe and Minckley 1997; Stockwell and Mulvey 1998).

The White Sands pupfish (*Cyprinodon tularosa*) and its associated parasites provide an excellent system for addressing these issues because salinity varies considerably within and among habitats (Stockwell and Mulvey 1998; Rogowoski and Stockwell 2006). Specifically, variation in salinity among habitats is likely to influence parasite-host relationships for this protected fish species, as salinity gradients are often responsible for shaping communities (Williams 1998; Wolfram et al. 1999; Costil et al. 2001). Rogowski and Stockwell (2006) found that salinity limits the distribution of trematode parasites infecting White Sands pupfish. Similarly, salinity could limit the distribution of the gyrodactylids within and among habitats of White Sands pupfish.

Earlier work showed gyrodactylids to co-occur with two native populations of pupfish (Salt Creek and Malpais Spring) and one non-native population of pupfish at Mound Spring that had genetically descended from Salt Creek. By contrast, gyrodactylids were not observed to co-occur with the non-native pupfish population at Lost River;

pupfish here were also genetically derived from Salt Creek (For introduction history, see Stockwell et al. 1998; Pittenger and Springer 1999). The apparent absence from Lost River could be explained by the fact that this population went through a severe bottleneck during founding (30 fish), or because of the unusually high levels of salinity at Lost River (see Stockwell and Mulvey 1998); however, the sampling of gyrodactylids within all populations has been relatively limited.

Worldwide, gyrodactylids live in fresh, brackish, and sea water, with varying levels of salinity tolerance among species (Malmberg 1970). Numerous marine species can tolerate a decrease in salinity from 35 to 6 ppt, while some freshwater species can survive salinity increases to at least 6 ppt (Malmberg 1970). Soleng and Bakke (1997) found that the freshwater species *Gyrodacylus salaris* can survive and reproduce at salinity levels of up to 7.5 ppt; however, at salinity levels of 10, 15, 20, and 33 ppt no reproduction occurred. Additionally, survival time was negatively associated with water temperature (Soleng and Bakke 1997).

Given the lack of knowledge regarding gyrodactylid communities within the habitats of White Sands pupfish, the objective of this study was to assess gyrodactylid prevalence (percent hosts infected) and intensity (parasites per host) in all four White Sands pupfish habitats. The following null hypotheses were assessed with this field survey:

H_{O1} – Parasite intensity is not correlated with fish size.

H_{O2} – Parasite intensity does not differ between male and female pupfish.

H_{O3} – Parasite intensity does not differ for fish occurring in different habitats.

Methods

Gyrodactylid prevalence and intensity were assessed for pupfish populations at Malpais Spring, Mound Spring, Salt Creek, and Lost River (Figure 1.2). A minimum of thirty fish were sampled within each of the following survey sites: (1) Lost River-upper, (2) Lost River-middle, (3) Salt Creek-upper, (4) Salt Creek at Range Road 316, (5) Salt Creek-lower at the "Cable Crossing", (6) Mound Spring-upper, (7) Mound Spring-lower, (8) Malpais Spring-middle, and (9) Malpais Spring-lower marsh (Table 2.1).

Beach seines were used to collect fish in all but two of the sites. The presence of submerged algae and detritus in Malpais Spring-middle, and the water depth in Mound Spring-lower, made it necessary to collect fish via minnow traps. Six traps were used at each location. Traps were set for seven hours at Malpais Spring-middle and ten hours at Mound Spring-lower. All captured fish were subsequently transferred to live-cars at a density of approximately one fish/gallon. Following capture and transfer to live-cars, fish were individually isolated in .5 liter cups. Parasite assessment was conducted streamside, as maintaining fish at high density in captivity would likely increase parasite transmission and alter patterns of parasite distribution patterns among fish.

Fish were individually anesthetized with MS-222 (100 mg/l) and their parasite loads evaluated with the aid of a dissecting microscope. The body, as well as caudal, anal, dorsal, pelvic, and pectoral fins, were observed for parasite occurrence. In addition, standard length (nearest 0.01 mm) and mass measurements (nearest 0.01 g), as well as sex, were recorded for each fish. After inspection, fish were placed in a recovery bucket and returned to the wild. Field sampling took place in May, 2005.

Parasite load aggregation was measured as described by Wilson et al. (2001). The

Table 2.1. Number of fish sampled, habitat characteristics at time of sampling, and minimum and maximum temperatures and salinities (from Stockwell and Mulvey 1998; Rogowski 2004).

Site	Number Sampled	Temperature (°C)	Salinity (ppt)	Min - Max Temp (°C)	Min - Max Salinity (ppt)
Lost River-middle	40	17.5	49.5	2.74 - 38.13	28.50 - > 88.00
Lost River-upper	40	29.6	31.4	11.90 - 33.44	23.00 - 40.00
Malpais Spring-lower	40	17.1	4.0	3.20 - 29.00	4.40 - 21.50
Malpais Spring-middle	40	17.5	3.5	13.20 - 19.72	2.10 - 4.00
Mound Spring-lower	30	24.0	3.0	4.69 - 30.81	1.70 - 4.00
Mound Spring-upper	40	20.2	2.7	9.40 - 26.30	1.50 - 4.00
Salt Creek-lower	30	23.0	40.8	3.00 - 36.16	7.40 - > 88.00
Salt Creek @ RR-316	40	23.7	25.0	2.73 - 36.92	10.20 - 28.10
Salt Creek-upper	40	19.0	25.3	0.34 - 33.40	13.50 - 32.00

inverse measure of parasite aggregation, *k*, was calculated using the following equation:

$$k = (m^2 - s^2/n)/(s^2 - m),$$

where *m* is the mean and *s* is the variance (Elliot 1977). A *k* value of 1 indicates a parasite population that is highly aggregated, whereas a *k* value greater than 20 indicates a population with a normal distribution (Wilson et al. 2001).

Fish body condition was evaluated using relative condition factor ($K_n = w/w'$), where *w* is an individual fish's weight and *w'* is the predicted weight of the fish, given its length (Bolger and Connolly 1989). Predicted weight was calculated using the following mass length regression: predicted wt. = $0.000012928*L^{3.23708}$ (Rogowski 2004).

Linear regression was used to test for relationships between parasite load (ln transformed) and fish length, mass, and body condition. Uninfected fish were excluded from analyses concerning parasite intensity. A regression was run for each sampling location, as well as for the pooled data. The pooled data were also used to compare parasite loads between males and females using ANOVA. In addition, parasite loads were compared between habitats as a whole (Salt Creek, Lost River, Malpais Spring, and Mound Spring), as well as by habitat type (saline rivers versus brackish springs), using ANOVA (post-hoc pair wise comparison with Bonferroni correction).

Results

Gyrodactylids were present in all nine locations that were sampled. Parasite prevalence at each site was nearly 100% (Table 2.2); however, intensity was more variable among populations (Table 2.2). The parasite populations, as measured by the corrected moment estimate of *k* (Elliot 1977), were highly aggregated in all sample locations (Table 2.3).

Table 2.2. Fluke prevalence (percent infected) and intensity (mean infection) at each sampling site.

Site	Fluke Prevalence (%)	ln (Fluke Intensity) (± SEM)
Lost River-middle	85	0.9882 (± 0.1312)
Lost River-upper	100	4.6482 (± 0.1341)
Malpais Spring-lower	92.5	1.8096 (± 0.1374)
Malpais Spring-middle	97.5	2.9421 (± 0.1601)
Mound Spring-lower	96.7	2.5352 (± 0.1599)
Mound Spring-upper	72.5	0.9896 (± 0.1619)
Salt Creek @ RR-316	100	4.2320 (± 0.1509)
Salt Creek-lower	100	2.8282 (± 0.1413)
Salt Creek-upper	97.5	2.9036 (± 0.1317)

Table 2.3. Measures of parasite aggregation (*k*) and summary of parasite load (ln transformed) regressions at each sampling site.

		Parasite Load Regressions		
Site	*k*-value	Standard Length	Mass	Condition
Lost River-middle	1.05	$F_{1,32} = 0.69$, $p = 0.41$, $R^2 = 0.0212$	$F_{1,32} = 1.39$, $p = 0.25$, $R^2 = 0.0415$	$F_{1,32} = 1.63$, $p = 0.21$, $R^2 = 0.0483$
Lost River-upper	1.30	$F_{1,38} = 6.80$, $p = 0.01$, $R^2 = 0.1518$	$F_{1,38} = 4.23$, $p = 0.05$, $R^2 = 0.1002$	$F_{1,38} = 2.36$, $p = 0.13$, $R^2 = 0.0585$
Malpais Spring-lower	1.15	$F_{1,35} = 1.52$, $p = 0.23$, $R^2 = 0.0417$	$F_{1,35} = 1.74$, $p = 0.20$, $R^2 = 0.0475$	$F_{1,35} = 0.96$, $p = 0.34$, $R^2 = 0.0266$
Malpais Spring-middle	0.72	$F_{1,37} = 0.54$, $p = 0.47$, $R^2 = 0.0144$	$F_{1,37} = 0.52$, $p = 0.48$, $R^2 = 0.0137$	$F_{1,37} = 0.03$, $p = 0.87$, $R^2 = 0.0007$
Mound Spring-lower	0.20	$F_{1,27} = 4.16$, $p = 0.05$, $R^2 = 0.1335$	$F_{1,27} = 2.41$, $p = 0.13$, $R^2 = 0.0819$	$F_{1,27} = 0.48$, $p = 0.49$, $R^2 = 0.0175$
Mound Spring-upper	0.51	$F_{1,27} = 20.41$, $p < 0.001$, $R^2 = 0.4305$	$F_{1,27} = 27.26$, $p < 0.001$, $R^2 = 0.5024$	$F_{1,27} = 0.01$, $p = 0.94$, $R^2 = 0.0002$
Salt Creek @ RR-316	1.02	$F_{1,38} = 9.64$, $p < 0.01$, $R^2 = 0.2024$	$F_{1,38} = 8.24$, $p < 0.01$, $R^2 = 0.1783$	$F_{1,38} = 3.68$, $p = 0.06$, $R^2 = 0.0882$
Salt Creek-lower	1.92	$F_{1,28} = 1.82$, $p = 0.19$, $R^2 = 0.0611$	$F_{1,28} = 0.91$, $p = 0.35$, $R^2 = 0.0314$	$F_{1,28} = 0.02$, $p = 0.88$, $R^2 = 0.0008$
Salt Creek-upper	1.63	$F_{1,37} = 6.25$, $p = 0.02$, $R^2 = 0.1445$	$F_{1,37} = 5.08$, $p = 0.03$, $R^2 = 0.1208$	$F_{1,37} = 1.32$, $p = 0.26$, $R^2 = 0.0343$

There was no correlation between parasite load and fish standard length ($F_{1,315}$ = 9.22, $P < 0.01$, $R^2 = 0.0284$), fish mass ($F_{1,315} = 4.21$, $P = 0.04$, $R^2 = 0.0132$), or fish condition factor ($F_{1,315} = 0.92$, $P = 0.34$, $R^2 = 0.0029$) across all sites (Figure 2.1). Similarly, for all but one of the sites there was no correlation between parasite load and fish standard length, fish mass, or fish condition factor (Table 2.3). Mound Spring-upper showed a significant correlation between parasite load and fish length ($F_{1,27} = 20.41$, $P < 0.001$, $R^2 = 0.4305$) and mass ($F_{1,27} = 27.26$, $P < 0.001$, $R^2 = 0.5024$), but not condition factor ($F_{1,27} = 0.005$, $P = 0.94$, $R^2 = 0.0002$) (Figure 2.2).

There was not a significant difference in mean infection levels for males and females across all sites ($F_{1,315} = 0.03$; $P = 0.85$) (Figure 2.3); however, there was a significant differences among populations ($F_{3,313} = 20.00$; $P < 0.0001$) (Figure 2.4). Further, there was no difference in parasite intensity within the two major habitat types; brackish springs (Malpais Spring and Mound Spring) and saline rivers (Salt Creek and Lost River). Thus, the two habitat types (brackish springs and saline rivers) were compared. Parasite loads were significantly higher for fish in saline rivers compared to fish from brackish springs ($F_{1,315} = 47.82$; $P < 0.0001$) (Figure 2.5).

Discussion

These data contrast with earlier surveys in which *Gyrodactylus tularosae* was not observed on Lost River fish. Others have shown gyrodactylid populations to vary in space and time (Cone and Cusack 1988; Mo 1992; Walberg et al. 2003). Thus, these data show the need for systematic and perhaps repeated sampling of gyrodactylids infecting White Sands pupfish. The successful introduction of *G. tularosae* from Salt Creek to Lost River and Mound Spring is interesting, as it suggests that these gyrodactylids have a wide salinity

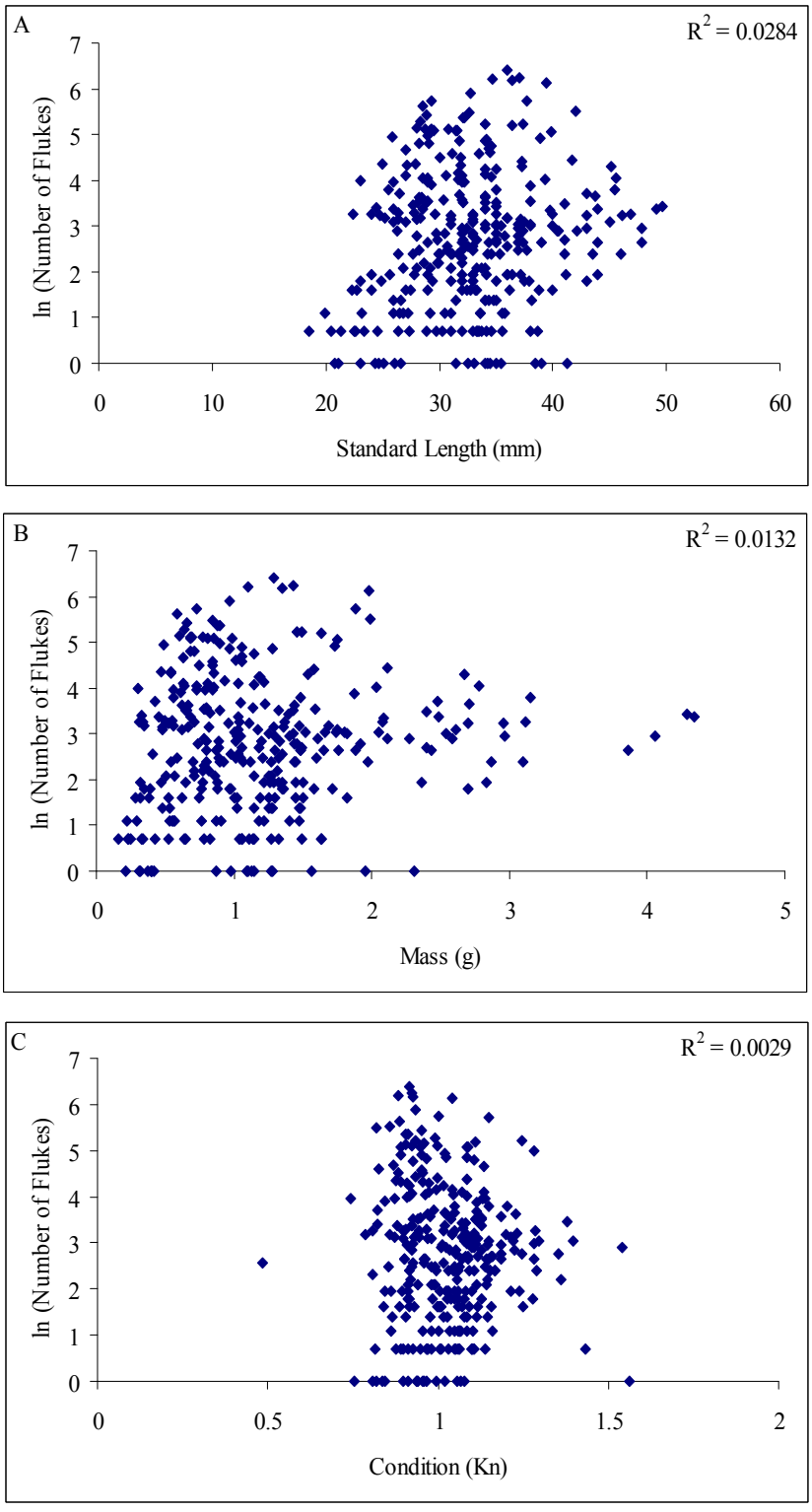

Figure 2.1. Linear regression of parasite load and fish standard length (A), mass (B), and condition (C) across all sites.

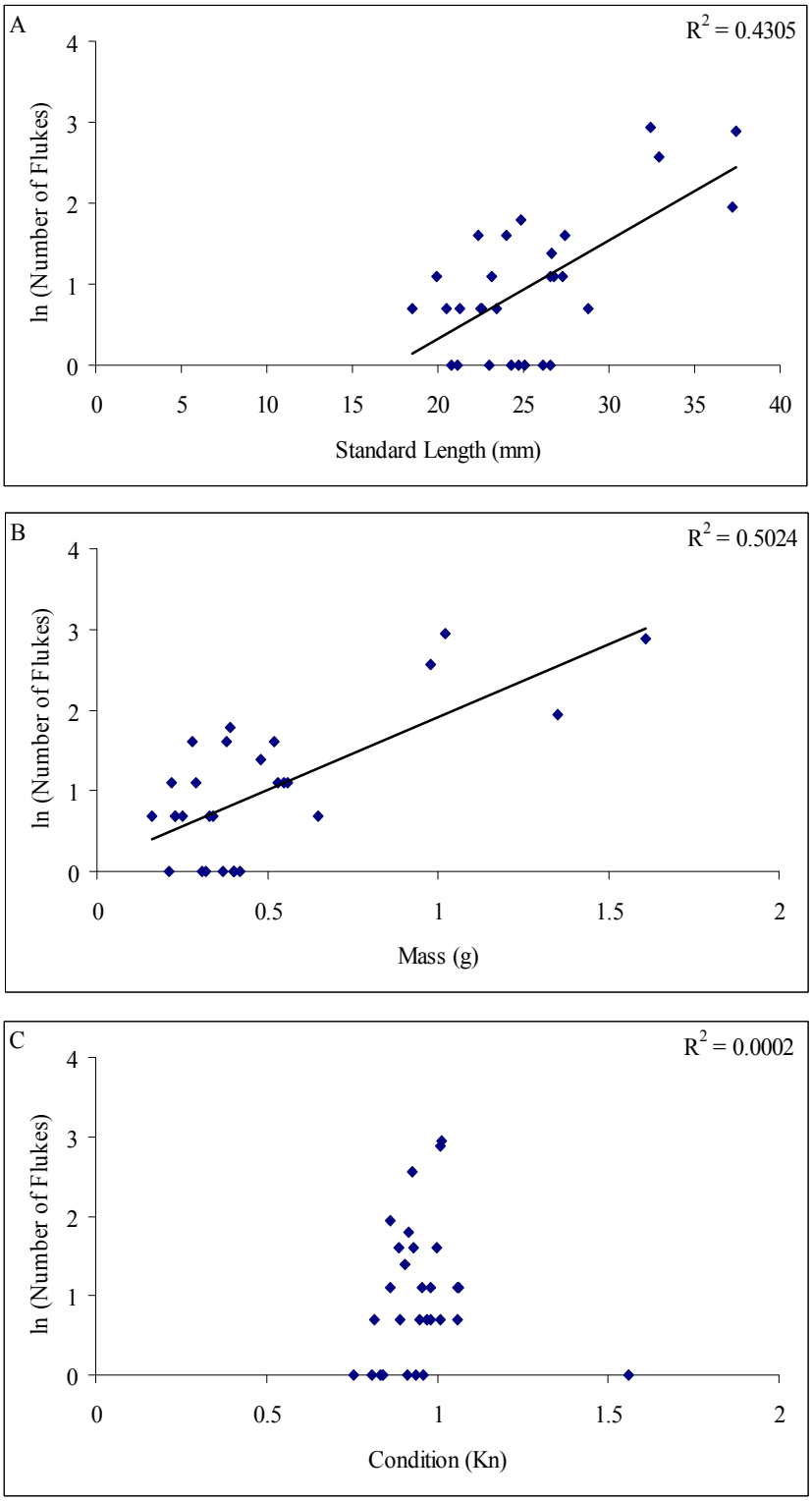

Figure 2.2. Mound Spring-upper linear regression of parasite load and standard length (A), mass (B), and condition (C).

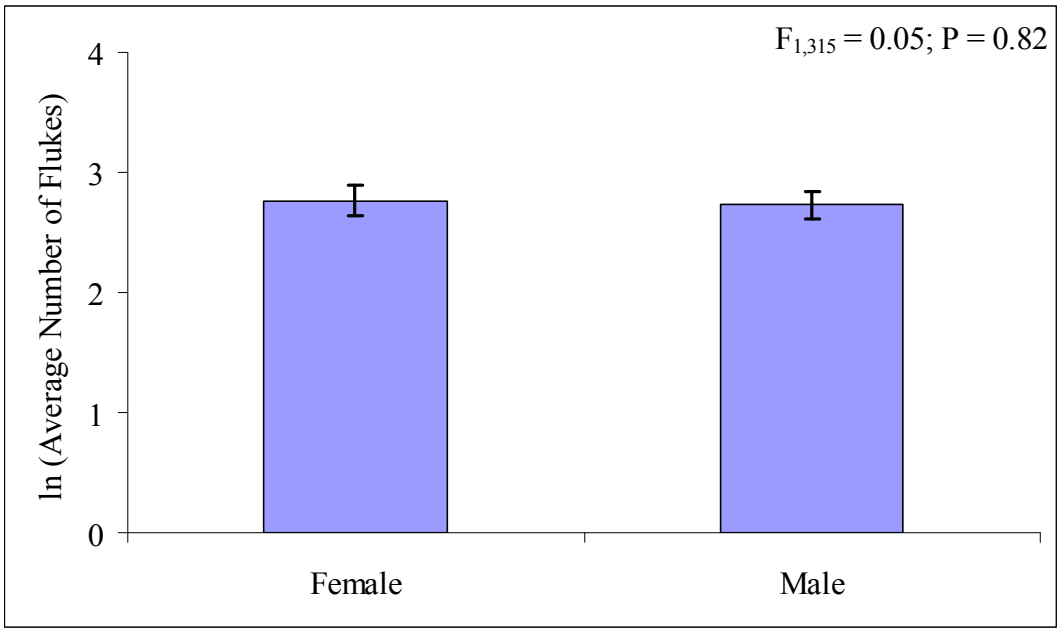

Figure 2.3. Average number of parasites for male and female fish across all sites. Error bars represent one standard error (SE) of the mean.

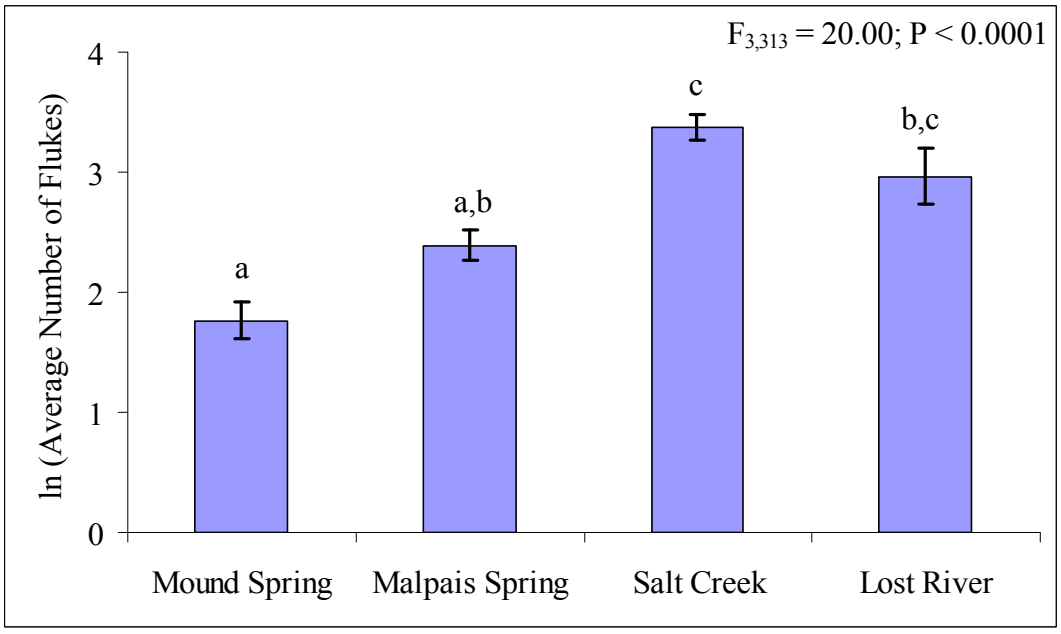

Figure 2.4. Comparison of average parasite loads across pupfish populations. Error bars represent one standard error (SE) of the mean. Groups sharing a letter are not statistically different.

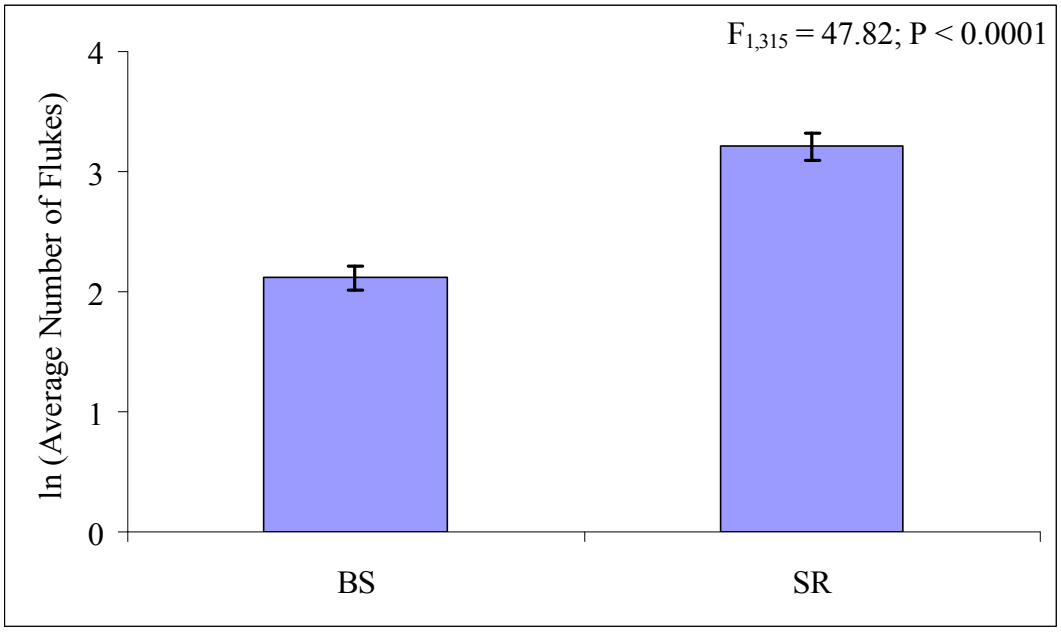

Figure 2.5. Comparison of average parasite loads between brackish springs (BS) and saline rivers (SR). Error bars represent one standard error (SE) of the mean.

tolerance. Salinity values at Lost River are high and variable (range: 7.4 ppt to > 88 ppt), whereas salinity is low and relatively constant at Mound Spring (range: 1.5 ppt to 4 ppt) (Stockwell and Mulvey1998; Rogowski 2004). This salinity tolerance may well reflect the variable conditions that can occur at Salt Creek. Flash floods at Salt Creek in 2005 and 2006 resulted in salinity dropping to approximately 3.8 ppt and 1.5 ppt, respectively, from recent readings of 25 ppt and 39 ppt. In both cases, salinities subsequently increased to 7.9 ppt and 5.1 ppt within 24 hours as flood waters receded.

These data suggest that gyrodactylids have much higher levels of salinity tolerance (> 88 ppt) than previously reported. This high level of salinity tolerance may well occur for other gyrodactylids that co-occur with pupfishes. Unfortunately, little data exist regarding pupfish gyrodactylids. In general, parasite surveys of the pupfishes have been rather limited (Hargis 1955; Mizelle and Kritsky 1967; Collyer and Stockwell 2004; Rogowski and Stockwell 2006), perhaps due in part to their protected status. In fact, during a parasite survey of fishes from the Salton Sea (Kuperman et al. 2001), desert pupfish (*Cyprinodon macularius*) were not surveyed, although they have historically been present there (Evermann 1916; Barlow 1958).

The level of parasite aggregation found in this survey was consistent with other animal parasites in general (Shaw et al. 1998; Wilson et al. 2001). Here, the high level of parasite aggregation was likely a result of host-parasite interactions in which individual hosts within the population are at differing levels of susceptibility. Uninfected fish, and those with low infection intensities, had likely mounted effective immune responses and were not immediately susceptible to reinfection (Lindenstrøm and Buchmann 2000). Scott and Robinson (1984) showed that guppies (*Poecilia reticulata*) subjected to reinfection by

Gyrodactylus bullatarudis had significantly lower establishment success, mean parasite population size, peak parasite burden, time to peak burden, and duration of infection.

Host immune response was likely one of several factors affecting fluke aggregation. In their study of rabbit gut helminths, Boag et al. (2001) found that parasite aggregation varied with year, season, age class, host sex, and myxomatosis. Of these variables, host sex was the only one in this White Sands pupfish *Gyrodactylus* survey with the potential of being analyzed; however, an unequal number of male and female pupfish were surveyed at each site, leading to unequal sample sizes. Further, analyzing aggregation with too small of a sample size can lead to aggregations being underestimated (Boag et al. 2001), so fluke aggregation between sexes was not evaluated in this study. Nonetheless, there was no evidence of differences in distribution between males and females in terms of mean intensities. There was also no significant difference between parasite loads with respect to fish size (standard length and mass) and condition.

The absence of a correlation between parasite load and fish sex, size, and condition is consistent with the lack of detectable costs for White Sands pupfish by *G. tularosae* (Chapter 4). Similarly, Moura et al. (2003) found no correlation between host sex, size, or developmental stage and the community structure of ectoparasitic flys (*Noctiliostrebla aitkeni* and *Paradyschiria fusca*) and their host, the fishing bat (*Noctilio leporinus*). Walberg et al. (2003) also found no correlation between arrow goby (*Clevelandia ios*) standard length and the number of *Gyrodactylus perforatus* per host; however, Pickering and Christie (1980) found that mature male brown trout (*Salmo trutta*) had significantly greater numbers of *Gyrodactylus* sp., as well as several other ectoparasites, compared to mature female brown trout. Similarly, Appleby (1996a) found that during the breeding

season, male sand gobies (*Pomatoschistus minutus*) had significantly higher *Gyrodactylus* sp. abundances compared to females; however, this trend was reversed at the end of the breeding season.

Even though there were no differences in mean intensities between males and females, size, or condition, there are likely to be seasonal differences in fluke infections. Mo (1992) found the highest infestation intensity of *G. salaris* on Atlantic salmon parr (*Salmo salar*) during the summer and early autumn, with infection intensities lowest during the winter and early spring. *Gyrodactylus callariatis* infecting juvenile Atlantic cod (*Gadus morhua*) also reached peak intensities during the summer (Appleby 1996b). Interestingly, Cone and Cusack (1988) found that infections of *Gyrodactylus colemanensis* and *Gyrodactylus salmonis* on hatchery reared brook trout (*Salvelinus fontinalis*), rainbow trout (*Oncorhyncus mykiss*), and Atlantic salmon increased during winter, with a peak in spring, followed by a decrease during the summer. Similarly, Dávidová et al. (2005) found that *Gyrodactylus rhodei* infecting bitterling (*Rhodeus sericeus*) had highest prevalence, abundance, and intensity of infection during autumn and winter, when water temperatures decreased.

Clearly, making generalizations as to the seasonal occurrences of gyrodactylids, as a whole, is impossible. Because of logistical constraints, gyrodactylid prevalence and intensity was assessed only once during this study. In order to gain a better understanding of how gyrodactylid numbers on White Sands pupfish fluctuate with changes in water temperature and salinity, surveys should take place in the fall, winter, spring, and summer. In order to provide further insights into the salinity tolerance of gyrodactylids infecting White Sands pupfish, assessment of fluke numbers preceding and following major flood

events is also advisable. Additionally, given the findings of Appleby (1996a) and Boag et al. (2001), it may be interesting to assess infection intensities during spawning periods and in the presence of other parasite outbreaks, respectively.

The significant difference in mean intensities between brackish springs (Malpais Spring and Mound Spring) and saline rivers (Lost River and Salt Creek) could be the result of salinity and temperature differences; another explanation involves differences in host population size and density (Reno 1998; Sterud et al. 2002; Bagge et al. 2004). Compared to brackish springs, saline river environments are much more stochastic in nature, at times resulting in high fish population sizes and densities in isolated pools (personal observation). Given the mode of *Gyrodactylus* transmission (Chapter 1), high host densities may be an important factor in the occurrence of high infection intensities. Krasnov et al. (2002) showed this to be true for the flea species *Xenopsylla dipodilli* and *Nosopsyllus iranus theodori* parasitizing the Wagner's gerbil (*Gerbillus dasyurus*).

Another aspect to consider here is that during high water and flood events, isolated pools become connected and fish are able to disperse and colonize new areas along with other previously isolated fish. This dispersion has the likelihood of bringing together individuals that are at differing stages of infection (infected vs. uninfected/low infection level) which is a requirement for the persistence of *Gyrodactylus* infections within a population (Sterud et al. 2002). This is not to say that brackish springs do not have infected and susceptible fish present at any given moment; however, fish densities in brackish springs, at the time of collection, appeared much lower (personal observation) which could be resulting in lower fluke infection intensities. Furthermore, there are likely to be fewer opportunities for stochastic events (i.e., floods and drought) to change the fish

population sizes and densities in brackish springs.

Ultimately, the infection dynamics of any parasite depend on interactions among the host, parasite, and environment (Reno 1998). The nature of the relationship between gyrodactylids and protected species is of some concern, as other studies have reported certain species of gyrodactylids to be pathogenic (Bakke et al. 1992b; Leberg and Vrijenhoek 1994; Soleng et al. 1998; Hedrick et al. 2001). In most of these instances, the pathogenicity is a result of novel host-parasite associations. This could be an issue where management plans for protected species, such as the White Sands pupfish, call for translocations. Given the ubiquity of gyrodactylids, translocations of fishes could easily result in gyrodactylids being introduced to novel host species. Thus, host-parasite dynamics should be thoroughly studied before introducing any species into a novel environment.

CHAPTER 3. LOCAL ADAPTATION

Introduction

Host-parasite systems provide an interesting setting in which to study local adaptation because host and parasite are coevolving, with host defenses imposing strong selection on parasites and parasites often imposing selection on their hosts (Kawecki and Ebert 2004). The extent of local adaptation in host-parasite systems is influenced by spatial scale, genetic aspects of resistance and pathogenicity, environmental stochasticity, and life histories of both host and pathogen (Thrall et al. 2002). The scale of local adaptation is also important and has implications for the management of rare and endangered fish species. For instance, if local adaptation occurs on a fine scale, then artificial gene flow among sites may be not advised (Currens et al. 1997).

The genetic divergence and habitat dissimilarities between Salt Creek and Malpais Spring strains of White Sands pupfish (*Cyprinodon tularosa*) make for an intriguing system in which to study parasite-host local adaptation. Specifically, the association between the monogenean ectoparasite *Gyrodactylus tularosae* and White Sands pupfish is of interest because gyrodactylid species such as *Gyrodactylus salaris* and *Gyrodactylus turnbulli* have been shown to be pathogenic to Atlantic salmon (*Salmo salar*) (Bakke et al. 1992; Soleng et al. 1998) and the endangered Gila topminnow (*Poecilipsis occidentalis*) (Hedrick et al. 2001), respectively. In both of these cases, the fish species affected is not the gyrodactylids' principle host species; rather, they constitute new parasite-host associations that are ultimately costly to the host. Furthermore, Moen and Stockwell (2006) recently reported evidence of local adaptation for *G. tularosae* (Kritsky and Stockwell 2005) to White Sands pupfish over a closely related congener of *Cyprinodon*. The scale of

adaptation could also exist among populations of White Sands pupfish, as this pupfish is comprised of two genetically isolated ESUs at Malpais Spring and Salt Creek (Stockwell et al. 1998). These populations have been shown to be infected with gyrodactylids, but *G. tularosae* was described only from the Salt Creek strain.

Given the threatened status of White Sands pupfish and the underlying uncertainties surrounding the geographic range of *G. tularosae*, the objective of this experiment was to assess local adaptation of *G. tularosae* on its native stock of pupfish at Salt Creek to fish from Malpais Spring, as these two populations have been isolated for 3,000 to 5,000 years (Miller and Echelle 1975; Pittenger and Springer 1999). The following null hypothesis was assessed in this study:

H_{O1} – There is no difference in *G. tularosae* prevalence and intensity between Salt Creek and Malpais Spring strains of White Sands pupfish.

Methods

This experiment included 24 lab reared (clean) fish from the two native pupfish populations at Salt Creek (12 fish) and Malpais Spring (12 fish). To provide a source of *G. tularosae*, 36 wild Salt Creek fish were collected by beach seine below Range Road 316 and transferred live to NDSU. Salinity in Salt Creek at the time of collection was 17.4 ppt. Thus, the experiment was conducted at a similar salinity level.

Focal fish had been maintained in aquaria with salinity at 3.5 ppt. Consequently, they were introduced to experimental aquaria one day prior to the initiation of the experiment. Each 38 L tank received two female focal fish (1 Salt Creek and 1 Malpais Spring). In order to distinguish between strains, the caudal fin on each focal fish was clipped. In tanks 1-6, Malpais Spring fish received an upper-caudal fin clip and Salt Creek

fish a lower-caudal fin clip. In tanks 7-12, Salt Creek fish received an upper-caudal fin clip and Malpais Spring fish a lower-caudal fin clip.

Introduction of source fish to their respective tanks was temporally staggered in order to make the parasite counts more manageable. On the first day of the experiment, 18 source fish were anaesthetized in MS-222 (80 mg/l) and examined for parasite loads under a dissecting microscope. Parasite loads for these source fish ranged from 2-17 flukes. Upon completing the assessments, three source fish were introduced along with two focal fish to tanks 1-6. Tanks 1-3 received three male source fish each. Tanks 4-6 received three female source fish each. On day two, 18 additional source fish were assessed for parasite loads and introduced to focal fish in tanks 7-12. Parasite loads for these fish ranged from 2-50 flukes. Tanks 7-9 received three male fish each. Tanks 10-12 received three female fish each. After the introduction of all source fish, fish in tanks 1-6 and 7-12 were assessed for parasite loads every 48 hours for a duration of 192 hours.

Data were log transformed (ln) and analyzed by repeated measure ANOVA (SYSTAT 2004) to evaluate population growth of *G. tularosae* between the two strains of pupfish.

Results

There was no evidence of local adaptation, as parasites loads on both strains were not significantly different ($F_{1,22} = 0.1580$, $P = 0.70$). Further, parasite population growth patterns were similar for both strains (Figure 3.1). Additionally, within 48 hours, parasite prevalence reached 100% and 91.67 % for the Salt Creek and Malpais Spring focal fish, respectively. Subsequently, prevalence was 100% from 96 hours to the end of the experiment for both pupfish strains (Figure 3.2).

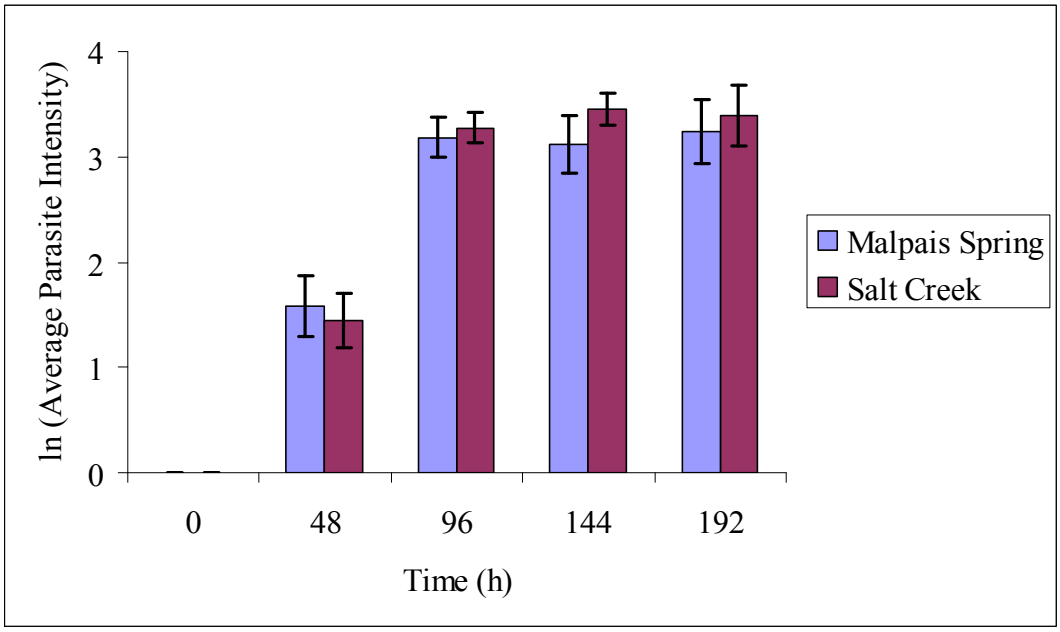

Figure 3.1. Average parasite intensity for Malpais Spring and Salt Creek fish infected with *Gyrodactylus tularosae* from Salt Creek fish. Error bars represent one standard error (SE) of the mean.

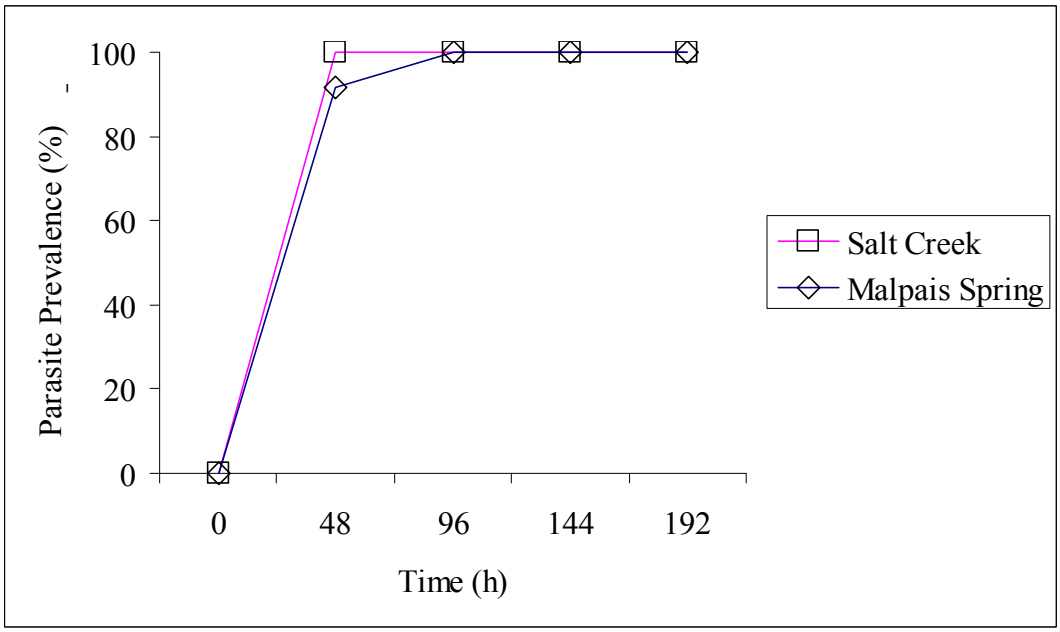

Figure 3.2. The prevalence (% fish infected) of *Gyrodactylus tularosae* across 12 replicates and 5 time periods during the Malpais Spring and Salt Creek challenge experiment.

Discussion

The lack of evidence for local adaptation of *G. tularosae* to the Salt Creek strain of White Sands pupfish is consistent with other studies that failed to detect parasite local adaptation. Host-parasite study systems ranging in focus from lizard-tick (Uller and Olsson 2004), plant-plant (Koskela et al. 2000), and fish-digenean (Sasal et al. 2000) all failed to detect local adaptation.

Interestingly, the results of this study were in contrast to those of Moen and Stockwell (2006), who found evidence of local adaptation of *G. tularosae* to White Sands pupfish, compared to its congener, the sheepshead minnow. Despite the different outcomes concerning local adaptation, several aspects of both studies were similar. The patterns of parasite growth, as well as parasite prevalence up to the time of peak infection, were almost identical. Time to peak infection varied slightly (96 vs. 192 hours). This was likely the result of 60-70 flukes per source fish used by Moen and Stockwell (2006), compared to 2-50 flukes per source fish used in this study. Finally, mean parasite loads at the time of peak infection were higher for Moen and Stockwell (2006), compared to the current study.

Failure to detect local adaptation between *G. tularosae* and White Sands pupfish may be partially explained by the length of isolation of Salt Creek and Malpais Spring strains of pupfish. In contrast to White Sands pupfish and sheepshead minnow which have been isolated for ca. 2 million years (Echelle et al. 2005), strains of White Sands pupfish have presumably been isolated for ca. 3,000-5,000 years (Pittenger and Springer 1999). Even though Salt Creek and Malpais Spring pupfish strains have diverged enough to be considered evolutionary significant units (ESUs) of White Sands pupfish (Stockwell et al. 1998), there may not be enough genetic divergence for *G. tularosae* to have become locally

adapted. Even with greater genetic divergence, local adaptation may not be shown, as genotypic variation among populations can make interpreting local adaptation difficult (Thrall et al. 2002). This is because when a number of different genotypes are present, levels of resistance and virulence of host and pathogen can differ (Bevan et al. 1993a; Bevan et al. 1993b; Thrall et al. 2001). Variation in host resistance can obscure studies that test the performance of a parasite on sympatric versus allopatric hosts (as was done here), whereas variation in pathogen virulence can obscure studies that test the performance of sympatric versus allopatric parasites on a single host population (Thrall et al. 2002).

Another factor that makes detecting local adaptation difficult is the population dynamics between host and parasite. Host-parasite systems that are ephemeral and experience high rates of local extinction, such as with White Sands pupfish and *G. tularosae*, are governed by migration-drift dynamics, and, therefore, unlikely to generate interactions leading to local adaptation (Kaltz and Shykoff 1998). Similarly, seasonal variation of parasite populations may hinder local adaptation (Thrall and Burdon 1997; Burdon and Thrall 2000).

Even though local adaptation was not shown in this experiment, it should not be entirely ruled out for this host-parasite system. Failure to detect local adaptation where it exists can be a result of weak statistical power, the scale of local adaptation (parasites adapting to individuals rather than populations), acquired immunity of the host, maternal effects on resistance, uneven gene flow between populations, or not enough time for adaptation to occur (Ebert and Hamilton 1996). Also, for local adaptation to be detected, parasite performance on its sympatric host must be compared to several allopatric host populations (as alluded to previously) (Kaltz and Shykoff 1998). There must also be

replication with sympatric parasite host combinations to account for temporal variations within populations (Kaltz and Shykoff 1998); however, in this specific case, only two native strains of White Sands pupfish exist.

As the results indicated, no evidence of parasite local adaptation was found within this study system; however, to take into consideration the multitude of factors that can influence the detection of local adaptation, this study should be repeated under varying environmental conditions (i.e., salinity, temperature, food availability, etc.) using Salt Creek flukes to infect Salt Creek and Malpais Spring fish and using Malpais Spring flukes to infect Salt Creek and Malpais Spring fish. Additionally, in order to further evaluate the time necessary for local adaptation to evolve, it would also be helpful for local adaptation to be tested for other species of *Gyrodactylus*.

CHAPTER 4. COSTS OF PARASITISM: ANALYSIS OF SURVIVORSHIP, GROWTH, AND FAT CONTENT

Introduction

It is believed that parasites impose a cost on their hosts which can be manifested as impacts on life history traits (e.g., reproductive success, survivorship, and growth), behavior, or morphology (Price 1980; Barber et al. 2000; Moore 2002); however, costs of parasitism, although generally assumed, are not always assessed (Møller 1997; Collyer 2000). Even when costs are assessed, they are not always demonstrated (Collyer 2000). Given the ubiquity of parasites and the dynamic nature of host-parasite interactions, it is important to assess the potential costs associated with parasitism.

Costs of parasitism may be particularly important for threatened species (Woodroffe 1999; Daszak et al. 2000; Cleaveland et al. 2001). For instance, a population decline of the threatened White Sands pupfish (*Cyprinodon tularosa*) coincided with an outbreak of white grub parasites (see Collyer and Stockwell 2004). Subsequent work showed that White Sands pupfish experimentally infected with white grubs (Diplostomatidae) had higher levels of mortality, decreased growth rates, decreased fat storage, decreased metabolic rates, altered coloration, and swollen eyes compared to non-infected fish (Collyer 2000; Harstad 2003; Collyer and Stockwell 2004). Similarly, Rogowski and Stockwell (2006) reported field evidence of a negative effect of trematode parasites on pupfish body condition.

All the previously described work has focused on digene trematodes, while monogene trematodes have not been similarly evaluated; however, White Sands pupfish are host to at least one monogenetic trematode (*Gyrodactylus tularosae*), known to infect Salt Creek, Lost River, and Mound Spring populations of White Sands pupfish (Chapter 3).

The Malpais Spring population is also host to a species of *Gyrodactylus*, but its identity has not been confirmed. Until now, the costs of *G. tularosae* have not been evaluated. Such work is important because other members of the genus *Gyrodactylus* have been shown to be pathogenic to some fish species (Bakke et al. 1992b; Leberg and Vrijenhoek 1994; Soleng et al. 1998; Hedrick et al. 2001) while many fish species appear to be unaffected by the flukes (MacKenzie 1970; Cone and Odense 1984; Bakke et al. 1991; Bakke et al. 1992a; Jansen and Bakke 1995; Bakke et al. 1996; Buchmann and Uldal 1997; Soleng and Bakke 2001; Sterud et al. 2002). Understanding the host-parasite association between the White Sands pupfish and *G. tularosae* is important to the management efforts of this threatened pupfish. Assessing the costs of gyrodactylids with field data is problematic because parasite loads are highly variable in space and time. Thus, the objective of this study was to experimentally determine the costs, in terms of survivorship, growth, and fat content, imposed by *G. tularosae* on its host, the White Sands pupfish. The following null hypotheses were assessed:

H_{O1} – There is no affect of *G. tularosae* infection on White Sands pupfish survivorship.

H_{O2} – There is no affect of *G. tularosae* infection on White Sands pupfish growth.

H_{O3} – There is no affect of *G. tularosae* infection on White Sands pupfish fat content.

Methods

To assess the costs of parasitism imposed by *G. tularosae* on White Sands pupfish, wild fish collected from upper Lost River on the Holloman Air Force Base were used as focal fish. Lost River fish were selected because in earlier surveys this population was

shown to lack gyrodactylids. These fish, 40 male and 40 female, were acclimated to experimental conditions for two weeks prior to the beginning of the experiment.

Wild Salt Creek fish, collected below Range Road 316 on the White Sands Missile Range, were used as a source of *G. tularosae*. To ensure that *G. tularosae* to be used in the experiment were acclimated to experimental conditions, Salt Creek source fish were also maintained in the lab for two weeks prior to the beginning of the experiment.

To ensure that initial size differences did not obscure differences in growth during the experiment, prior to the commencement of the experiment fish in each replicate were size-matched to the nearest 1 mm whenever possible. At the beginning of the experiment, fish in both treatments were anaesthetized in MS-222 (100 mg/l). Fish assigned to the parasite treatments were then manually infected with six flukes on the caudal fin. Infections were conducted under a dissecting microscope using a forceps and a scale from the source fish. Flukes were "coaxed" onto the source fish scale. The scale was then held on the caudal fin of the focal fish until the flukes transferred. Control fish were handled in the same manner, except for fluke infection.

During the experiment, fish were housed in 15 L aquaria at 10 ppt salinity and maintained on a 14:10 light/dark cycle. This salinity level was selected as it reflects the conditions at Salt Creek where source fish were collected. Water nitrate levels were assessed every four days until it was determined that nitrate levels were no longer increasing. In the event that nitrate levels increased, half of the water was exchanged (and salinity maintained at 10ppt). Fish were fed 5% body mass of TetraMin® flake food daily. This was a relatively high feeding regime, thus a demonstration of any costs would be conservative.

Every seven days, all fish were assessed for parasite loads until they lost their infections. Parasite loads were assessed by first anaesthetizing fish in MS-222 (100 mg/l), then counting the total number of parasites on the caudal, anal, pectoral, and pelvic fins, as well as the body, using a dissecting microscope. In addition to parasite loads, length and mass measurements were taken weekly. At the conclusion of the experiment, fish were fasted for 24 hours and sacrificed in MS-222 (500 mg/l). Length, mass, and fat content of the fish were then analyzed.

Techniques for fat extraction followed those used by Collyer and Stockwell (2004). Fish carcasses were first weighed for wet mass, and then dried at 56°C for 48 hours and weighed for dry gross mass. Fish carcasses were then placed in vials containing 20 ml of anhydrous ether. After 24 hours, ether was replaced, and at 48 hours, it was discarded. Fish were then dried overnight at 56°C and reweighed for dry net mass. Fat content was computed as a percentage of mass allocated to fat.

During the course of the experiment, 23 of the 40 control fish became infected with flukes. As a result, data analyses included only those control fish that did not become infected.

All analyses were conducted using SYSTAT ® v. 11. The initial analysis called for a full ANCOVA model which included treatment, sex, and appropriate covariates. Covariates included initial mass and somatic mass for the analyses of growth rate and fat content, respectively. The final model only included the effects of sex and covariates that were significant. ANOVA was used to compare percent fat and growth between treatment and control groups.

Parasite data were log transformed (ln). Analyses involving parasite loads included

only those fish with one or more parasites. Data for fat mass, fat percent, and change in mass and standard length were transformed and visually inspected using probability plots. Data appeared normal and thus were not transformed.

Results

Parasite Infection Rates

Mean parasite intensity for the treatment group varied significantly throughout the study ($F_{2,109} = 34.75$, $P < .0001$) (Figure 4.1). The number of flukes per fish ranged from 1 to 56, 1 to 387, and 0 to 360 on days 7, 14, and 21, respectively (Figure 4.2). Flukes were present on all observed areas of the body. Overall, there was a significant difference in fluke location on all days of observation (Day 7: $F_{5,159} = 11.10$, $P < 0.0001$; Day 14: $F_{5,204} = 56.41$, $P < 0.0001$; Day 21: $F_{5,113} = 15.68$, $P < 0.0001$), with the body being the primary area of infection (Figure 4.3).

Survivorship

Survivorship for both treatment and control groups was 100% over the duration of the experiment. Additionally, there were no external signs of fluke infection, such as increased mucus production or lesions on the skin.

Growth Rate and Fat Content

There were no differences in initial size among the control and treatment groups for mass ($F_{1,55} = 1.02$, $P = 0.32$) or for standard length ($F_{1,55} = 0.95$, $P = 0.33$) (Table 4.1). Fish from both control and treatment groups exhibited growth during the experiment. Significant differences in mass and standard length gained were observed from Day 0 to Day 22 (Mass: $F_{1,112} = 32.76$, $P < 0.001$; SL: $F_{1,112} = 12.15$, $P < 0.001$) (Figure 4.4). On average, fish increased in mass by 22.72% and increased in standard length by 4.28%.

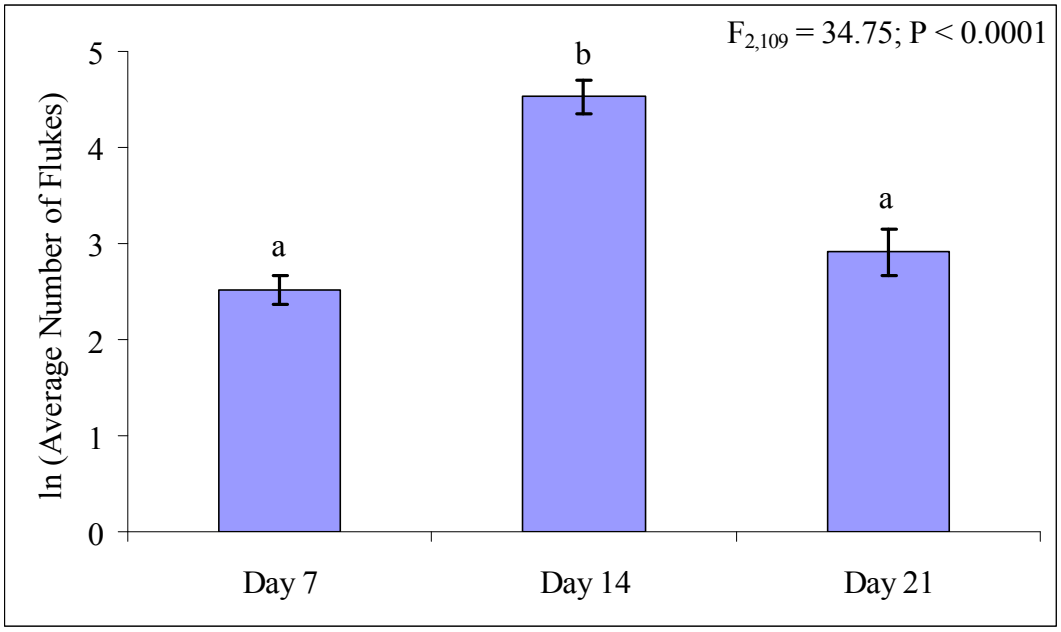

Figure 4.1. Average parasite loads on days 7, 14, and 21. Error bars represent one standard error (SE) of the mean. Groups sharing a letter were not statistically different.

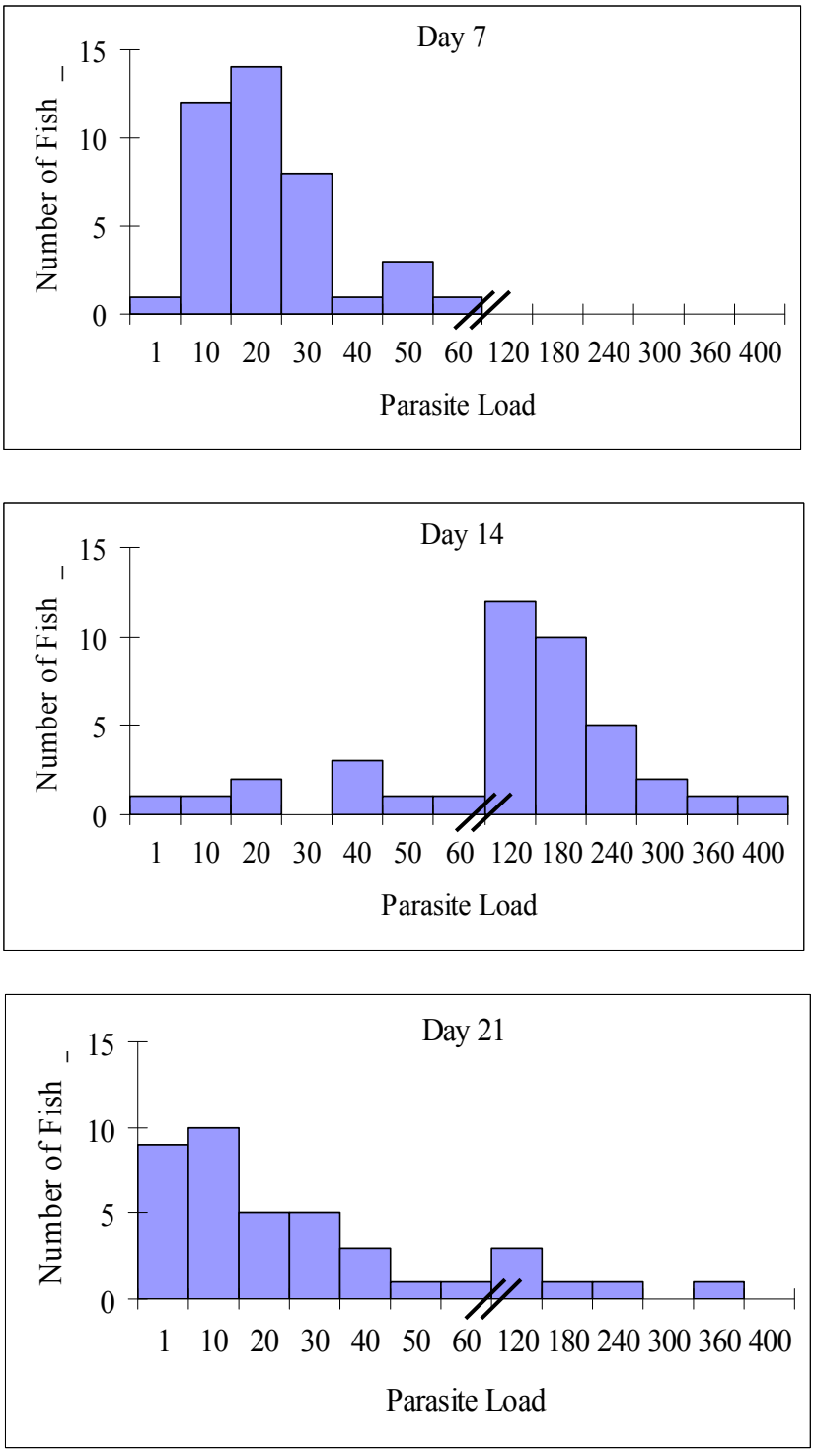

Figure 4.2. Distribution of parasite loads on treatment group on days 7, 14, and 21.

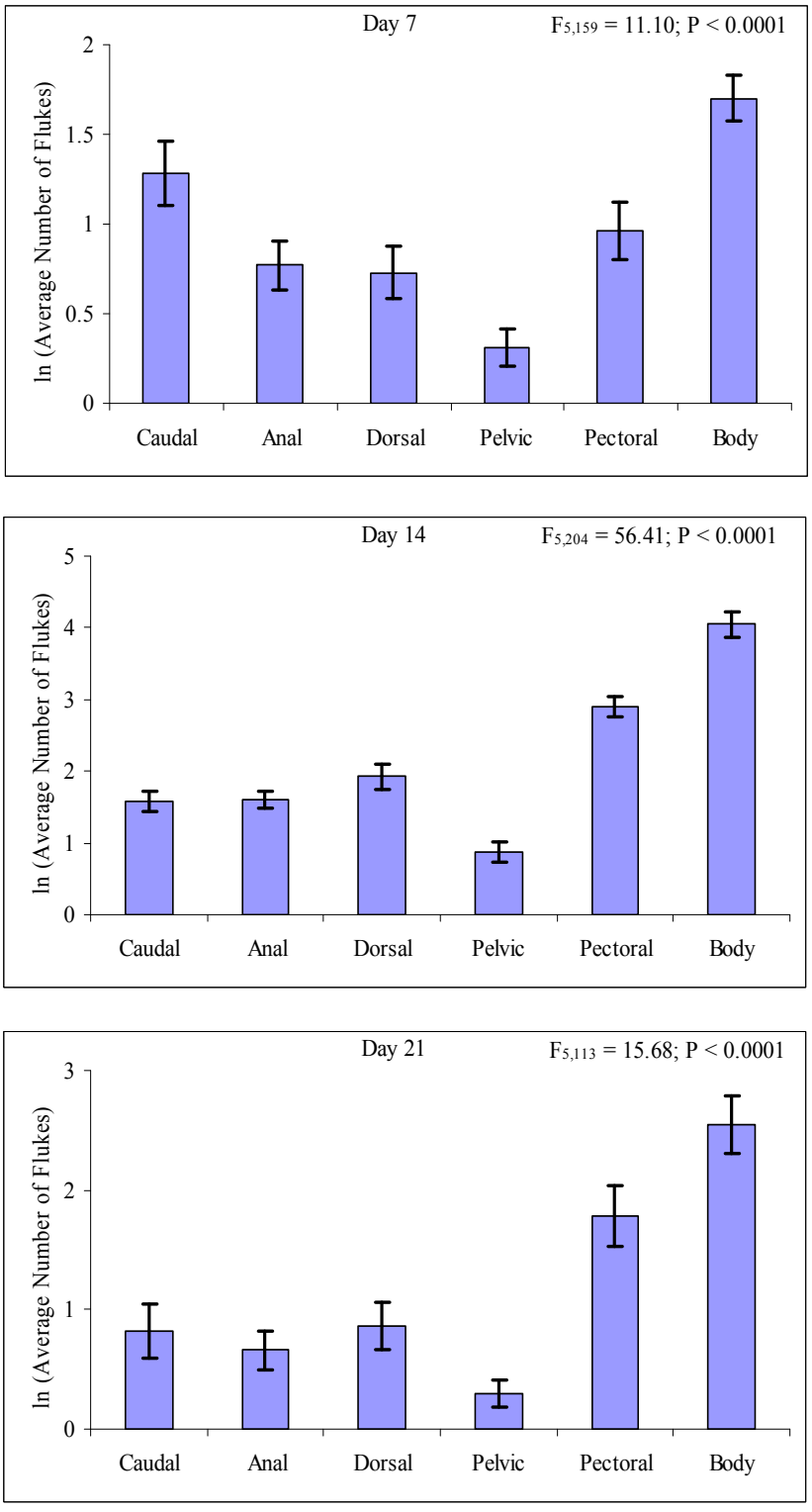

Figure 4.3. Average parasite load for each observed anatomical location on days 7, 14, and 21. Error bars represent one standard error (SE) of the mean.

Table 4.1. Comparison of initial mass (g) and standard length (mm) for treatment and control groups.

Group	Mean Initial Mass (± SEM)	Mean Standard Length (± SEM)
Treatment	1.147 (± 0.037)	33.820 (± 0.344)
Control	1.226 (± 0.083)	34.476 (± 0.646)

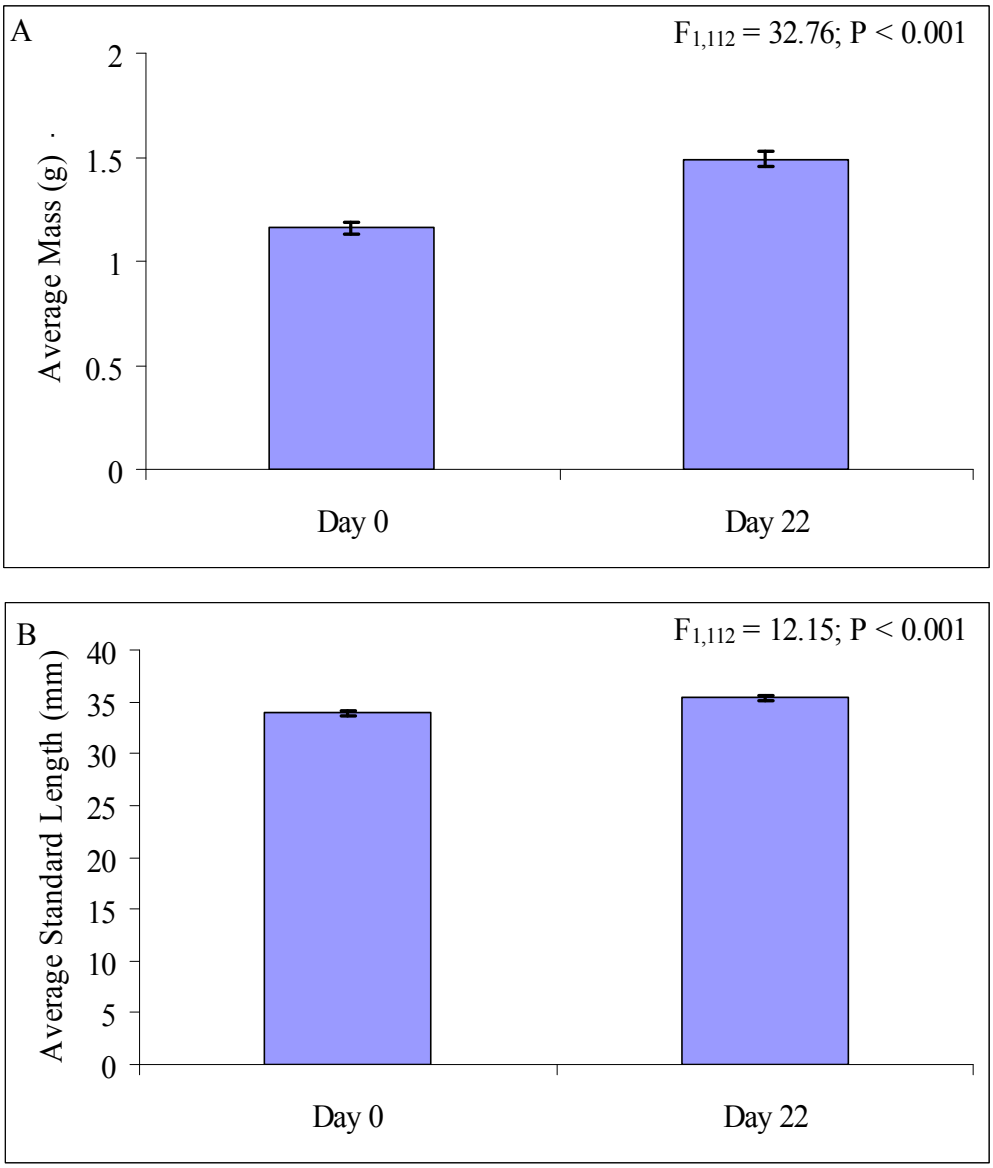

Figure 4.4. Comparison of average mass (A) and average standard length (B) for all fish, from Day 0 to Day 22. Error bars represent one standard error (SE) of the mean.

There was no effect of *G. tularosae* on growth rates in terms of mass or standard length (Mass: $F_{1,55} = 0.24$, P = 0.63; SL: $F_{1,55} = 0.79$, P = 0.38) (Figure 4.5). Likewise, fat stores did not differ between treatment (23.75% ± 0.58 SEM) and control groups (24.44% ± 0.68 SEM) ($F_{1,55} = 0.48$, P = 0.49) (Figure 4.6).

Discussion

At the individual level, parasitism by *G. tularosae* was not costly for White Sands pupfish in terms of survivorship, growth, or fat content. These results were consistent with most other species of *Gyrodactylus* (MacKenzie 1970; Cone and Odense 1984; Bakke et al. 1991; Bakke et al. 1992; Jansen and Bakke 1995; Bakke et al. 1996; Buchmann and Uldal 1997; Soleng and Bakke 2001; Sterud et al. 2002). Instances where gyrodactylids were found to be costly were generally the result of novel host-parasite associations, such as occurred with the introduction of *Gyrodactylus. salaris* to the Norwegian strain of Atlantic salmon (*Salmo salar*) (Heggberget and Johnsen 1982; Johnsen and Jensen 1986). This phenomenon has also been demonstrated with *Gyrodactylus turnbulli* infecting novel hosts such as the Gila topminnow (*Poeciliopsis occidentalis*) (Hedrick et al. 2001) and various other species of desert topminnow (Leberg and Vrijenhoek 1994). *Gyrodactylus turnbulli* is native to the guppy *Poecilia reticulata* (Leberg and Vrijenhoek 1994).

The overall course of infection in this study was similar to that of other *Gyrodactylus* studies. Moen and Stockwell (2006), in a study of *G. tularosae* infecting White Sands pupfish and sheepshead minnows (*Cyprinodon variegatus*), observed a rapid increase in fluke numbers, followed by an even more rapid decline by hour 120 post-infection. Bakke et al. (1992a) observed a similar trend, albeit longer, with *G. salaris* infecting brook trout (*Salvelinus fontinalis*); fluke infections continued to increase until

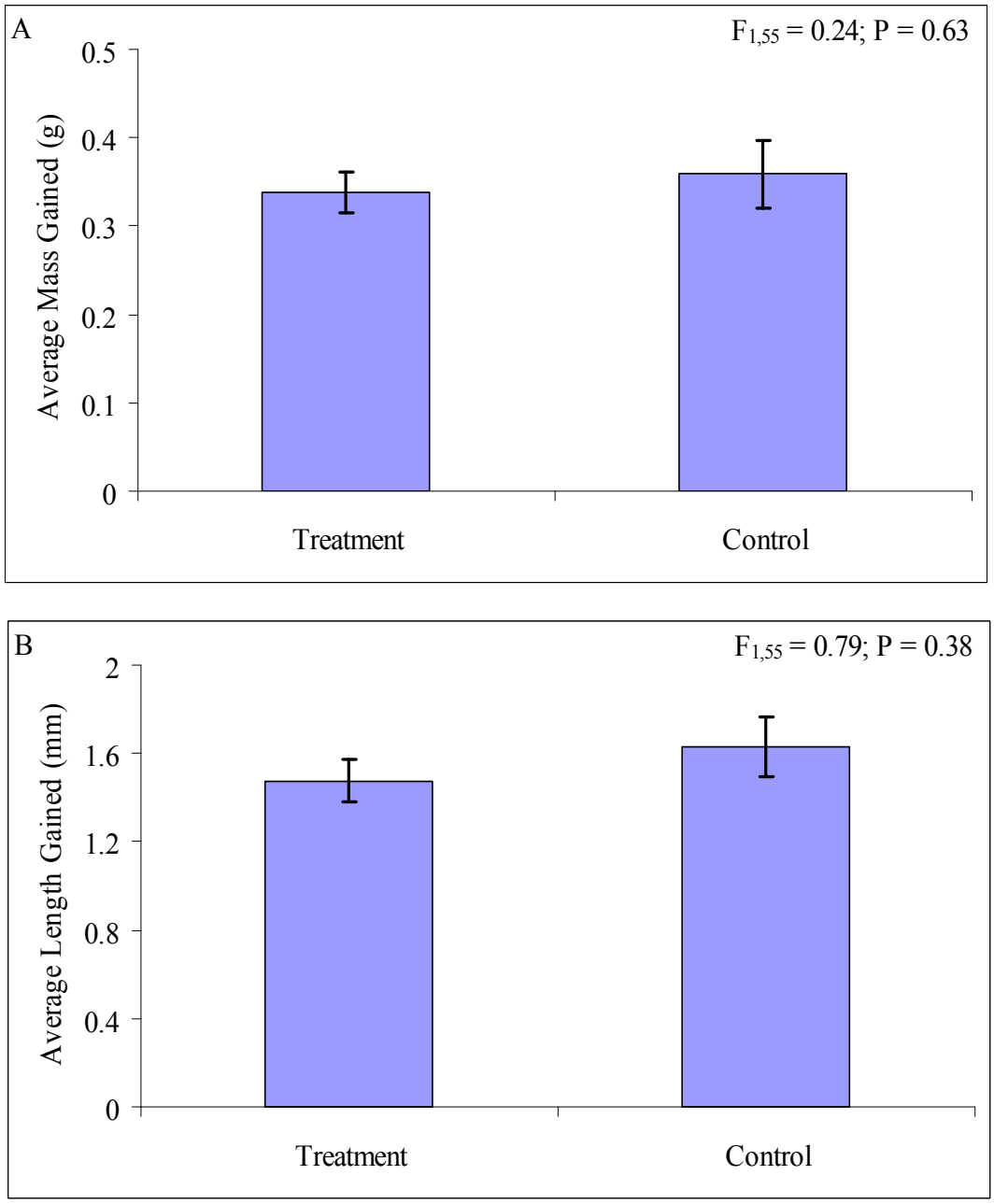

Figure 4.5. Average mass (A) and average standard length (B) gained for treatment and control groups on Day 22. Error bars represent one standard error (SE) of the mean.

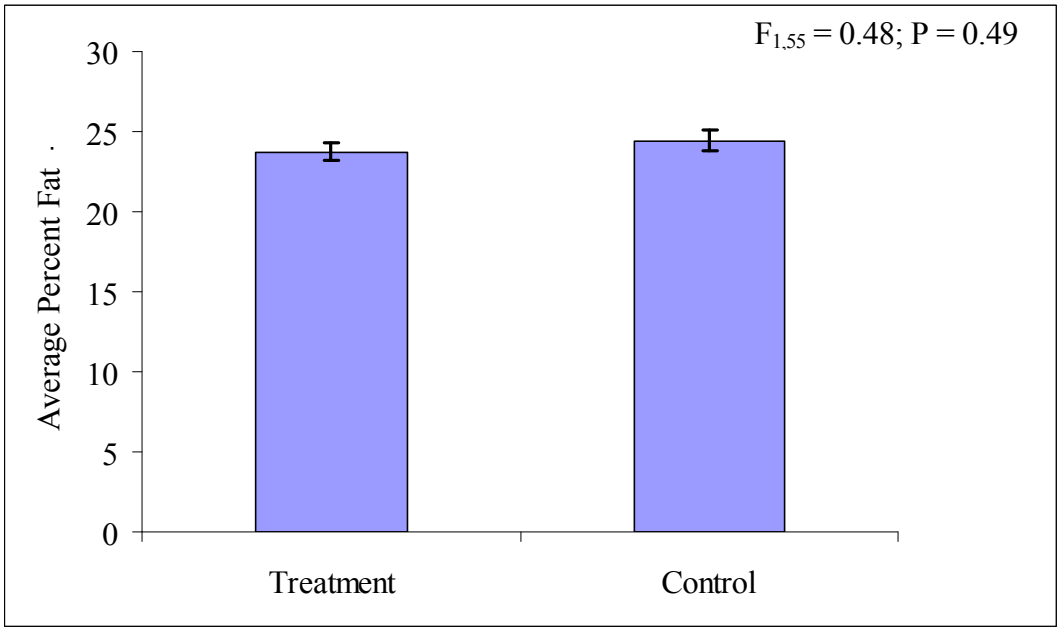

Figure 4.6. Average percent fat for treatment and control groups at the conclusion of the experiment. Error bars represent one standard error (SE) of the mean.

approximately day 30, at which point they began to decline.

Throughout this study, the highest average number of flukes was located on the body, with fins being infected to a lesser extent (Figure 4.2). It should be noted that some species of *Gyrodactylus* have been found to infect the gills of their host (Ergens 1983). Because fish would need to be sacrificed in order to effectively locate and count flukes infecting the gills, gills were not assessed for the presence of parasites during this experiment. Nonetheless, the distribution of flukes in this study was in contrast to other studies that found the fins, specifically the caudal, were more heavily infected. Cone and Cusack (1989) demonstrated that *Gyrodactylus colemanensis* infecting the fry of rainbow trout (*Oncorhyncus mykiss*) occurred most frequently on the caudal fin, followed by the pectoral and pelvic fins. Similarly, guppies experimentally infected with *G. turnbulli* had 42% and 40% of flukes located on the caudal peduncle and caudal fin, respectively, compared to only 8% on the pectoral fins, 5% on the dorsal fins, 2% on the pelvic fins, and 1% on both the anal fins, head, and flanks (Harris 1988).

It has been suggested that haptoral specializations (Harris 1988), host immune response, and crowding (Buchmann and Uldal 1997) could be responsible for changing the distribution of flukes on a host. In the instances where flukes tended to congregate on the fins, they were likely escaping from localized epithelial immune reactions (Buchmann and Uldal 1997) or increasing their chances of transfer to a new host (Cone and Cusack 1989). Since *G. tularosae* did not seem deterred from colonizing the body of its pupfish host, perhaps it did not invoke a strong enough immune response. This would be an interesting topic to address in future studies of this host-parasite system.

Closely related to host immune response is the possible pathology associated with

Gyrodactylus infections, commonly known as gyrodactyliasis. Fish in this experiment did not appear to exhibit any of the clinical symptoms described by Cone and Odense (1984) in their observations of rainbow trout infected with *Gyrodactylus salmonis*. Signs of infection included profuse mucous production, skin discoloration, frayed fins, and open sores; however, there were no signs of gyrodactyliasis associated with the four other *Gyrodactylus* species studied by Cone and Odense (1984). Clearly, the outcome of *Gyrodactylus* infections varies from (fish) species to species. A closer evaluation of *G. tularosae* pathology and host immune response may be helpful in better understanding how White Sands pupfish interact with *G. tularosae*.

Even though there was no evidence of direct costs associated with parasitism by *G. tularosae*, indirect costs may still exist. For example, swimming performance could be affected at high parasite loads. Coleman (1993) showed that parasitism by the heterophyid trematode *Ascocotyle pachycyctis* in the bulbous arteriosus of sheepshead minnows, leads to decreased swimming performance. If *G. tularosae* had a similar impact on the swimming ability of White Sands pupfish, infected individuals could be at a disadvantage when it comes to feeding ability or escaping predators and competition. Experimentally evaluating the potential influence of gyrodactylids on host swimming ability could be of potential importance where hosts are likely to encounter lotic habitats, such as White Sands pupfish often experience in Salt Creek and Lost River (Chapter 2).

Because environmental conditions can play a role in how parasites affect their hosts (Coleman 1993), it would be interesting to repeat the current study with added stressors to the fish, including different temperatures, salinities, host densities, and feeding regimes. Furthermore, parasites can affect host fitness without their effects being obvious or

regulatory (Coleman 1993). For example, Ballabeni and Ward (1993) found that the trematode *Diplostomum phoxini*, infecting the European minnow (*Phoxinus phoxinus*), did not affect host mortality, but did influence juvenile fish growth. Similarly, Lemly and Esch (1984) showed that under certain environmental conditions, the trematode *Uvulifer ambloplitis* was responsible for a 10-20% over-winter reduction of young-of-the-year juvenile bluegill sunfish (*Lepomis macrochirus*) in a small (2 ha) North Carolina pond.

Studies have also been conducted addressing the effects of gyrodactylids on juvenile fish. Cusack (1986) showed that *G. colemanensis* infecting rainbow trout fry did not influence growth or survival when compared to uninfected controls. Conversely, Cusack and Cone (1986) demonstrated that *G. salmonis* infecting brook trout fry significantly reduced fry survival. Clearly, juvenile fish infected with parasites may be affected differently than adults. Thus, it may be pertinent to assess juvenile White Sands pupfish for any costs associated with *G. tularosae* infection.

Costs of parasitism may indeed be subtle, may only be manifested under certain environmental conditions, or may affect individuals differently. Nonetheless, the approach in this study was powerful, as multiple response variables were measured, yet no evidence of a cost for any of the measured traits was found.

CHAPTER 5. CONCLUSIONS

Understanding the dynamic associations that exist between parasites and their hosts is essential to the conservation of threatened and endangered species. This is especially relevant where management protocols call for the establishment of refuge populations via translocations. The potential exists for translocations to create novel host-parasite associations which may result in increased parasite virulence (Esch and Fernandez 1993). Given its threatened status and historical (and probable future) translocations, the White Sands pupfish (*Cyprinodon tularosa*) and its associated monogenean ectoparasite *Gyrodactylus tularosae* provide an excellent system in which to study host-parasite interactions.

Prior to this study, little was known about the interactions between White Sands pupfish and *G. tularosae*. Intensive field sampling had not been conducted to assess gyrodactylid distributions within all four pupfish populations. There was also nothing known regarding fluke specificity between pupfish strains or the potential costs associated with fluke infections.

The results of field sampling in this study revealed the presence of gyrodactylids in all four pupfish populations. This finding is somewhat surprising, as fish transferred from Salt Creek to Lost River were thought to have lost their flukes because of the high levels of salinity often present in Lost River. This indicates that *G. tularosae* has a wider range of salinity tolerance than previously expected.

Even though parasite prevalence and intensity was sampled only once, in May, patterns of fluke infection did emerge among habitats. On average, the saline rivers (Lost River and Salt Creek) had higher infection intensities compared to the brackish springs

(Mound Spring and Malpais Spring); however, there was no significant difference found between parasite load and fish size, sex, or condition. Additionally, fluke prevalence was similar among sample sites, ranging from 72.5 % to 100%, and parasites were highly aggregated.

The assessment of parasite local adaptation, where Malpais Spring and Salt Creek fish were infected with Salt Creek flukes and maintained at a salinity level similar to that found in Salt Creek, revealed no preference for either strain. Infection levels on both strains were maintained until hour 192, at which point parasite loads decreased rapidly. This shows that in this host-parasite system, an isolation period of ca. 3,000-5,000 years (Pittenger and Spring 1999) and significant genetic divergence between host populations (Stockwell et al. 1998) have not been accompanied by parasite local adaptation.

No costs were found associated with parasitism of White Sands pupfish by *G. tularosae*. Variables considered in the study included survivorship, growth rate (standard length and mass), and fat content. Infections followed a pattern of rapid increase in fluke numbers, followed by a rapid decline. These results indicate there is no innate pathogenicity associated with *G. tularosae*.

The overall relationship between White Sands pupfish and *G. tularosae* appears benign. Nonetheless, the dynamic interactions in host-parasite systems, together with the threatened status and management protocols of White Sands pupfish, merit the continued study of this relationship. Given the ubiquity of gyrodactylids and the continued introduction of exotic species, any translocations have the potential to produce novel host-parasite associations. The potential outcomes of these situations should be well understood to avoid costly management mistakes.

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