IMPACT OF AN UNDESCRIBED HETEROPHYID TREMATODE ON THE FOUNTAIN DARTER *ETHEOSTOMA FONTICOLA*

THESIS

Presented to the Graduate Council of
Southwest Texas State University
in Partial Fulfillment of
the Requirements

For the Degree of

Master of SCIENCE

By

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San Marcos, Texas
May, 2000

ACKNOWLEDGEMENTS

I would like to thank the U.S Fish and Wildlife Service for funding this project, with special thanks to the San Marcos National Fish Hatchery and Technology Center for the use of their equipment, facilities and personnel. Thank you, Dr. T. M. Brandt for the advice, time and effort you have put into making this project a success, and for talking me into going to graduate school in the first place. Thanks to Dr. David Huffman for chairing my committee, your guidance, attention and patience have been priceless, thank you for being a friend. And to Dr. Bobby Gene Whiteside, thank you for the fishing stories and the incredibly frank editing comments. Thanks also to A. J. Mitchell and A. E. Goodwin for collaborating on the report for this study and to R M. Overstreet and H. D. Murray and his students for providing valuable background information.

Thanks to all the professors in the SWT biology program who have made this an incredible learning experience, and to all the graduate students at SWT who have made this an experience Thanks also to J. N. Fries and M. V. Estrada for all the help out at the hatchery. Special thanks to the "gang", C. A. Kaczmarek, C. M. Cardenas, L. R. Lindsay IV, V Cantu, J. W. Miller, K. S McDermott, D. M. Levine and C. J. Stanley for helping me with this project and for helping me recover from it Even more special thanks to P E Schaefer for helping me retain enough of my sanity during that final semester to get this thing done and turned in Most of all, thanks to Noel, Pat and the boys for your support and encouragement. Thank you, thank you all.

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ABSTRACT

An undescribed heterophyid trematode causes considerable pathogenicity in fountain darters *Etheostoma fonticola* collected from the Comal River, Texas Survivorship was monitored under refugium conditions and compared between wild fish collected from two sections of the Comal River, where the parasite is well established, and from two sections of the San Marcos River where it is not yet epizootic. Significantly lower survival was seen for fish collected from one section of the Comal River Wild-infected fish were challenged in a sealed-jar hypoxia test and no correlation was found between lethal dissolved oxygen (DO) values and parasite load. Also, five groups of lab-infected fish were challenged in identical hypoxia tests at various times post-exposure to see if DO tolerance changes as time progressed after exposure The correlation for the lab-infected fish between decreasing mean lethal DO and increasing time post infection was significant, as well as the correlations between increasing parasite load and lethal DO for each group. Uninfected lab-reared fish were also tested in the sealed-jar hypoxia test in order to ascertain that the scattering of lethal DO values immediately after exposure was not due to handling stress The mean lethal DO of the infected fish was significantly higher than the mean lethal DO of the uninfected fish immediately after exposure. It was determined that this gill parasite does have an adverse affect on the fountain darter and that heavy parasite loads could impact the health of the fish

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Introduction

General background.---The fountain darter *Etheostoma fonticola* is federally listed as endangered [U.S. Office of the Federal Register 35 (13 October 1970)· 16047] It is endemic to two Central Texas rivers, the San Marcos and the Comal, which are both spring outflows from the Edwards Aquifer. During periods of normal and above normal flow, both rivers have year-round temperatures of ~22-23 °C (U.S. Fish and Wildlife Service 1995). Concern for the survival of the fountain darter has increased due to declining Edwards Aquifer water levels and the resulting reduced spring flows during the late 1980's and the 1990's

Refugia for fountain darters were established at the National Fish Hatchery and Technology Center, San Marcos, Texas, (NFHTC), and at the Uvalde National Fish Hatchery, Uvalde, Texas, in response to the 1996 Central Texas drought A collection program was initiated to maintain and supplement refugium populations using wild fish from both rivers During a collection from the Comal River in October 1996, NFHTC staff members noticed that the majority of the fish had swollen gills. A preliminary examination of the gills revealed heavy infections of trematode metacercarial cysts. These fish appeared to be severely affected, which caused concern since there was no indication from the literature that parasites had ever before threatened the fountain darter There was speculation that this abrupt epizootic could have been triggered by a variety of factors associated with low water conditions caused by the 1996 Central Texas drought. It was further speculated that reduced respiratory efficiency of the affected gills, in combination with degraded water quality due to low-water conditions, represented a serious threat to

the survival of wild populations of the fountain darter

Fountain darters from both the Comal and San Marcos rivers were shipped to the Bozeman Fish Technology Center for examination. All of the fish from the Comal River were moderately to severely infected with the trematode metacercariae, but no cysts were found on the gills of the fish from the San Marcos River. The infection in some of the Comal fish had progressed to such a degree that proliferation of tissue had occurred, resulting in a pronounced deformity of gill filaments and loss of normal gill structure (B. MacConnell, Bozeman Fish Technology Center, personal communication)

The taxonomic identity of this trematode has not yet been established. A similar trematode was reported in Florida by Blazer and Gratzek (1985), but there are some discrepancies between the physical description of their unidentified trematode and that of the Central Texas species. Knott and Murray (1991) reported a trematode in the San Antonio River identical to the trematode in the Comal River. It is undescribed, but it can be keyed using Schell (1970) to the family Heterophyidae Based on what is currently known about the life cycle, morphology and pathology of the Central Texas trematode, it appears similar to *Centrocestus formosanus*, a heterophyid trematode which has been reported to cause considerable pathology in the gills of cultured cyprinids (Lo and Lee 1996a; Velez-Hernandez et al. 1998). Because of the similarities between *C. formosanus* and the undescribed heterophyid, *C. formosanus* was used as a model to guide the design of this study.

Generalized heterophyid life cycle.---The typical heterophyid digenetic trematode life cycle, according to Schmidt and Roberts (1989), involves alternation of generations and utilizes two or three different hosts. The sexually mature adult fluke is parasitic in a vertebrate definitive host. The trematode reproduces in the definitive host and the eggs are shed from the host's body, usually in the feces. The eggs either hatch in the water, in which case the free-swimming miracidia actively seek out and penetrate a snail, or they are taken

up by the snail during feeding and hatch in the intestine. Depending on the species, a successful miracidium may develop into a sporocyst or a redia stage, which begins producing cercariae, a free swimming stage. The cercariae emerge from the snail and enter the second intermediate host, usually a vertebrate, and develop into the next larval stage, the metacercaria The mature metacercaria awaits ingestion by the definitive host where the worms mature and reproduce.

Centrocestus formosanus ---The first intermediate host of *C. formosanus* is usually a snail of *Melania* species (Nishigori 1924) in which a sporocyst stage and one redial generation were observed (Martin 1958). *Centrocestus* cercariae exit the snail through its exhalant current (Lo and Lee 1996b) and enter the gills of the second intermediate host, usually a fish, by being sucked into the mouth with the respiratory current (Salgado-Maldonado et al. 1995) Nishigori (1924) found *Centrocestus* metacercariae to attain infective maturity at 18-20 d post-infection, and Chen (1948) reported the disappearance of eyespots and development of an X-shaped excretory vesicle, which signals infective maturity, after a period of 1 month Possible definitive hosts obtained through feeding experiments include dogs, cats, rabbits, guinea pigs, white rats, mice and humans. Natural infections were found in *Nycticorax nycticorax* (Nishigori 1924), *Rana limnocharis*, *Bufo melanostictus,* and *Rattus rattus* (Chen 1942).

First intermediate host.---The mollusk host for the Central Texas trematode has been identified as *Melanoides tuberculata,* a widely distributed thiarid snail (Knott and Murray 1991). This snail was introduced from the Orient, probably through the aquarium trade, and was reported in the San Antonio River for the first time in 1964 (Murray 1964). It can serve as a first intermediate host to many different larval trematodes, some of which are detrimental to man. Ismail (1990) cites research showing that more than 50 different forms of cercariae have been recovered from *M. tuberculata.* Cercariae of the Central Texas trematode have been found in 1,008 out of a sample of 3,600 *M. tuberculata* taken

from the Comal River during 1997 and 1998, but in only 1 out of 3,600 snails examined from the San Marcos River during that same time period (Mitchell et al. 2000).

Second intermediate host ---H D. Murray (Trinity University, personal communication) successfully infected local fishes of the families Characidae and Poeciliidae with this parasite and subsequently used these metacercariae to infect domestic chicks. Metacercariae have been found in the gills of many different species of fish from the Comal River, in addition to the fountain darter (S. J Bergin, U.S. Fish and Wildlife Service, personal communication). However, metacercariae encysted in fountain darter gills are almost always found dead and encased by thick layers of host reaction tissue (Mitchell et al 2000) Also, no cysts have been found without eyespots in the darter, which means that the metacercariae do not live long enough in the darter to develop infective morphology. This indicates that the parasite cannot complete its life cycle utilizing the fountain darter as a second intermediate host.

Definitive host ---It is believed that the principal definitive host for this trematode in Central Texas is the Yellow-crowned Night-Heron *Nyctanassa violacea* High prevalence of the parasite has been found in snails and fish in close vicinity to Yellow-crowned Night-Heron nests in the San Antonio Zoo waterways (Kothari and Murray 1992), and rookeries for these birds have been found along the banks of the San Antonio and Comal rivers. There is some concern that rookeries may ultimately be established on the San Marcos River, thus potentially threatening the population of fountain darters there as well

Pathogenicity ---Trematode metacercariae can cause considerable damage in the gills of a piscine host, both mechanically, because of a reduction in gill surface area available for gas exchange, and pathologically, because of adverse host reactions to encystment. Mass mortalities have been caused by *C. formosanus* infections in the gills of elvers *Anguilla japonica* (Yanohara and Kagei 1983), loaches *Misgurnus anguillicaudatus* (Tung et al. 1989), and of carp fry and fingerlings *Cyprinus carpio*

(Subasinghe 1992). Velez-Hernandez et al (1998) reported *C. formosanus* cysts associated with moderate to severe hyperplasia of primary gill lamellae and lymphoid tissue in *C. carpio*. Metacercariae can be particularly pathogenic to the second intermediate host at the moment of contact because of damage done by penetration organs and enzymes secreted by the parasite upon attaching to the host. Ginetsinskaya (1968) reports death from tissue damage in insect larvae and tadpoles immediately after exposure to styleted cercariae

The Central Texas trematode ---It is possible that the encystment of the metacercariae in the gills of the fountain darter may cause some initial damage that surviving fountain darters may adapt to over time, and so fish with more recently acquired metacercariae may be in a higher state of stress than fish which have had a sufficient recovery period. On the other hand, the hyperplasia that follows death and encapsulation of the parasite may later interfere with respiratory flow to an extent that the stress resistance of the fish may again decline, this time without recovery.

Preliminary findings by Mitchell et al (2000) suggest that continued survival of the wild fountain darter population may be jeopardized by the presence of this parasite in the Comal River. Histological examinations have shown parasites encased in masses of cartilage which caused the swelling of gill filaments to several times their normal thickness The number of lamellae was greatly decreased and filaments were short, thick and distorted. Wet-mount microscopy demonstrated extensive proliferation of gill epithelium and cartilage deformity. If this parasite population continues to expand in the Comal River, and eventually spreads to the San Marcos River, the fountain darter population in the wild could be compromised, and management of the refugium populations would have to be adjusted accordingly

Objectives.---There are two objectives in this study. The first is to estimate the impact of this trematode on the fountain darter in the wild. Hypoxia tests will be run on

wild-infected fish in order to determine if the presence of the trematode increases the darter's vulnerability to low dissolved oxygen (DO) levels. In addition, survivorship will be compared between wild-caught fountain darters from both rivers to see if the river of origin (and presence or absence of trematode infection) influences survival of fish under typical refugium conditions. The second objective is to investigate the parasite's effect on the host after a single heavy exposure Uninfected fish will be exposed to a brief pulse of cercarial infection pressure and the variation in their vulnerability to low DO will be followed as it progresses over time using hypoxia tests.

Methods

Survivorship of refugium fish.---Survivorship of refugium fountain darters collected from populations known to be heavily infected with the trematode (Comal River and Landa Lake) was compared to fountain darters from populations in which the parasite is not epizootic (San Marcos River) Fifty fountain darters 20-32 mm in total length were collected from two sections on the Comal River and two sections on the San Marcos River, for a total of 200 fish The sections (See Mitchell et al. 2000) are as follows. 1) Comal River, 2) Landa Lake, 3) upper San Marcos River and 4) middle San Marcos River

All fish were collected with dip nets over a 3 d period , with 10 fish taken from each of the four sections on the first and second day in the morning and in the afternoon, and 10 fish taken from each section on the third day in the morning The fish were transported to the NFHTC in 0.5% saline river water (40 g NaCl / 8 L water from the collection site) in separate 15 L ice chests (two ice chests of five fish per section per trip) Since the Comal River is further from the NFHTC than the San Marcos River, transport times varied with the longest collection and transport time being 2 h. Therefore, the fish in each ice chest were held for 3 h from the time of capture of the first fish in that ice chest

to equalize the transport-stress variable across the sections.

Before being transferred into the experimental aquaria, all fish were prophylactically treated in the ice chests for external parasites and fungi. The treatment for each ice chest began 3 h from the capture of the first fish in that ice chest. The treatment protocol consisted of adding 2 mL of formalin to the 8 L of water in each ice chest (250 ppm formalin) and then aerating for 1 h At the end of the 1 h treatment, the fish were dipped out of the ice chests with aquarium nets and transferred to their respective experimental aquaria.

Four 720-L fiberglass tanks (Frigid Unit, Toledo, Ohio) were used as recirculation reservoirs for the experimental aquaria, with each reservoir tank serving water to ten 10-L glass aquaria mounted over the reservoir tank and holding 50 fish from one of the four sections On the first day of collection, the five fish from one of the ice chests from the Landa Lake section were placed into aquarium 1 and the five fish from the other ice chest from the Landa Lake section were placed into aquarium 2 on the reservoir tank serving the Landa Lake section. The same pattern was used to distribute the fish from the other three sections that day into aquaria 1 and 2 being served by the corresponding reservoir tank for that section. Fish were collected again in the afternoon of that same day, and aquaria 3 and 4 on each reservoir tank received the fish from the appropriate section. This procedure was repeated for aquaria 5, 6, 7 and 8 for sampling day 2, and for aquaria 9 and 10 for sampling day 3

The fish were fed either blackworms (Aqualife, Friant, CA) or zooplankton (from hatchery ponds) and waste was siphoned from the tanks three times a week

Water temperature in each reservoir tank was maintained at 21 ±1 °C using a temperature control unit linked to a 0.5 horse-power chiller and a 1000-W heater (Universal Marine Industries, Inc , San Leandro, CA). The aquaria and recirculating tanks were filled with Edwards Aquifer water from the NFHTC wells (total hardness, 270 mg/L as $CaCo_3$; pH 7.1; Ogden et al. 1985). Temperature, total dissolved gases (Sweeney

Aquametrics Saturometer Model Ds-1B, Stoney Creek, Connecticut), pH (Unifet FieldLAB-100, San Diego, California) and DO (YSI Model 95 Dissolved Oxygen Meter, Yellow Springs, Ohio) were monitored at least weekly and tank systems were adjusted accordingly to maintain uniform water quality among tanks

A second formalin prophylaxis treatment was performed on the fish after 5 weeks in the aquaria. Formalin was mixed into the reservoirs at a 250 ppm concentration, recirculated for 1 h, and then drained and refilled with fresh water from the well and recirculation restarted. After 1 h, the reservoirs were drained and refilled once more.

The aquaria were checked for mortalities daily and dead fish were removed immediately and preserved in 10% formalin. No less than 24 h after preservation, the gill arches on the right side of the fish were removed and examined microscopically at 100X magnification for metacercarial cysts Data recorded for mortalities included: date fish were collected, site of origin, date of death, number of days survived in captivity, total length, sex, number of trematodes per gill arch on the right side and an estimated number of trematodes per fish Raw data for this experiment are listed in the appendix in Table 1

The experiment ran for 70 d for all groups of fish. A one-way ANOVA (α=0.05) was run among the four river sections to determine if fish from sections where the parasite is prevalent (Comal River and Landa Lake) have a lower survival rate than fish from sections where the parasite is not epizootic (upper and middle San Marcos River). After the survivorship test, the living fish became part of the refugium at the NFHTC.

Hypoxia test on wild fish ---Lethal DO was determined for naturally infected wild fish from the Comal River using a sealed-jar hypoxia test (Mazik et al. 1987). The purpose of this test was to describe any relationship between parasite load on the gills and intolerance for low DO.

Twenty fish 30-35 mm in total length were collected 240 to 370 m downstream of the Elizabeth Street low water crossing (a site where the undescribed trematode was known to have high intensity in fountain darters) and transferred to the lab in ice chests

filled with water from the Comal River. Water from the collection site was filtered through 12 5-cm ashless filter paper (Whatman, Maidland, England) and used in the experiment to reduce water quality stress. Twenty 60-mL jars were filled with water from the site, excluding all air. One fish was placed into each jar, and timing began when the lids were sealed onto the jars. The approximate time of death and lethal DO (mg/L) were recorded for each fish The "death" endpoint for each fish was defined as cessation of gill movement and lack of response to lifting and inversion of the jar The jars were checked at least every 30 min, and for the most part the actual death "endpoint" was known to have occurred within a 10 min time period. For the lethal DO reading, the jar lid was removed and replaced quickly with an otherwise identical lid fitted with the DO probe The dead fish remained in the jar until the reading on the instrument stabilized, at which time the lethal DO value was recorded The fish were then removed from the jar and preserved in 10% formalin The fish were dissected no less than 24 h after preservation for parasite counts All gill arches on the right side of the fish were removed and examined for parasites under 100X power on a compound microscope. The number of trematode metacercariae per fish was estimated by doubling the number found on the right side Raw data are listed in Table 2 in the appendix. A simple linear regression (Zar 1999) was performed with metacercariae per fish as the independent variable and lethal DO as the dependent variable to see if there is a relationship between lethal DO levels and parasite load

Progression of host reaction in lab-reared fish.---The progression of host reaction to encystment of trematode metacercariae was investigated by determining lethal DO at various times post-infection using a sealed-jar hypoxia test identical to the test used for the wild-infected fish Lab-reared lab-infected fountain darters 30-34 mm in total length were used in this experiment. The fish were exposed to a high number of cercariae to increase the likelihood that a detectable response would be elicited.

A preliminary trial-and-error assay was conducted to estimate the maximum sub-

lethal density of simultaneously acquired metacercariae a typical lab-reared fountain darter could tolerate. Collection of cercariae and exposure of fish to the cercariae were done using methods adapted from Lo and Lee (1996a). Through trial and error it was estimated that a parasite load of 450 cysts per fish caused sufficient stress to impact the health of the fish. In order to obtain a load of 450 cysts from one exposure under laboratory conditions 120 fountain darters were exposed to approximately 660,000 cercariae (5,500 per fish) in 25 L of well water in an aquarium with aeration. After 1 h, one fish was removed from the aquarium and sacrificed to see if it had acquired a sufficient parasite load. The fish had less than 100 cysts, so the exposure continued. Another fish was removed and sacrificed after 2 h and again did not have the desired number of cysts so the exposure continued for another h After the fish had been exposed to the cercariae for 3 h, about half of the fish began to exhibit loss of equilibrium. Many also exhibited listlessness and a few began piping for air at the surface. All of the fish were removed from the exposure tank at this time and placed into a 20-L bucket in 10 L of fresh well water.

The 120 fish were distributed from the bucket one at a time into ten 10 L aquaria using a distribution procedure designed to avoid any sequential sampling bias. The first 10 fish were distributed into 10 separate aquaria so that the aquaria at first had only 1 fish each The next 10 fish were distributed the same way so that each aquarium had two fish, and so on, until all 120 fish were distributed and each aquarium had 12 fish. The two extra fish in each aquarium were maintained as spares in case of mortality before hypoxia tests were run.

Immediately after the fish were distributed to the 10 aquaria, two aquaria were randomly chosen from the 10, and 10 infected fish were removed from each aquarium and simultaneously tested for low DO tolerance in a sealed-jar hypoxia test. Twenty more of the fish were tested for low DO tolerance 1 d post-infection in the same manner as for the 0 d post-infection test. The test was repeated again after 3, 7 and 14 d.

The lethal DO at the death of each fish was recorded along with the total length

of the fish in mm and the time from the start of the experiment to death. Within 30 min of death, the fish were preserved in a 10% formalin solution. The formalin was allowed to penetrate for at least 24 h before the gills were removed for metacercarial counting to determine parasite load and distribution Raw data for each run are listed in Tables 3-7 in the appendix.

Simple linear regression was performed between groups with time post-infection as the independent variable and lethal DO as the dependent variable The effects of parasite load on tolerance to low DO were analyzed by regressing lethal DO against parasite load at each time post-infection. Parasite load was also regressed against total length of fish to check for confounding effects due to host size. Correlation values were also derived for every regression.

A set of 120 uninfected lab-reared fountain darters was used as a control to determine if any variation in lethal DO on the first day of the experiment could be due to handling stress. The fish were held in the same aquarium used for the exposure for 3 h and then distributed into aquaria in the same manner as the infected fish. Twenty of these fish were tested in a hypoxia test identical to the one used for the lab-infected fish Their mean lethal DO at 0 d was compared to the mean lethal DO for the lab-reared lab-infected fish at 0 d using a t-test at $\alpha = 0.05$.

Results

Survivorship of refugium fish

During collection, many of the fountain darters taken from the Comal River had bloody, swollen, inflamed gills extending beyond the operculum Some of the fish showed visible signs of distress during the transport and treatment such as loss of equilibrium and a lack of reaction when the cooler was moved. None of the fish taken from the San Marcos River had any inflammation or swelling in the gills. Some showed visible signs of distress like the fish from the Comal, but not to the same extent. Dissections of fish that had died during the study showed that the gills of the Comal River fish appeared as

described by Mitchell et al (2000) and the gills of the fish from the San Marcos River appeared healthy The 18 fish from the Comal River section had a mean parasite load of 109 cysts per fish with a range of 22-330, while the only fish that died from the Landa Lake section had 26 cysts. None of the 10 dead fish from the San Marcos River were infected

At the end of the 70 d quarantine period, the Comal River section had the fewest survivors (32 of 50), followed by the upper San Marcos River (44 of 50), the middle San Marcos River (46 of 50) and Landa Lake (49 of 50) (Figure 1) The Comal River section had a significantly lower survival rate than all of the other sites (P=0 003) The other sites were not significantly different from one another Five of the six fish that died from the middle San Marcos River site were from the same aquarium.

Hypoxia test on wild-infected fish

All 20 of the fish taken from the Comal River had bloody, inflamed gills which extended beyond the operculum In most of the heavier infections, the gill lamellae appeared fused and the swelling and inflammation made the actual gill structure difficult to ascertain. The correlation between lethal DO and parasite load had an r^2 value of 0.14 (P=0.1002) (Figure 2), which does not show a relationship between lethal DO and parasite load. The mean lethal DO was 1.53 with a range of 0 96-2.25. The fish had a mean parasite load of 710.7 cysts per fish with a range of 396-1614 cysts per fish, 1614 being the highest recorded parasite load on a fountain darter to date in this study and in Mitchell et al. (2000). The range of time to death for the fish in the jars was approximately 7-11 h ± 30 min after beginning the test. There did not seem to be any relationship between time of death and lethal DO.

Progression of host reaction in lab-reared fish

Five fountain darters died during the exposure and two fish were found dead in their aquaria the first day after the exposure. The estimated numbers of metacercariae on the five fish which died during the exposure were 292, 348, 378, 428, and 464 The

estimated numbers of metacercariae on the fish which died the next day were 274 and 350 The estimated mean number of metacercariae on all lab-infected fish in the single exposure was 262.7 with a range of 156-544 cysts per fish. None of the lab-infected fish had inflamed or bloody gills as seen in the wild-infected fish. The correlation between mean lethal DO and time post-infection (Figure 3) showed a broad scattering of lethal DO values immediately after exposure followed by less variance and lower mean lethal DO values over time post-infection (r^2=0 26; P<0 0001). The correlations of lethal DO and parasite load for each run (Figures 4-8) had significant r^2 values of 0.29 (P=0.0142), 0.34 (P=0 0075), 0.32 (P=0 0089), 0 59 (P<0 0001) and 0 31 (P=0.0106) for 0, 1, 3, 7 and 14 d post-infection, respectively The slopes for all regression equations were positive which showed reduced tolerance to low oxygen with an increase in parasite load. The correlation of parasite load with total length had an r^2 value of 0 01 (P=0 3229) which means that there were no size effects (Figure 9) The mean DO of 2 62 for the group of infected fish (range of 1 52-6.31) which were run immediately after the exposure was significantly higher than the mean DO of 1 03 (range of 0 87-1.24) for the group of uninfected lab-reared fish (P<0.0001) (Figure 10).

Discussion

Survivorship of refugium fish

The significant difference in survival for the fish from the Comal River section shows that these fish may be less fit than the fish from the other three sections Since only one fish died from the Landa Lake section, parasite load cannot be compared for the two sections to see if the difference in survival was due to cysts in the gills. However, since there is still a standing stock of fish at the NFHTC for fish from both sections, mortalities from the Comal River and Landa Lake section could be monitored continuously over time and parasite loads counted for all of the fish which die in refugium. It was unexpected that mortality for the fish from Landa Lake was so low. The mean parasite load of fish in the

size group collected is not known, but this area was seen to have a high load from previous sampling for other studies and for fish in a different size group for this study The fact that 5 of the 6 fish which died from the middle San Marcos River section suggests that these mortalities may have been due to some factor not related to sampling location, such as inadequate water flow to the aquarium or handling stress during collection

To avoid mortality due to old age in refugium and during this study, longer fish were rejected and it was assumed that the collected fish were younger Younger fish were expected to have lighter parasite loads since they were exposed to infective pressure for a shorter amount of time Since all of the fish from the Comal River that died during the study had only 22-330 cysts, which is a light to medium load, it is possible that this test was not sensitive enough to observe impact due to parasite load in both sections. If older fish with more parasites had been used in this study, perhaps a much lower survival in the Landa Lake fish would have been seen. In future collections for the refugium, it might be prudent to take smaller fish in order to assure higher survival in refugium

Hypoxia test on wild fish

It was unexpected that the correlation between lethal DO and parasite load was so low and that there was not a much steeper slope of the regression line. From the outward appearance of the gills, it would be assumed that these fish would be much more sensitive to low DO than the hypoxia test illustrated. It was hypothesized at the beginning of this experiment that fountain darters in the wild acquire the cysts a few at a time over an extended time period, and the fish may have physiologically adapted to using less gill tissue for gas exchange.

Progression of host reaction in lab-reared fish

It was hypothesized that the fish would experience a dramatic increase in sensitivity to low DO immediately after a pulsed exposure to infective cercariae due to penetration and initial encystment with a decrease in trauma after the initial stress subsided. It was also expected that sensitivity to low DO would increase again once

inflammatory duress had abated but hyperplasia had progressed The fact that the group of infected fish had a mean lethal DO which was significantly higher immediately after exposure than the mean lethal DO for the uninfected fish suggests that the higher lethal DO values in the infected fish was due strictly to stress of infection and not handling There was a drop in the mean lethal DO of the infected fish after 24 h, and the mean DO remained low for the duration of the test sequence. It appears that the fish are under the most stress immediately after exposure to cercariae, and that recovery continues through the first 14 d of the infection. Dissections of the fish which were tested 14 d post-infection did not reveal the hyperplasia seen in the wild-caught fish, so no conclusions can be drawn from this experiment concerning the effects of hyperplasia in the gill cartilage on sensitivity to low DO. Future experimentation might include exposure to infective cercariae with longer intervals between hypoxia tests to see if the mean lethal DO begins rising as hyperplasia progresses. It was surprising that there were no visible signs of trauma to the gills in the experimental fish It may have been that if the fish had been dissected immediately after death instead of after having been preserved for 24 hours that swelling and inflammation might have been more readily noted Gill trauma had been very obvious in preserved wild-infected fish, but preservation might have disguised some of the more subtle effects such as redness and irritation, and swollen and bloody gills.

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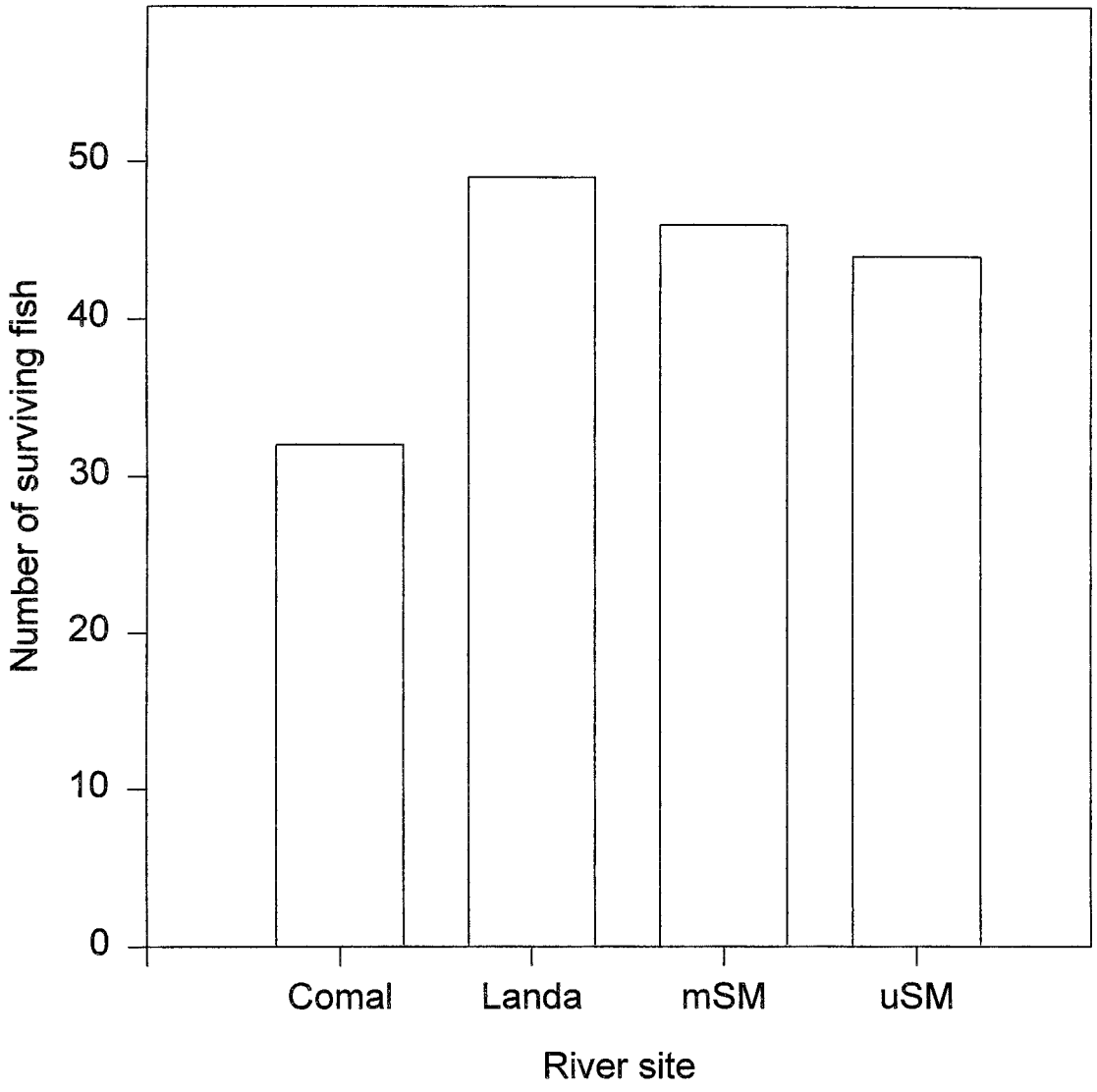

FIGURE 1.---Survival in refugium of groups of 50 fountain darters from the Comal River, Landa Lake, the middle San Marcos River (mSM) and the upper San Marcos River (uSM), collected August, 1999.

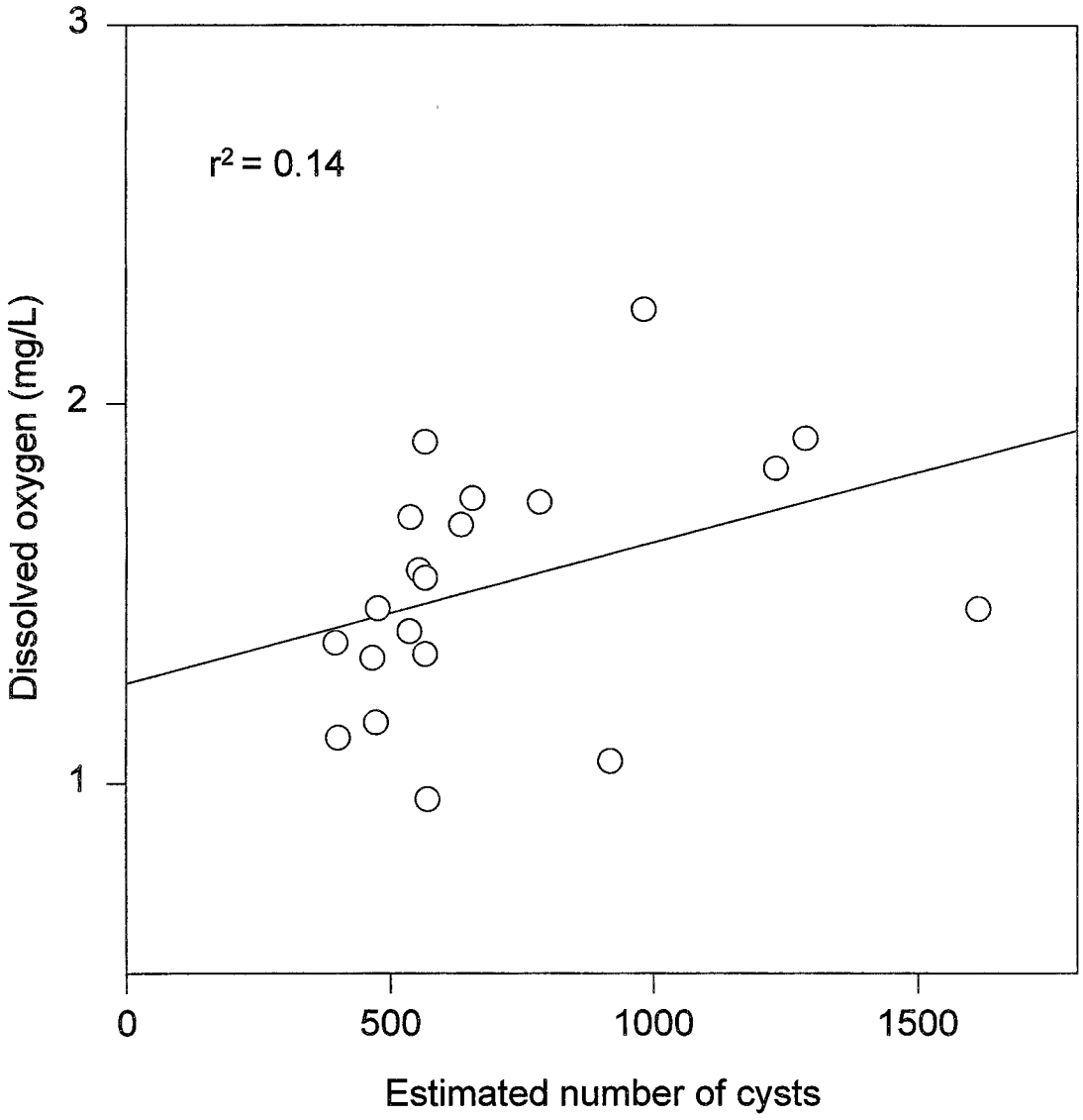

FIGURE 2.---Relation between lethal dissolved oxygen levels and parasite load for wild fountain darters from the Comal River, Comal County, Texas (P=0.1002).

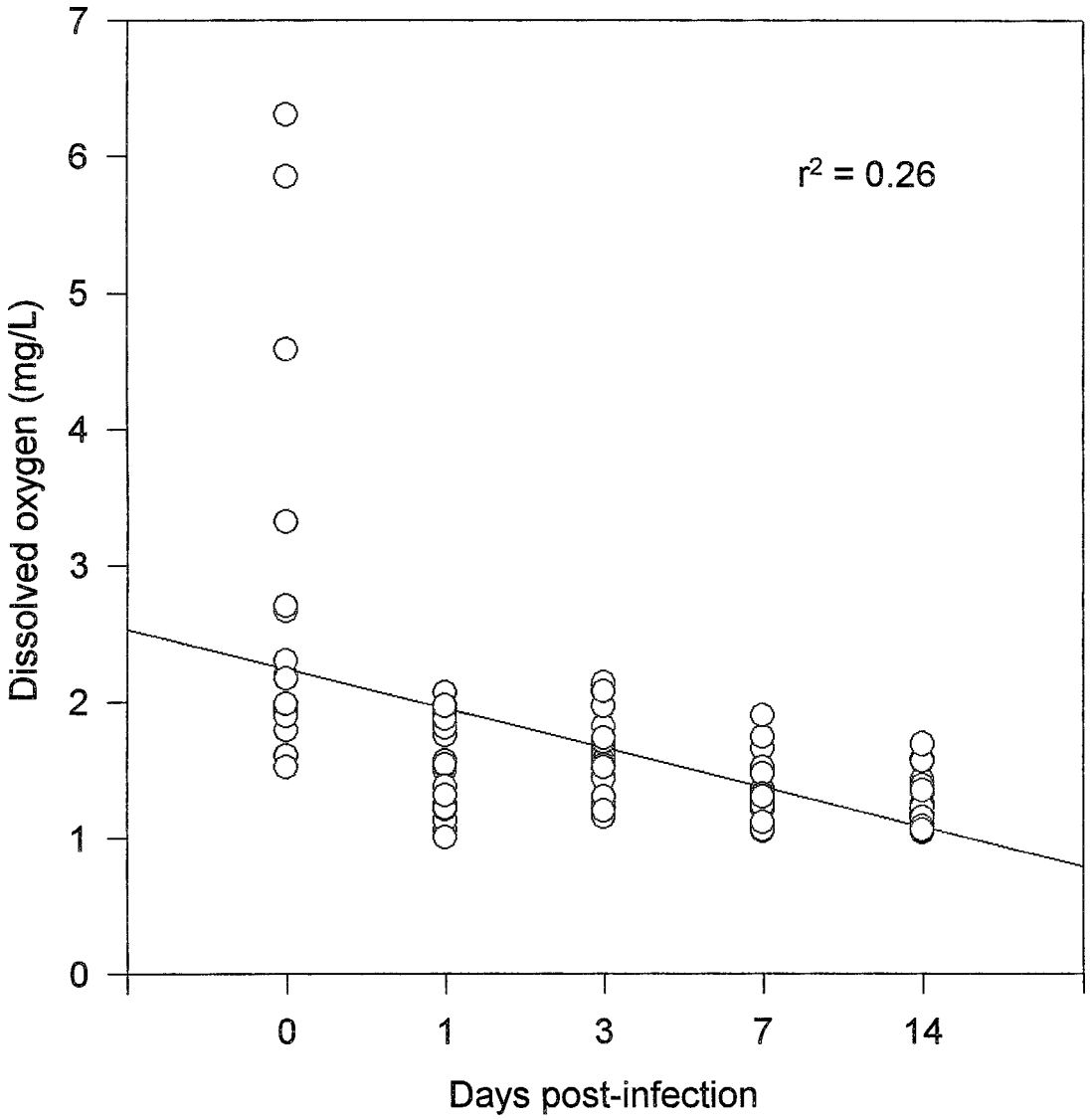

FIGURE 3.---Relation between mean lethal dissolved oxygen levels for lab-infected fountain darters over time ($P<0.0001$).

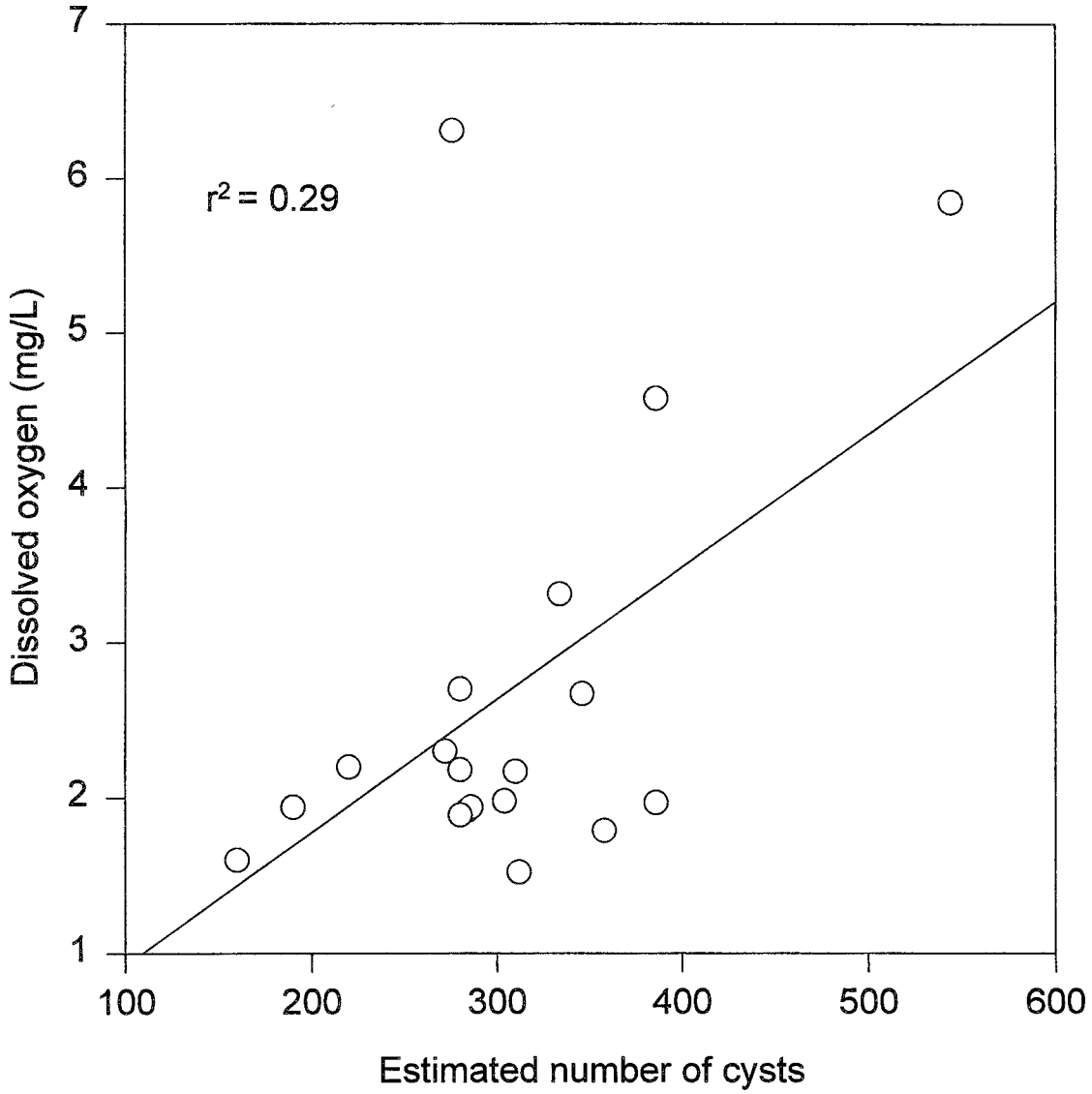

FIGURE 4.---Relation between lethal dissolved oxygen levels and parasite load in lab-infected fountain darters immediately (0 d) after exposure (P=0.0142).

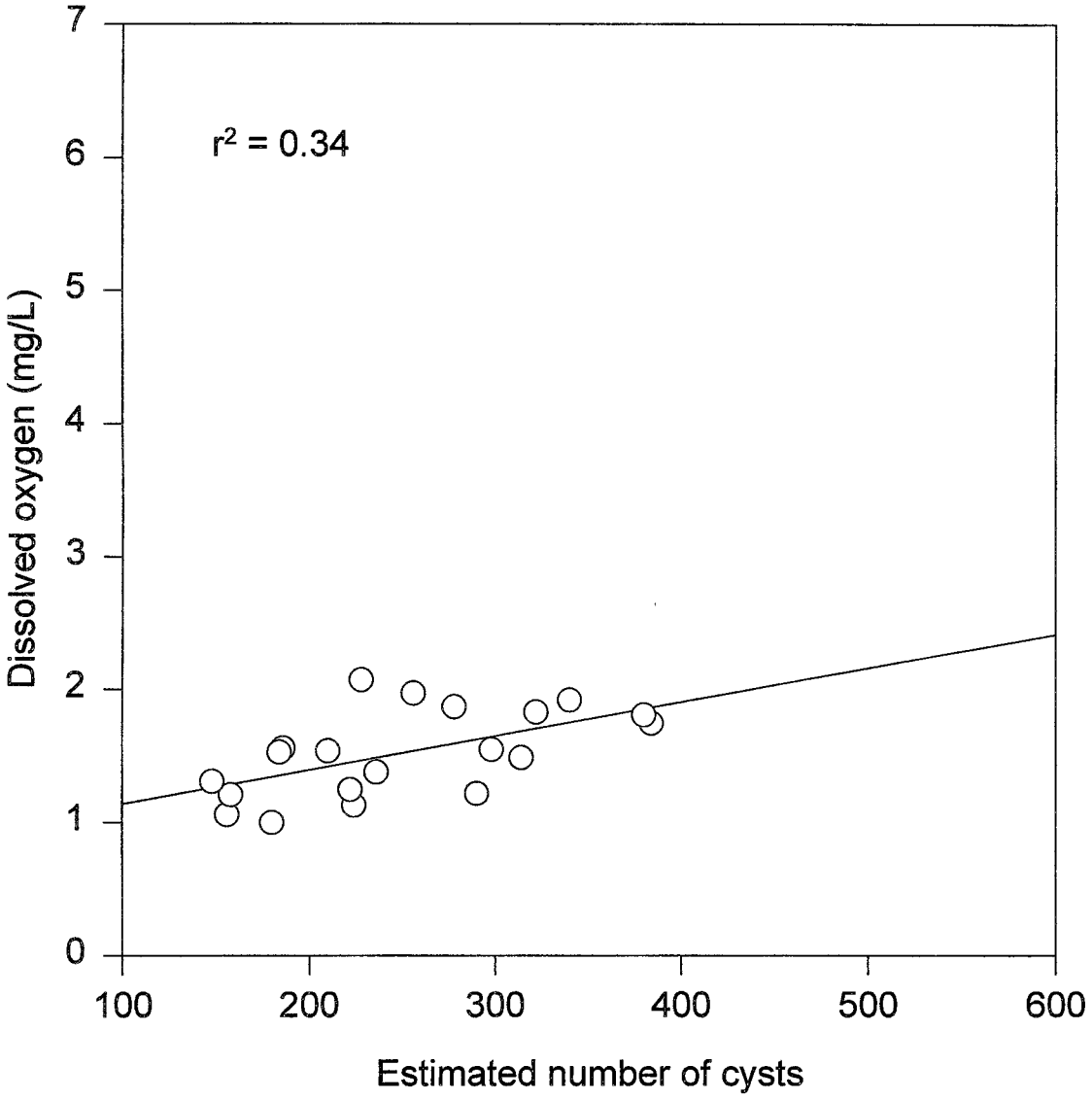

FIGURE 5.---Relation between lethal dissolved oxygen levels and parasite load for lab-infected fountain darters 1 d post-infection (P=0.0075).

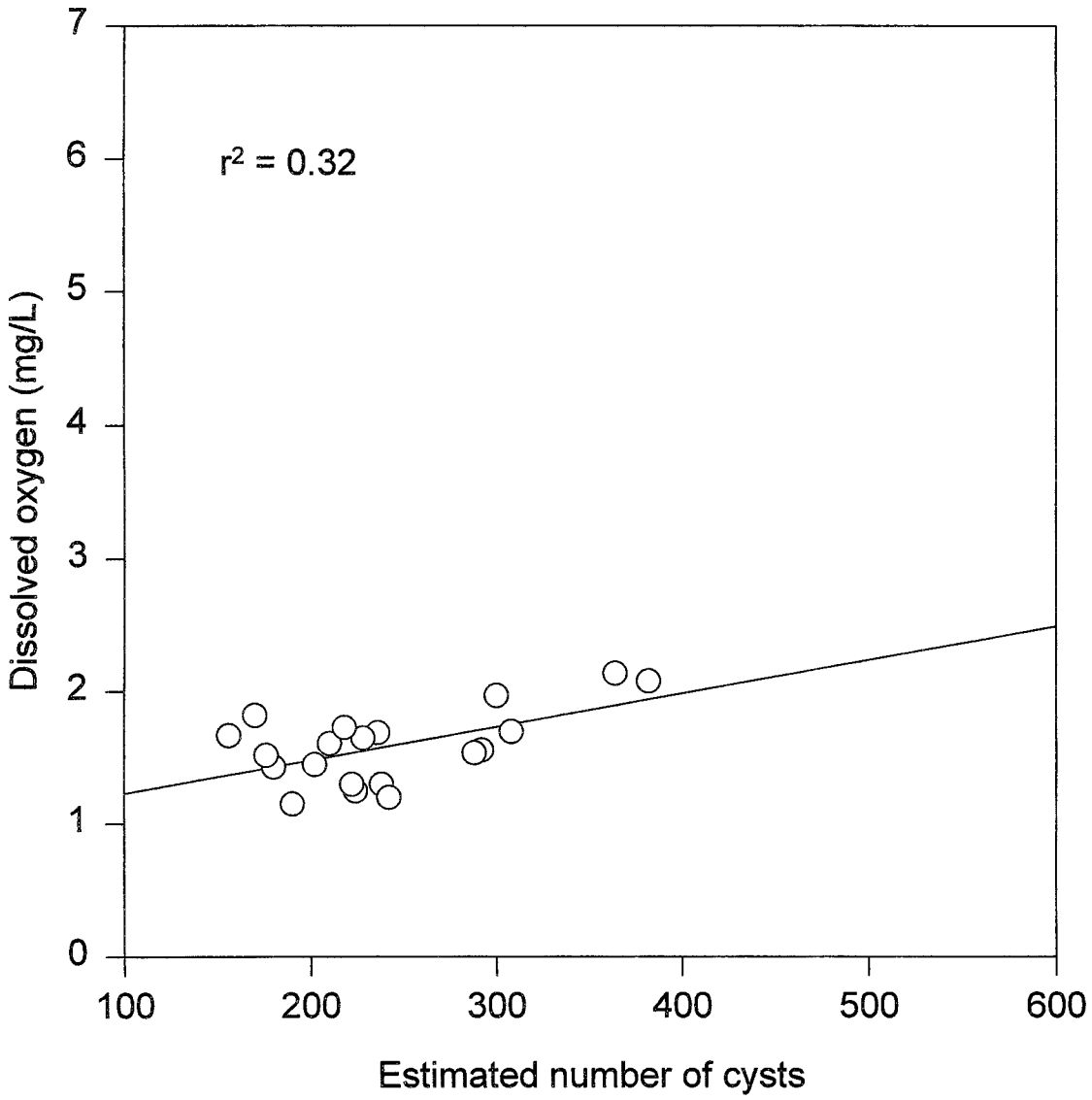

FIGURE 6.---Relation between lethal dissolved oxygen levels and parasite load in lab-infected fountain darters 3 d post-infection (P=0.0089).

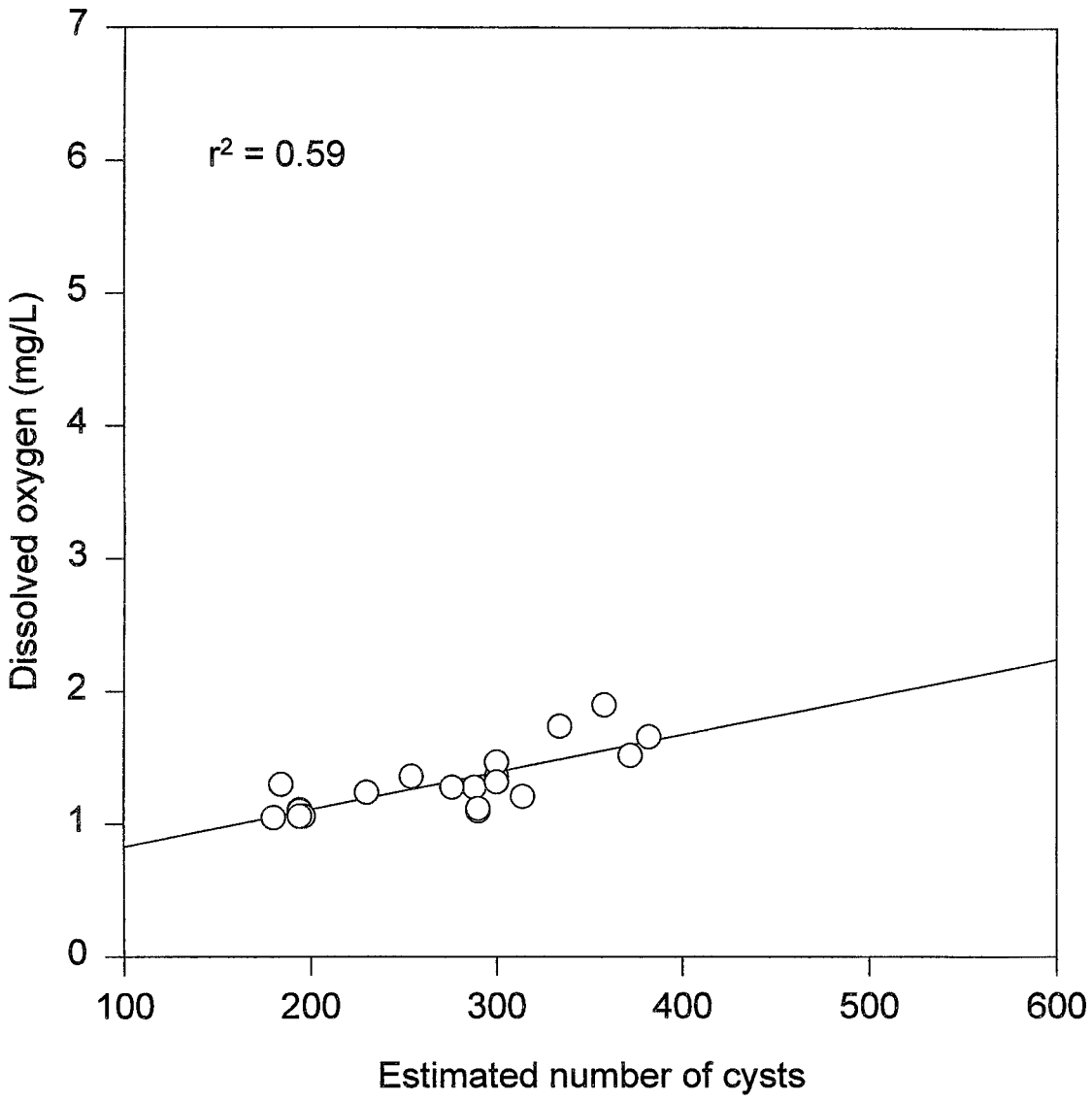

FIGURE 7.---Relation between lethal dissolved oxygen levels and parasite load for lab-infected fountain darters 7 d post-infection ($P<0.0001$).

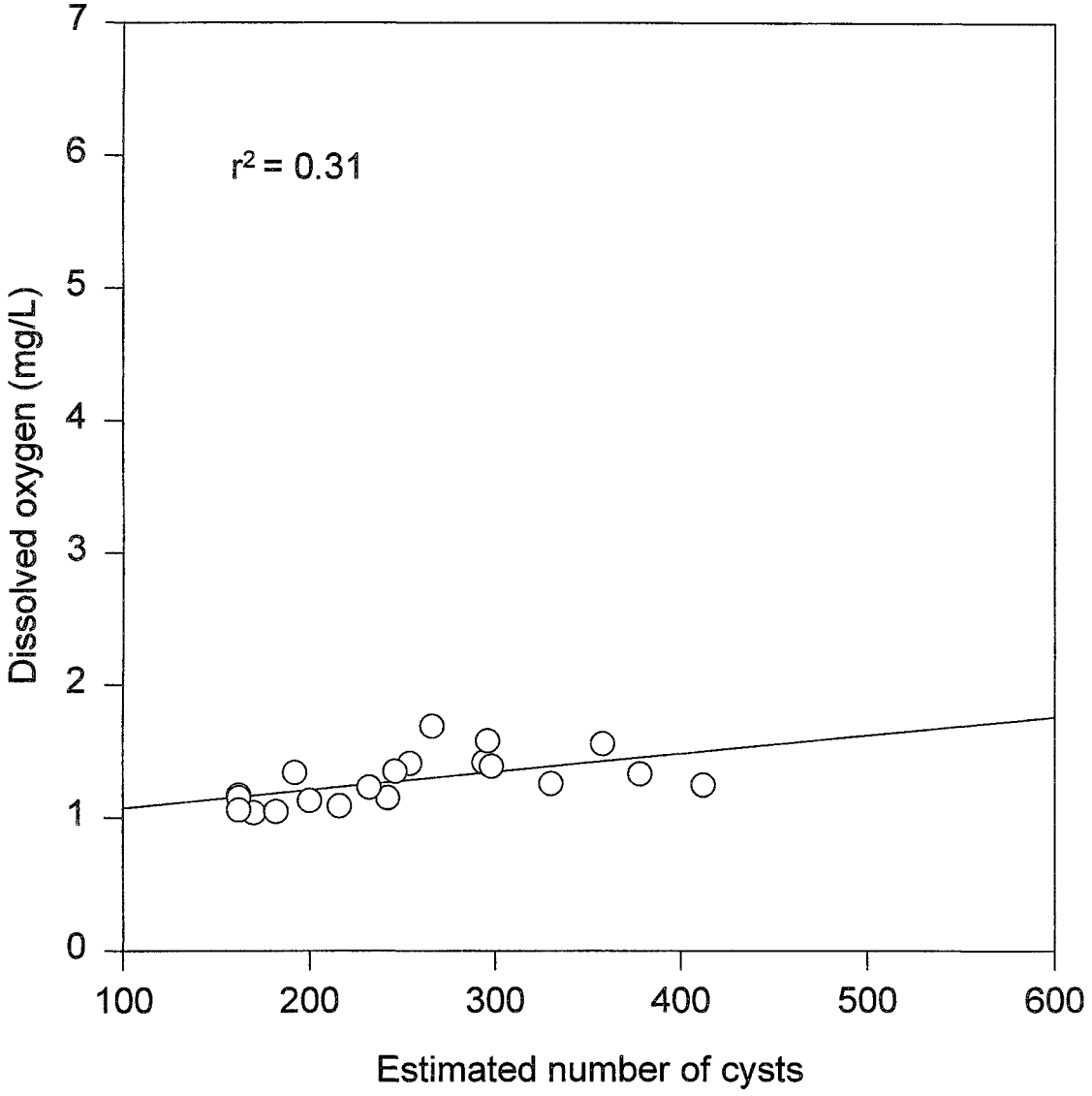

FIGURE 8.---Relation between lethal dissolved oxygen levels and parasite load in lab-infected fountain darters 14 d post-infection (P=0.0106).

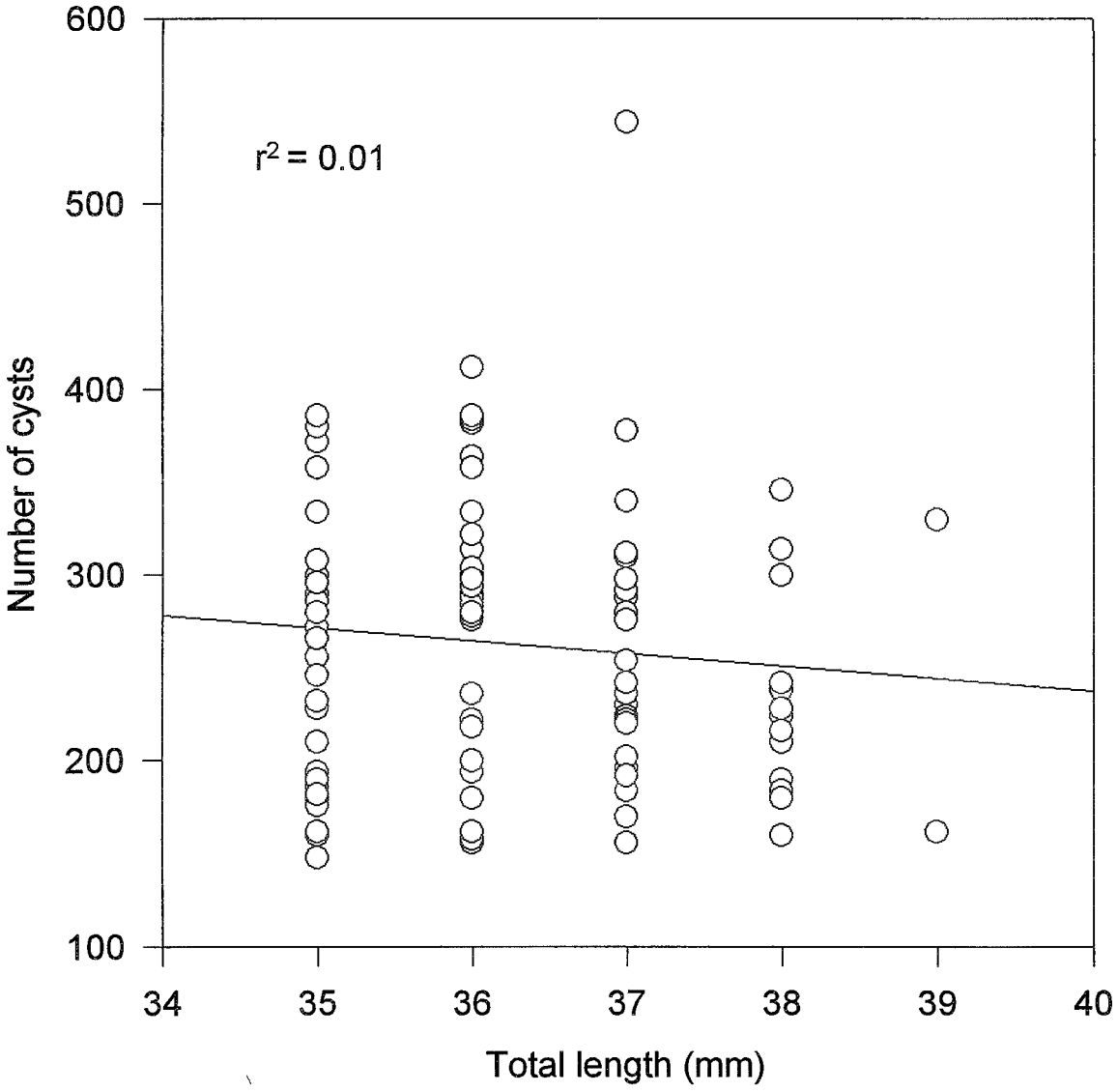

FIGURE 9.---Relation between parasite load of lab-infected fountain darters and total length of fish in mm (P=0.3229).

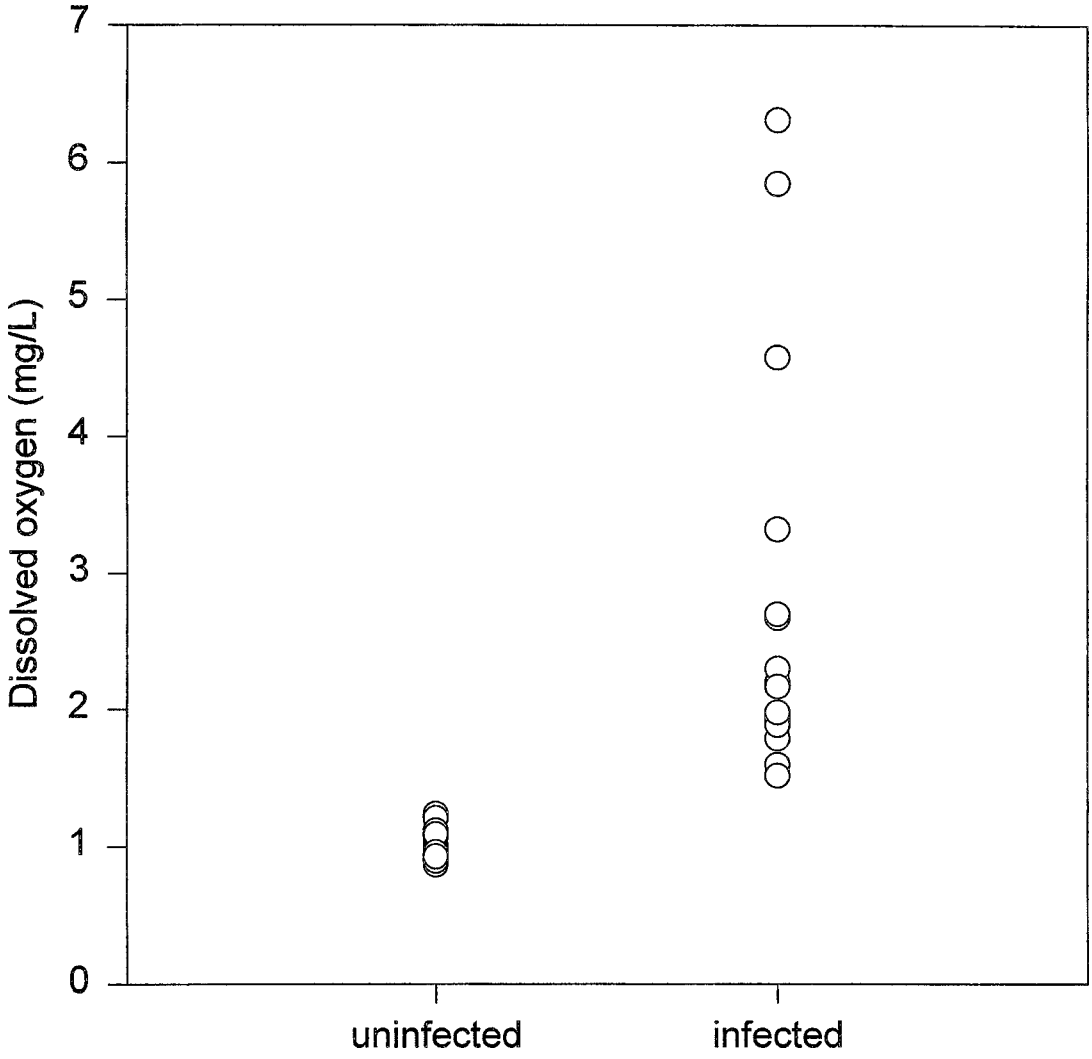

FIGURE 10.---Relation between lethal dissolved oxygen levels for uninfected lab-reared fountain darters and lab-infected lab-reared fountain darters immediately after exposure.

TABLE 1.---Raw data for mortalities of fountain darters collected from four different sections on the San Marcos and Comal Rivers The sites were the Comal River (Comal), Landa Lake (Landa), the upper San Marcos River (uSM) and the middle San Marcos River (mSM) The number of metacercariae per fish was estimated from a four-arch count on the right side of the fish

collection date	death date	days survived	site	total length (mm)	sex	estimated number of cysts
8/17	8/18	1	Comal	17	?	52
8/18	8/18	0	Comal	20	f	24
8/18	8/18	0	Comal	25	f	66
8/18	8/18	0	Landa	21	f	26
8/18	8/19	1	uSM	20	f	0
8/18	8/19	1	Comal	23	f	26
8/19	8/19	0	uSM	27	f	0
8/19	8/22	3	mSM	23	f	0
8/19	8/24	5	mSM	28	m	0
8/19	8/26	7	mSM	30	f	0
8/19	8/26	7	mSM	25	f	0
8/18	9/17	30	mSM	29	f	0
8/19	9/24	36	mSM	27	m	0
8/19	9/26	38	Comal	21	f	62
8/19	9/28	40	Comal	21	f	88
8/18	9/28	41	Comal	27	f	90
8/18	10/1	44	Comal	26	f	52
8/18	10/1	44	uSM	28	m	0
8/18	10/1	44	uSM	26	m	0
8/17	10/2	46	Comal	23	f	88
8/17	10/10	54	Comal	22	f	216
8/17	10/16	60	Comal	29	f	330
8/18	10/16	59	Comal	30	f	148
8/17	10/18	62	Comal	31	f	190
8/19	10/18	60	Comal	30	m	76
8/19	10/20	62	Comal	26	f	22
8/19	10/22	64	Comal	24	f	56
8/17	10/24	68	Comal	28	f	204
8/17	10/28	72	Comal	25	f	174

TABLE 2 ---Raw data for fountain darters collected from the Comal River 240-370 m downstream of the Elizabeth Street low water crossing These fish were used in the sealed-jar hypoxia test for wild-infected fountain darters

Fish	Total length (mm)	Sex	Subjective grade[a]	Estimated cysts per fish[b]	DO (mg/L)
1	35	m	2	566	1 34
2	32	m	2	1288	1 91
3	35	m	3	1614	1 46
4	33	m	2	918	1 06
5	32	f	3	1232	1 83
6	32	m	2	570	0 96
7	30	m	3	784	1.74
8	30	f	2	554	1 56
9	31	f	3	634	1 68
10	30	m	3	538	1.70
11	31	f	2	566	1 54
12	31	f	3	656	1 75
13	32	m	3	466	1 33
14	31	f	2	982	2 25
15	30	m	2	396	1 37
16	32	f	3	536	1 40
17	33	m	2	472	1 16
18	30	f	2	566	1 90
19	30	m	2	476	1 46
20	33	f	3	400	1 12

[a] 1 = does not appear affected, 2 = swollen, bloody gills, 3 = swollen, bloody gills, and fish appears emaciated or in distress

[b] Number is estimated by doubling a four-arch count on the right side of the fish

TABLE 3.---Raw data for lab-reared lab-infected fountain darters 0 d after exposure to infective trematode cercariae

Fish	Total length (mm)	Sex	Estimated cysts per fish[a]	DO (mg/L)
1	36	m	284	1 92
2	35	f	190	1 94
3	36	m	358	1 79
4	35	m	160	1 60
5	35	f	286	1 94
6	37	f	220	2 20
7	36	m	280	2 18
8	35	f	272	2 30
9	37	m	310	2 17
10	36	m	386	1 97
11	38	m	160	1 60
12	36	f	334	3 32
13	37	m	280	1 89
14	36	m	304	1 98
15	37	m	312	1 52
16	38	f	346	2 67
17	35	m	386	4 58
18	35	m	280	2 70
19	37	f	276	6 31
20	37	f	544	5 85

[a] Number of cysts is estimated by doubling a four-arch count on the right side of the fish

TABLE 4.---Raw data for lab-reared lab-infected fountain darters 1 d after exposure to infective trematode cercariae

Fish	Total length (mm)	Sex	Estimated cysts per fish[a]	DO (mg/L)
1	36	m	314	1 49
2	37	m	156	1 06
3	37	m	224	1 13
4	37	m	298	1.55
5	35	m	186	1 56
6	37	m	340	1 92
7	36	m	384	1 75
8	36	m	158	1 21
9	36	f	322	1 83
10	35	m	380	1 81
11	38	f	184	1.53
12	38	f	228	2 07
13	38	f	210	1 54
14	37	m	236	1 38
15	36	m	278	1 87
16	35	f	256	1 97
17	37	m	222	1 25
18	38	m	182	1 00
19	35	m	290	1 22
20	35	m	148	1 31

[a] Number of cysts is estimated by doubling a four-arch count on the right side of the fish

TABLE 5 ---Raw data for lab-reared lab-infected fountain darters 3 d after exposure to infective trematode cercariae

Fish	Total length (mm)	Sex	Estimated cysts per fish[a]	DO (mg/L)
1	37	m	170	1 82
2	36	f	364	2 14
3	36	f	236	1 69
4	36	m	156	1 67
5	35	m	228	1.65
6	38	m	238	1 30
7	35	m	210	1 61
8	38	m	190	1 15
9	37	m	292	1 56
10	37	f	202	1 45
11	35	m	180	1 43
12	36	f	300	1 97
13	38	f	224	1 25
14	36	m	222	1 30
15	36	f	288	1 54
16	35	f	308	1 70
17	36	m	382	2 08
18	35	f	176	1 52
19	38	f	242	1 20
20	36	f	218	1.73

[a] Number of cysts is estimated by doubling a four-arch count on the right side of the fish

TABLE 6 ---Raw data for lab-reared lab-infected fountain darters 7 d after exposure to infective trematode cercariae

Fish	Total length (mm)	Sex	Estimated cysts per fish[a]	DO (mg/L)
1	38	m	300	1 36
2	36	m	382	1 66
3	36	m	180	1 05
4	37	m	254	1 36
5	36	m	194	1 11
6	35	m	194	1 10
7	37	f	196	1 06
8	37	f	288	1 28
9	35	f	334	1 74
10	35	f	372	1.52
11	36	f	290	1.10
12	36	m	276	1.28
13	35	m	194	1 06
14	35	f	300	1 47
15	38	f	314	1 21
16	36	m	300	1 32
17	37	m	230	1 24
18	35	f	290	1 12
19	35	f	358	1 90
20	37	f	184	1.30

[a] Number of cysts is estimated by doubling a four-arch count on the right side of the fish

TABLE 7 ---Raw data for lab-reared lab-infected fountain darters 14 d after exposure to infective trematode cercariae

Fish	Total length (mm)	Sex	Estimated cysts per fish[a]	DO (mg/L)
1	37	f	254	1 41
2	36	m	200	1 13
3	36	m	294	1 42
4	37	m	378	1 33
5	39	f	330	1 26
6	37	f	192	1 34
7	35	f	296	1 58
8	37	m	242	1 15
9	36	f	162	1 17
10	37	m	170	1 04
11	36	m	412	1 25
12	35	m	232	1 23
13	36	f	298	1 39
14	39	f	162	1 15
15	35	m	182	1.05
16	38	f	216	1 09
17	35	m	358	1 56
18	35	f	162	1 06
19	35	f	246	1 35
20	35	m	266	1 69

[a] Number of cysts is estimated by doubling a four-arch count on the right side of the fish