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Title: Host Responses of English Sole (Parophrys vetulus) to Infection by the Monogenetic Trematode Gyrodactylus stellatus

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The infection intensity of the monogenetic trematode Gyrodactyus stellatus on laboratory held English sole (Parophrys vetulus) appeared to be influenced by handling stress, substrate, and starvation. In bioassays testing G. stellatus survival times in mucus and serum collected from laboratory held sole at different times during trematode infection, trematode survival times were significantly reduced in serum and mucus samples collected from sole at the later, recovering stages of infection. Ouchterlony gel diffusion tests of rabbit antiserum against English sole serum diffused with English sole mucus samples showed that the mucus of G. stellatus

infected English sole contained factors antigenically similiar to factors in English sole serum. These factors were not present in the mucus of uninfected sole. Precipitin bands in the gel diffusions tests appeared to be the strongest in mucus samples from sole at later, recovering stages of infection.

The results of the serum and mucus bioassays and the Ouchterlony tests suggest the possible presence of resistance factors in both the serum and the mucus of English sole at later stages of trematode infection, and that these factors may result in recovery from the infection. The results also suggest that resistance factors in the mucus may originate from the serum. This study did not attempt to characterize these resistance factors.

Host Responses of English sole (*Parophrys vetulus*) to Infection by the Monogenetic Trematode *Gyrodactylus stellatus*

by

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HOST RESPONSES OF ENGLISH SOLE (PAROPHRYS VETULUS) TO INFECTION BY THE MONGENETIC TREMATODE GYRODACTYLUS STELLATUS

I. INTRODUCTION

Monogenetic trematodes are small to medium sized (.03-20 mm) ectoparasites of poikilothermous vertebrates, usually fishes. They have a direct life cycle on the gills or skin of their hosts, are often highly host specific and most are blood or mucus feeders. The major attachment structure is the opisthaptor, a posterior disk which bears hooks, suckers or both (Smyth 1966, Sproston 1946, Bychowsky 1961, Dawes 1968).

The monogenetic trematode genus Gyrodactylus includes approximately 350 species parasitic on the gills and body surfaces of marine and freshwater teleosts (Cone and Odense 1984). Gyrodactylids are small (less than 1 mm), elongated monogeneans which feed on the host's epidermis and epidermal secretions (Smyth 1966). They have a well developed opisthaptor bearing a pair of large anchor hooks and 15-16 marginal hooklets (Sproston 1946, Bychowsky 1961).

The subject of this study is Gyrodactylus stellatus, a parasite of pleuronectid flatfishes (Kamiso and Olson 1986). Like all members of the family Gyrodactylidae, G.

stellatus is viviparous. This contrasts with all other monogenetic trematode groups which are oviparous and possess a ciliated larval stage (Sproston 1946).

Reproduction in the genus Gyrodactylus has been described by Bychowsky (1961). Up to four embryos may result from a single egg. The first born individual has in its uterus three embryos lying one inside the other, the second born has two embryos, the third has one, and the fourth none. The first three individuals differ from the fourth in that they give birth to embryos from the same egg cell as themselves. In the fourth individual, a new egg enters the uterus so that it gives birth to an embryo arising from an egg it has produced. That embryo (containing three embryos) develops within 4 to 5 days and is born, thus repeating the cycle. The lifespan of an individual Gyrodactylus sp. is at least 12-15 days (Bychowsky 1961). Lester and Adams (1974) recorded two births per worm at 15°C with the first birth at 1.6 days and the second birth after 6.9 days. They recorded a longest life-span of 28 days at 15°C and 71 days at 7°C. Gyrodactylids do not live longer than 48 hours away from the host and usually perish within 24 hours (Bychowsky 1961).

Since members of the genus Gyrodactylus are viviparous and capable of rapid reproduction, they may increase to dangerous levels of infection under hatchery

conditions that often crowd the fish under culture (Mizelle and Kritsky, 1967). The only known cases of mortality due to Gyrodactylus sp. in natural waters occurred in Atlantic salmon (Salmo salar) in Norwegian rivers (Heggberget and Johnsen, 1982), where G. salaris was thought to be recently introduced (Bakke et al., 1990). In this instance, high frequencies and intensities of Gyrodactylus infection were seen in conjunction with evidence of high mortalities (Heggberget and Johnsen, 1982).

Epizootics of gyrodactylids in North American fish hatcheries were reported as early as 1899 (Mizelle and Kritsky, 1967). The resulting disease condition, gyrodactyliasis, has caused losses in a wide variety of captive fish species (Cone and Odense, 1984). Losses due to gyrodactyliasis have been reported in rainbow trout, Onchorhynchus mykiss (formerly Salmo gairdnerii) (Mizelle and Kritsky, 1967); black bullhead, Ictalurus melas (Mizelle and Kritsky, 1967); golden shiner, Notemigonus crysoleucas (Lewis and Lewis, 1970); English sole, Parophrys vetulus (Kamiso and Olson, 1986); threespine stickleback, Gasterosteus aculeatus (Lester and Adams, 1974); Atlantic salmon, Salmo salar (Bakke et al., 1990); guppy, Poecilia reticulata (Scott 1985); bluegill, Lepomis macrochiri (Hoffman and Putz, 1964); and plaice,

Pleuronectes platessa (Mackenzie 1970).

Gyrodactylus sp. may live almost anywhere on the host but are usually most abundant on the fins. The affected surfaces may become covered with a bluish grey slime due to increased mucus production. When the parasites are very abundant, the fins can become frayed and may eventually be eroded. Infected fish often rub themselves against the sides of a tank or bottom of a pond in an apparent effort to dislodge the parasites (Davis 1965).

English sole, Parophrys vetulus, are a major contributor to Pacific Ocean trawl fisheries off the United States and Canada and, in Oregon, they rank third in annual landings (Toole et al., 1987). Juvenile English sole utilize Yaquina Bay as a nursery ground for their first year of life (Olson and Pratt, 1973), during which time they are commonly parasitized by Gyrodactylus stellatus (Olson 1978; Kamiso and Olson, 1986).

Juvenile English sole held in the laboratory have been observed to become much more heavily infected than those in the estuary. Kamiso and Olson (1986) found that fish from the estuary never averaged greater than 5.5 trematodes per fish while those held in the laboratory were observed to undergo a logarithmic increase during the first 9 weeks, peaking at over 1000 trematodes per fish, then decreasing rapidly over the following 3 week

period. This suggests that there may be a host response that regulates the levels of G. stellatus on English sole, a response that is temporarily lost when fish are subjected to capture and laboratory holding stresses.

In estuaries, juvenile English sole are found in areas with sand or mud substrate (Toole et al., 1987). Previous studies monitored G. stellatus levels on laboratory held English sole kept in tanks without substrate (Kamiso and Olson, 1986). The presence or absence of substrate may have an effect on infection intensities in laboratory held fish.

Kamiso and Olson (1986) observed that fish dying with heavy infections had ceased feeding and were emaciated. They suggested that death may have been the result of the combined effects of starvation and heavy parasitism. They also found that the rate of parasite increase on unfed fish was significantly higher than on fed fish, but they did not separate the effects of starvation and trematode infection on mortality.

The host response that regulates G. stellatus may be affected by the stresses associated with capture and laboratory holding conditions. Stress is known to have physiological effects on fish; these effects are termed primary, secondary and tertiary. The primary effects of stress are increased production of corticosteroids and catecholamines of the neuro-endocrine system (Schreck et

al., 1976; Schreck 1981; Mazeud et al., 1977). These primary responses bring about the biochemical, physiological, and immunological changes which are described as secondary responses (Mazeaud et al., 1977). Tertiary responses include changes in behavior, decreased growth rate, and increased susceptibility to disease (Wedemeyer and McLeay 1981).

Factors known to induce a stress response in fish include osmotic and ionic changes, pollutants, temperature changes, anesthetics and handling (Eddy 1981). In fish, the primary response to stress occurs quickly and is of short duration relative to secondary and tertiary responses (Schreck 1981; Mazeaud and Mazeaud, 1981). Increased catecholamine levels occur within minutes at the onset of stress and may last hours after cessation (Mazeaud and Mazeaud, 1981). The primary responses to multiple acute handling stresses have been shown to be cumulative and consist of stepwise increases of plasma cortisol and glucose concentrations (Barton et al., 1986; Flos et al., 1988).

The increased hormone levels associated with the primary response to stress have been shown to effect antibody producing cells. Cortisol implants resulted in increased plasma cortisol levels, decreased levels of antibody-secreting cells and decreased disease resistance in juvenile coho salmon, O. kisutch (Maule et al., 1987).

Stress, even a relatively mild handling stress of 30 second duration, suppressed the ability of lymphocytes to generate antibody-producing cells for at least 7 days in spring chinook salmon, O. tshawytscha (Kaattari and Schreck, 1987).

Fish respond to parasitic infections by the production of antigen specific IgM-like antibodies as well as by the elaboration of nonspecific cellular immunity, expressed either as phagocytes (Ellis et al., 1974) or cytotoxic cells (Evans et al., 1984); and nonspecific soluble factors such as lysozyme (Fletcher and White, 1976), C-reactive protein (Fletcher et al., 1977), transferrin (Suzumoto et al., 1977), and interferon-like molecules (Evans and Gratzek, 1989). In immunity to helminth infections, the host response appears to be mediated via antibodies, plus complement in most cases (Evans and Gratzek, 1989). Cellular immunity has not yet been shown to mediate anti-helminth responses in fish (Evans and Gratzek, 1989).

The external body surface of fish is covered by a mucoid layer that is secreted from goblet cells in the epidermis. Mucus provides a mechanical and chemical barrier to infection (Ingram 1980), and immunoglobulins have been isolated from the mucus of some species of fish (Bradshaw et al., 1971; Fletcher and Grant, 1969). Nigrelli (1935) studied the effects of marine fish mucus

on the monogenetic trematode Epibdella melleni and found that mucus from fish with natural immunity to the parasite had an effect on trematode survival. Hanson (1973) found that serum and mucus from fish species with natural resistance decreased survival times of adult Diclidophora embiotoci (Monogenea).

The purpose of this study was to explore the mechanism of English sole resistance to G. stellatus by examining the basis for the transitory loss of resistance in the laboratory. Specific objectives were to: Determine if the intensity of G. stellatus on laboratory held fish was influenced by handling stress, the presence or absence of substrate, and/or starvation; Determine if the mucus layer of the fish played a role in resistance to the trematode; and Determine if serum factors were involved in resistance.

II. MATERIALS AND METHODS

Collection and Maintenance of English Sole:

Juvenile English sole (Parophrys vetulus) were collected from Yaquina Bay, Oregon by towing a 16 foot otter trawl from the O.S.U. research vessel Sacajawea, or by beach seine. Fish were immediately transported to the Fish Disease Laboratory at the Oregon State University Hatfield Marine Science Center in Newport where they were held in fiberglass tanks provided with sand filtered, ultraviolet-light-treated salt water originating from Yaquina Bay. Water temperature was ambient and measured every other day. Most sole were initially fed frozen krill, then gradually switched to a diet of commercial moist salmon feed over a period of three weeks. Fish acclimating to laboratory conditions and commercially prepared food were fed ad libidum every other day and were available for use in experiments. Sole used in some experiments were not fed after capture or were fed only krill.

Infection Levels:

The number of trematodes per fish was determined at the termination of each experiment, with the exception of the starvation experiment in which sole were not fed and

infection levels were not determined. Fish were transferred to individual pint-size, plastic freezer containers where they were treated for 30 minutes in a 1:5500 dilution of formalin in sea water to kill and detach trematodes (Parker and Haley, 1960). Following the formalin treatment, the fish were euthanized with 2-phenoxyethanol, weighed and measured. Formalin concentrations were brought up to 10% in each container and the fish in the container was examined under a dissecting scope to make sure all trematodes were removed. Any attached trematodes were dislodged by a stream of formalin solution through a pasteur pipet and collected in the container. After removal of attached trematodes, the fish was discarded and the trematodes retained in 10% formalin in the containers.

The number of trematodes per fish was determined by counting the number of worms in each container. Trematodes were counted in a 10-30 ml aliquot in a petri dish backed with a transparent grid (1/4") under a dissecting scope. Worm numbers for each aliquot were added to get the total number of worms per fish.

When worm numbers were too high to count directly, estimates of total numbers were based on the number of trematodes in three aliquots (5-30 ml) of a homogeneous mixture of the trematodes in each container.

Condition Factors:

A unit-less condition factor (Fox 1962) was determined for each fish from length and weight measurements using the equation:

$$CF = (w \times 100{,}000)/l^3$$

where l=length and w=weight. The condition factor was determined for all English sole used in laboratory experiments.

Natural Gyrodactylus Stellatus Infection Levels:

Natural trematode infection levels and English sole condition factors were determined for a collection of 60 fish captured in October 1989 and used as a basis of comparison with data from experiments.

Experimental Design of Handling Stress, Substrate, and Starvation Experiments:

Experiments to assess the effect of handling stress, substrate, and starvation on G. stellatus infection levels on laboratory held English sole employed an experimental design that consisted of two treatments, with four test chambers per treatment and 10 fish per replicate. In the third handling stress experiment, due to the number of fish available, each treatment had three test chambers holding 3-5 fish each. Test chambers were aerated, flow-through, plastic 16 x 11 x 6" containers

that held 5.8L each. Fish were impartially distributed into test chambers and the treatment each chamber was to receive was determined with a random numbers table.

Handling Stress Experiments:

The effect of handling stress was tested in several experiments by holding test fish out water in a dipnet for various time intervals and then allowing infections to develop for four weeks before the number of worms per fish and condition factors were determined. Control fish did not receive the stress treatment.

Substrate Experiment:

The influence of substrate on G. stellatus infection levels on laboratory held P. vetulus was tested by holding newly captured naturally infected fish in test chambers with or without a sand substrate for two weeks before determination of the number of worms per fish and condition factors. The fish were fed frozen krill during the experiment.

Starvation Experiment:

To determine the effect of G. stellatus on the survival of unfed English sole, survival times of naturally infected English sole were compared to those of formalin-disinfected English sole. The fish were not fed

during the experiment, and dead fish were removed and recorded daily. Each fish was examined under a dissecting scope to confirm whether or not it was a G. stellatus infected fish. The experiment ran until no fish remained.

Statistical Analyses of Handling Stress, Substrate, and Starvation Experiments:

The number of worms per fish data had a Poisson distribution and were normalized with a square-root transformation. The assumption of normality was confirmed for the transformed trematode counts and condition factor data with a Kolmogorov-Smirnov one-sample goodness-of-fit test.

Student's t-test was used to determine if there were significant differences between test treatments. To determine if the data from each fish in a test chamber could be analyzed independently, a simple linear regression analysis was done to determine if the number of worms per fish was influenced by the number of fish per test chamber. If there was no association, the fish in each test chamber were assumed to be independent of one another. If a correlation existed, fish in a test chamber were not considered independent of one another and the data was analyzed with each test chamber considered as a replicate.

Laboratory Held, Uninfected English Sole:

For use as a comparison in bioassays, gel diffusion tests and agglutination tests, uninfected English sole were obtained. Uninfected sole were formalin-disinfected sole held in the laboratory without trematode infection for two months.

Serum and Mucus Sample Collection for Bioassays and Immunological Tests:

Fish from which mucus and serum were collected were anesthetized in a 1:1500 dilution of 2-phenoxyethanol, rinsed in seawater and drained. Skin mucus was obtained by gently scraping the surface of the fish with a glass slide and collecting the mucus in a petri dish. Blood was then collected from the dorsal aorta by severing the caudal fin or by cardiac puncture. Mucus was kept on ice during collection, refrigerated at 4°C overnight, centrifuged for 15 minutes at 1500 x g to remove scales and other debri and the supernatant collected and stored at -70°C in plastic 5 ml test tubes. Blood was allowed to clot for 1-2 hours at room temperature, refrigerated at 4°C overnight, centrifuged for 15 minutes at 1500 x g and the serum collected and stored at -70°C in microcentrifuge tubes.

Serum and mucus for use in bioassays, gel diffusion tests and agglutination tests were collected from natur-

ally infected juvenile English sole. Two collections of sole were made and samples from 25 fish were taken on the date of capture and then every two weeks following until no fish remained. This allowed samples to be collected from the first group of fish for six weeks and from the second group of fish for 10 weeks. Each fish was examined under a dissecting scope to determine the trematode infection level before sampling. The serum and mucus were pooled for each sampling unless there appeared to be a marked difference in infection levels, then serum and mucus samples were separated by infection level, either high or low.

Serum and mucus was also collected from uninfected laboratory held English sole, Buffalo sculpin (Enophrys bison) collected from Yaquina bay, and chum salmon (Onchorhynchus keta) hatched and raised at the Fish Disease Laboratory.

Gyrodactylus Stellatus Immunized English Sole:

In an effort to obtain English sole serum and mucus that contained antibodies against G. stellatus, 15 formalin-disinfected, laboratory held English sole (172.8 ± 23.4 S.E. mm) were anesthetized and injected interperitoneally with 0.1 ml of a 1:1 volume of whole, formalin-killed G. stellatus in Fruend's complete adjuvant (FCA). Booster injections were administered in the same

manner two weeks later. Four weeks after the booster, blood was collected by cardiac puncture and mucus was also collected.

Mucus Bioassays:

Bioassays testing the survival times of G. stellatus in English sole mucus samples were performed in 96-well polystyrene flat bottomed plates. Two bioassays were performed. Mucus from naturally infected sole held in the laboratory for varying periods of time, as well as mucus from uninfected and G. stellatus injected sole were compared. Buffalo sculpin mucus and seawater were used as controls. Bioassays were carried out in a 15°C constant-temperature room.

Live G. stellatus were obtained by treating infected sole with 2-phenoxyethanol for 30 s to 1 min, after which anesthetized trematodes were removed from the fish surface by a stream of water through a pastuer pipet (Lester and Adams 1973). Worms were filtered out of the anesthetic on a 53 μm nitex screen, rinsed in seawater, and collected in small glass crystallizing dishes.

One to two trematodes were placed in each well of a 96-well plate and brought to a 5% concentration of mucus in seawater. Control wells contained seawater and no mucus. Seawater used in the tests was from the same source as the holding water of G. stellatus source fish,

and varied between 29-32 $^{o}/_{oo}$ salinity and 10-16 °C. Samples were randomly assigned to the rows of the 96-well plate using a random numbers table, and three replicate plates were tested. Trematodes were monitored every 3-4 hours under a dissecting scope until all worms in the controls wells had died.

Serum Bioassays:

Bioassays to determine the survival time of G. stellatus in English sole serum were conducted in the same manner as the mucus bioassays. Serum from two groups of naturally infected sole held in the laboratory for varying periods of time, as well as sera from uninfected and G. stellatus injected sole were compared. Seawater, buffalo sculpin and chum salmon sera were used as controls. Trematode survival was monitored every 0.5-2 hours until the worms in all serum samples were dead.

Statistical Analysis of Mucus and Serum Bioassays:

Wells in which trematode births occurred were not analyzed. The mean and standard error of survival time were calculated for worms in each treatment replicate. The assumptions of normality and homogeneity of variance were tested for with the Shapiro-Wilkes and Bartlett's test respectively. When necessary, data were transformed to meet the assumptions and a one-way analysis of vari-

ance (ANOVA) was performed. Dunnett's test was used to compare the control mean to each of the sample means. Mucus and serum from newly captured fish were analyzed as the controls. In cases of unequal numbers of replicates, the Bonferroni t-test was used in place of Dunnett's test. Student's t-test was used to compare paired samples.

Preparation of Trematode Antigen for Agglutination and Gel Diffusion Tests:

Gyrodactylus stellatus for antigen preparation were obtained by treating infected sole with a 1:4000 dilution of formalin in seawater for 1 hour. Trematodes were filtered out of solution on a 53 μm nitex screen, rinsed and preserved in 10% formalin or 70% ethyl alcohol.

For gel precipitin tests, approximately 1 ml of a packed volume of trematodes was centrifuged for 15 minutes at 1500 xg, washed in PBS three times and homogenized in 2 ml PBS with a Brock tissue grinder. The BioRad protein assay was used to determine the concentration of soluble protein in the supernatant of centrifuged homogenate.

For agglutination tests, the trematode homogenate was centrifuged and washed three times in PBS to remove soluble proteins, and the pellet was retained. A 1% w/v concentration of antigen in PBS was used.

Rabbit Antiserum:

A rabbit antiserum against English sole whole serum was obtained by injecting a 2-2.5 kg female, New Zealand White rabbit with a 1:1 volume, 400 µg/ml protein, of English sole whole serum in FCA. The rabbit was injected with 0.1 ml of antigen intermuscularly in each leg, and 0.1-0.2 ml subcutaneously in five places along the back. A 10 ml sample of normal rabbit blood was collected by cardiac puncture before the injections. A booster of English sole serum in Freund's incomplete adjuvant, 400 µg/ml protein, was given two weeks later following the same injection regime. Two weeks following the booster injections, 10 ml of blood was collected from the rabbit by cardiac puncture. All injections and bleedings were performed by trained staff at the O.S.U. Lab Animal Resources facility in Corvallis.

The presence of anti-English sole serum antibodies in the rabbit antiserum was confirmed in Ouchterlony gel diffusion tests. The tests revealed 4-5 precipitation bands in serum dilutions of 1:1 and 1:2, but none at higher dilutions.

Gel Diffusion Tests:

An agarose gel, double diffusion precipitation test (Ouchterlony) was used to detect specific antibodies in rabbit antiserum, fish serum and fish mucus. Tests were

done in 5.0 cm diameter Gelman plates holding 5 ml of 1% agarose in .01 M PBS at pH 7.2. Seven 5 mm diameter wells placed 10 mm apart (from center to center) were cut out of the gel, with one well surrounded by six wells. Each well held approximately 25 µl of sample. Following the addition of the samples, plates were incubated in a humidity chamber at room temperature and read after 24 and 48 hours (Anderson and Dixon, 1981).

Precipitin tests were performed with normal and immunized rabbit serum against English sole serum; English sole serum and mucus samples against homogenized G. stellatus in PBS; and English sole and buffalo sculpin mucus samples against rabbit antiserum.

Microtiter Agglutination Test:

A microtiter agglutination test to determine if agglutinating antibodies against G. stellatus could be detected in English sole serum and mucus was performed in 96-well, u-bottom, polystyrene plates following proceedures described by Anderson and Dixon (1981). Buffalo sculpin and chum salmon serum samples were tested as controls. Serum from G. stellatus injected English sole was used as the positive control. The antigen was a 1% w/v suspension of homogenized trematodes in PBS.

Samples were assigned to rows on a plate with a random numbers table. Each row held eight wells, and two

replicate plates were tested. Two-fold serial dilutions of a sample in PBS were made in the eight wells (50 µl per well) of a row. The first well held undiluted serum and the eigth held PBS only. Following the serum dilutions, 50 µl of the antigen suspension was added to each well of the plate. Plates were gently agitated to mix the contents of each well, covered and set aside for readings at 1, 6, and 24 hours.

Macroscopic Slide Agglutination Tests:

Slide agglutination tests to determine if agglutinating antibodies against G. stellatus could be detected in English sole serum and mucus samples were performed following the proceedures described by Anderson and Dixon (1981). Buffalo sculpin, and chum salmon serum samples were also tested as controls. Serum from G. stellatus injected English sole was used as the positive control. The antigen was a 1% w/v suspension of homogenized trematodes in PBS.

One drop of antigen suspension was placed in each well of an agglutination test slide with a pastuer pipet. One drop of sample was added to each well on the slide, one well was left without sample. Wells were mixed with wooden applicator sticks and observed for 20 minutes.

III. RESULTS

Gyrodactylus Stellatus on English Sole from Yaquina Bay:

The prevalence and intensity of G. stellatus on 60 juvenile English sole (112 ± 14.6 S.E. mm) collected in Yaquina Bay in October 1989 was determined and the condition factor of those fish was calculated. Condition factors averaged 0.98±0.121 S.E.. The prevalence of G. stellatus was 86.7% and average intensity of infection was 3.8 ± 3.55 S.E. worms/fish. The heaviest infection found was 18.0 worms on one sole.

Handling Stress Experiments:

The influence of handling stress on the infection of juvenile English sole by G. stellatus was tested in three experiments.

In the first experiment, 80 sole were formalin-disinfected, acclimated in laboratory holding tanks for 11 days and test chambers for 15 days. At test initiation, 40 fish in the treatment group were stressed by holding them out of water in a dipnet for two minutes, a procedure repeated three times over the course of an hour. Following exposure to the handling stress, all fish were re-infected with the parasite via two fin-clipped G. stellatus infected sole placed in each test

chamber for 72 hours. The infection intensity levels and condition factors were determined for the fish that survived to end of the experiment. Eighty-five percent of the unstressed sole survived and only 47% of the stressed sole survived.

The results of the experiment are presented in Table 1. Infection intensity levels were 1766.1 $\pm$ 1281.63 S.E. worms/fish in the unstressed treatment and 2566.1 $\pm$ 2004.60 S.E. worms/fish in the stressed treatment (Figure 2). In the unstressed treatment, ANOVA and regression analyses showed the number of worms per fish was influenced by the number of fish per test chamber ($0.05 \geq P > 0.01$, R=96.7%), with higher infection levels on fish in tanks with higher densities due to higher survival rates (Figure 1). This prevented the data from each fish from being compared individually, requiring instead analysis of the averaged data from the fish in each test tank. There was no significant difference between stressed and unstressed fish in infection intensity levels ($0.10 \geq P > 0.05$) or condition factors ($0.10 \geq P > 0.05$).

Figure 1: Regression of the mean number of worms/fish versus the number of fish per test chamber in the unstressed treatment of the first handling stress experiment.

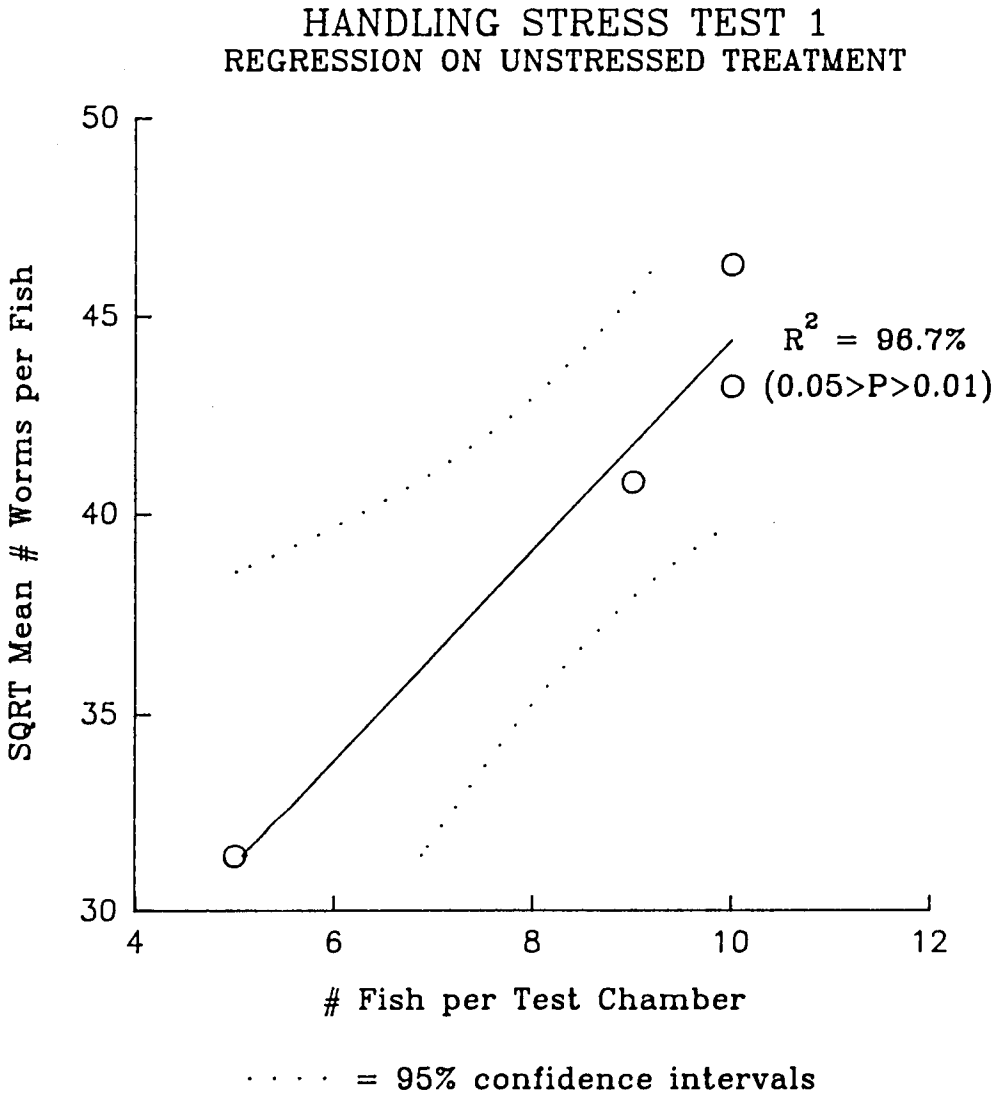
HANDLING STRESS TEST 1
REGRESSION ON UNSTRESSED TREATMENT
SQRT Mean # Worms per Fish
50
45
40
35
30
4
6
8
10
12
Fish per Test Chamber
R^2 = 96.7%
(0.05>P>0.01)
= 95% confidence intervals

Figure 1

Table 1: Handling stress experiment 1; the effect of handling stress on intensity of *Gyrodactylus stellatus*, condition factors and survival of laboratory held English sole after four weeks (1,2).

Treatment	N	Surviving N	Worms/Fish±S.E. (Range)	C-factor±S.E. (Range)
Unstressed	40	34	1766.1±1281.63 (380-6319)	0.90±0.043 (0.72-1.23)
Stressed	40	19	2566.1±2004.60 (232-6164)	0.89±0.032 (0.76-1.01)
Student t-test Significance Level			(0.10<P)	(0.10<P)

1. Disinfected fish were stressed three times then reinfected.

2. Seawater temperature during the test was 13.9±1.2 °C.

The second handling stress experiment was similiar to the first except that formalin-disinfected fish were acclimated longer (holding tanks, 51 days; test chambers, 87 days) and upon test initiation, fish in the stressed group were held out of water in a dipnet for a single two-minute treatment. Thirty-six sole were in the unstressed group and 37 sole were in the stressed group. Survival at the end of the experiment after four weeks was 100% in both treatments.

Results of the second stress experiment are given in Table 2. Infection intensity levels were 926.2 ± 374.73 worms/fish in the unstressed group and 628.2 ± 424.27 worms/fish in the stressed group (Figure 3). Infection

Table 2: Handling stress experiment 2; the effect of handling stress on intensity of Gyrodactylus stellatus, condition factors and survival of laboratory held English sole after four weeks (1,2).

Treatment	N	Surviving N	Worms/Fish±S.E. (Range)	C-factor±S.E. (Range)
Unstressed	36	36	926.2±374.73 (312-1924)	1.02±0.072 (0.87-1.18)
Stressed	37	37	628.2±424.30 (117-2151)	1.00±0.100 (0.85-1.26)
Student's t-test Significance Level			(P<0.001)	(0.10<P)

1. Disinfected fish were stressed once and then reinfected.

2. Seawater temperature during the test was 9.9±1.6 °C.

intensity levels between treatments were significantly different ($P \leq 0.001$) with unstressed fish having heavier infections. Fish condition factors were not significantly different between treatments ($0.10 \leq P$).

The third handling stress experiment was again similiar except that test fish were not formalin-treated and reinfected but rather carried a low level of infection that was observed to be typical for fish that survived a laboratory epizootic (≤300 worms/fish). General infection intensity levels at the beginning of the test were estimated by examining two sole under a dissecting scope. Sole were held in holding tanks for 6 months and acclimated to test chambers for 6 weeks. The unstressed

treatment had 12 sole and the stressed treatment had 13 sole. Ninety-two percent of the unstressed sole survived and 85% of the stressed sole survived four weeks to the end of the experiment.

Results of the third handling stress experiment are presented in Table 3. Infection intensity levels were 292.9 ± 315.90 S.E. worms/fish in the unstressed treatment and 1637.9 ± 2673.20 S.E. worms/fish in the stressed treatment (Figure 4). Sole in the stressed treatment had significantly higher infection levels than sole in the unstressed treatment ($0.01<P\leq0.05$). Condition factors between treatments were not significantly different ($0.01\leq P$).

Table 3: Handling stress experiment 3; the effect of handling stress on intensity of Gyrodactylus stellatus, condition factors and survival of laboratory held English sole after four weeks (1,2).

Treatment	N	Surviving N	Worms/Fish±S.E. (Range)	C-factor±S.E. (Range)
Unstressed	12	11	292.9±315.90 (70-1016)	0.92±0.077 (0.83-1.02)
Stressed	13	11	1637.9±2673.20 (123-9249)	0.92±0.059 (0.79-0.99)
Student's t-test Significance Level			(0.05>P>0.01)	(0.10>P)

1. Test fish were survivors of a laboratory epizootic, not disinfected and were stressed three times.

2. Seawater temperature during the test was 13.1±1.5 °C.

Figure 2: Handling stress experiment 1; the effect of handling stress on intensity of Gyrodactylus stellatus on laboratory held English sole after four weeks. Formalin-disinfected fish were stressed by holding them out of water in a dipnet for two-minutes three separate times over the course of an hour. Fish were then re-infected via two fin-clipped G. stellatus infected sole. Infection intensity levels of the two treatments were not significantly different (Student's t-test, $0.10>P$).

Figure 3: Handling stress experiment 2; the effect of handling stress on intensity of Gyrodactylus stellatus on laboratory held English sole after four weeks. Formalin-disinfected fish were stressed by holding them out of water in a dipnet for two-minutes. Fish were then re-infected via two fin-clipped G. stellatus infected sole. Infection intensity levels between the two treatments were significantly different (Student's t-test, $P\leq0.001$).

Figure 4: Handling stress experiment 3; the effect of handling stress on intensity of Gyrodactylus stellatus on laboratory held English sole after four weeks. Sole maintained low infection intensities from a previous laboratory epizootic. Test fish were stressed by holding them out of water in a dipnet for two-minutes three separate times over the course of an hour. Infection intensity levels between the two treatments were significantly different (Student's t-test, $0.05\geq P>0.01$).

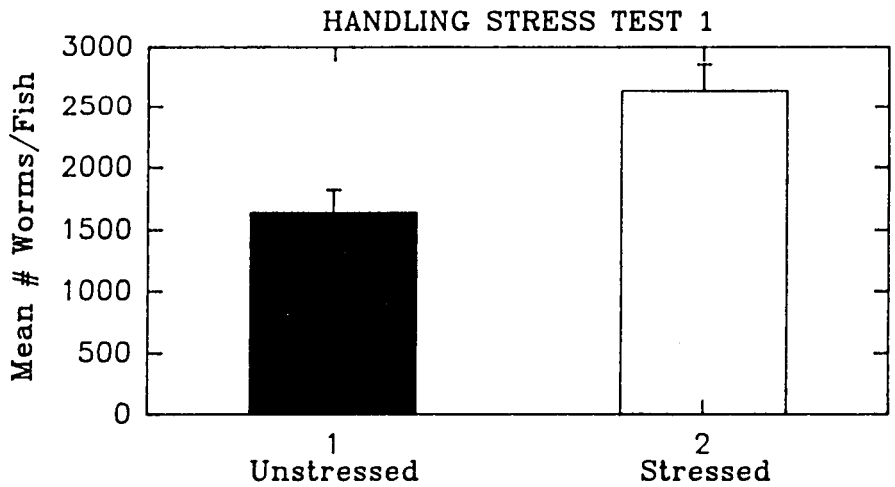

Figure 2

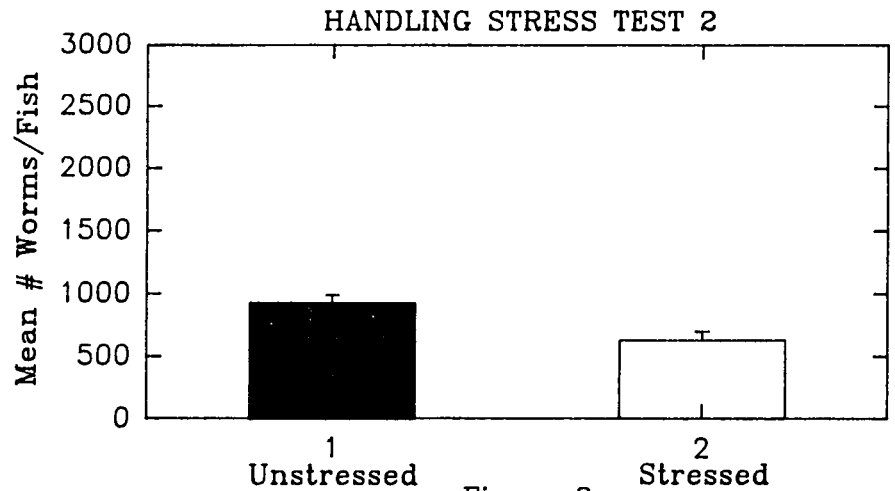

Figure 3

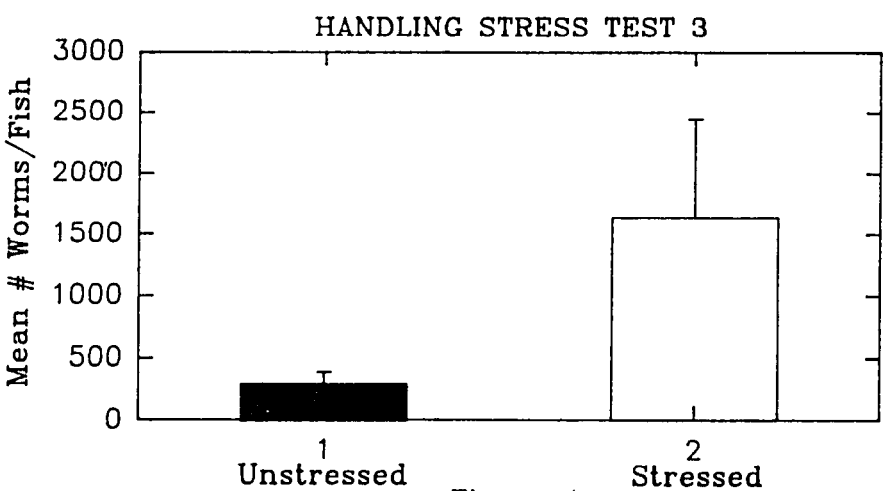

Figure 4

To determine how infection intensity levels and condition factors of sole used in the handling stress experiments compared to sole in Yaquina bay, infection intensity levels and condition factors were compared. Infection intensity levels on fish in all of the handling stress experiments were significantly higher than infection intensity levels of newly captured sole (Student's t-test, $P \leq 0.001$). Condition factors were not significantly different with the exception of the condition factors of the stressed treatment in the second handling stress experiment which were significantly higher than those of newly captured fish ($0.05 \geq P > 0.01$) (see page 22).

Substrate Experiment:

The influence of substrate on G. stellatus infection levels on laboratory held English sole was tested by holding seventy-nine (95.5 ± 9.25 S.E. mm) newly captured, naturally infected sole in tanks with or without a sand substrate for two weeks. Forty sole were placed in tanks without substrate and 39 sole were placed in tanks with substrate. Survival at the end of the experiment was 100% in both treatments.

Results are given in Table 4. Infection intensity levels were 54.9 ± 32.44 S.E. worms/fish on the fish in tanks without substrate and 37.3 ± 21.97 S.E. worms/fish on the fish in tanks with substrate (Figure 5). Infec-

tion intensity levels and condition factors were both significantly different between treatments, with fish in tanks with substrate having higher condition factors ($P \leq 0.001$) and lower infection rates ($P \leq 0.001$) than fish in tanks without substrate.

To determine how infection intensity levels and condition factors of sole used in the substrate experiment compared to sole in Yaquina bay, infection intensity levels and condition factors were compared. Infection intensity levels were significantly higher than infection intensity levels of newly captured sole ($P \leq 0.001$) and condition factors were significantly lower than those of newly captured sole ($P \leq 0.001$) (see page 22).

Table 4: The effect of substrate on intensity of *Gyrodactylus stellatus*, condition factors and survival of laboratory held English sole after two weeks (1,2).

Treatment	N	Surviving N	Worms/Fish±S.E. (Range)	C-factor±S.E. (Range)
No Substrate	40	40	54.9±32.44 (10-179)	0.77±0.046 (0.69-0.92)
Substrate	39	39	37.3±21.97 (5-106)	0.86±0.074 (0.73-1.09)
Student's t-test Significance Level			(P<0.001)	(P<0.001)

1. Test fish were newly captured, naturally infected and held in test chambers with or without a sand substrate.

2. Seawater temperature during the test was 12.9±0.8 °C.

Figure 5: The effect of substrate on infection intensity levels of Gyrodactylus stellatus on laboratory held English sole after two weeks. Infection intensity levels between the two treatments were significantly different (Student's t-test, $P \leq 0.001$).

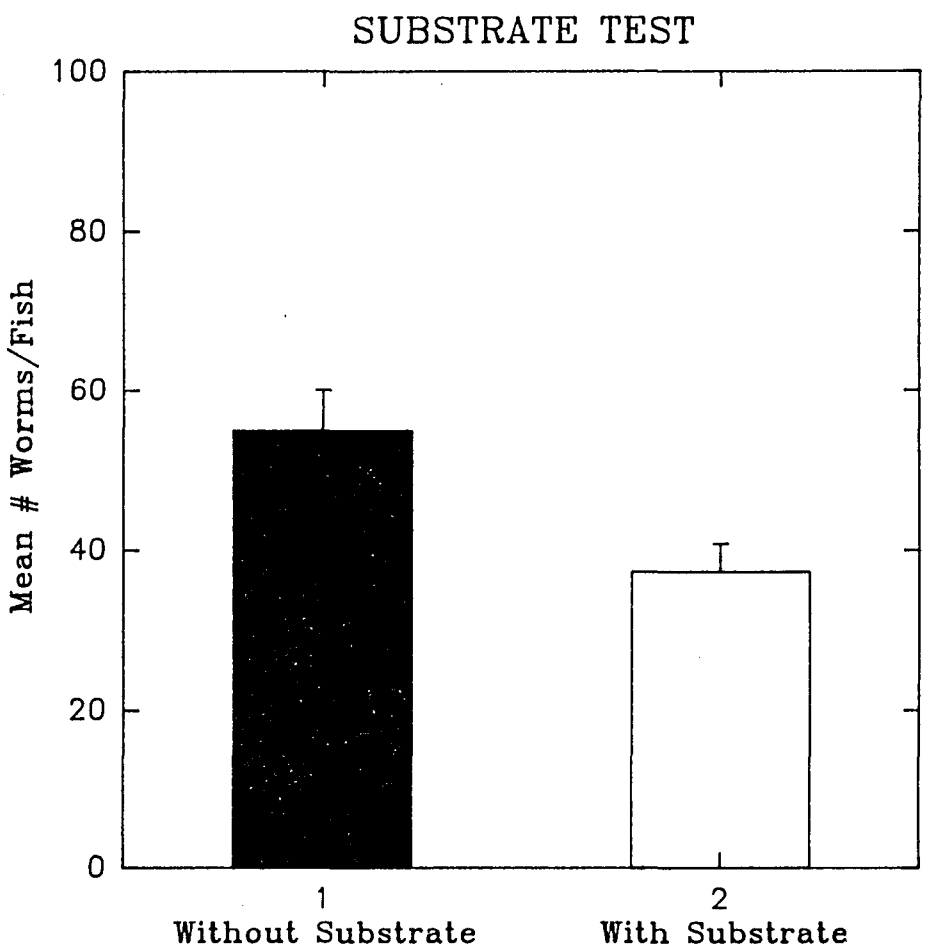
SUBSTRATE TEST
Mean # Worms/Fish
100
80
60
40
20
0
1
Without Substrate
2
With Substrate

Figure 5

Starvation Experiment:

To determine if G. stellatus had an effect on the survival of unfed English sole, the survival times of unfed sole that were naturally infected with the parasite were compared to the survival times of disinfected, unfed sole. Each treatment had 40 fish and dead fish were observed under a dissecting scope to confirm the presence or absence of worms depending on the treatment. Worms were found to be present on fish in one test chamber that contained disinfected fish, data from those fish were not included in statistical analyses.

Results of the starvation experiment are presented in Table 5. The mean survival time of the infected sole was 77.5 $\pm$ 7.37 S.E. days and mean survival time of the disinfected sole was 136.5 $\pm$ 34.58 S.E. days (Figure 6). Mean survival times ($P \leq 0.001$) and condition factors ($P \leq 0.001$) were significantly different between infected and disinfected fish, with disinfected fish living longer and having higher condition factors.

To determine how condition factors of sole used in the starvation experiment compared to sole in Yaquina bay, condition factors were compared. Condition factors of fish in the experiment were significantly lower than condition factors of newly captured sole ($P \leq 0.001$).

TABLE 5: The effect of Gyrodactylus stellatus on survival time and condition factors of unfed, newly captured, naturally infected and disinfected English sole held in test chambers until death (1).

Treatment	N	Days Survival± S.E. (Range)	C-factor±S.E. (Range)
Infected	40	77.5±7.37 (58-97)	0.62±0.070 (0.51-0.77)
Disinfected	28	136.5±34.58 (75-199)	0.50±0.072 (0.36-0.64)
Student's t-test Significance Level		(P<0.001)	(P<0.001)

1. Seawater temperature during the test was 12.3±8.9 °C.

Figure 6: The effect of Gyrodactylus stellatus on survival time of unfed, newly captured, naturally infected and disinfected English sole held in test chambers until death. Mean survival times between the two treatments were significantly different (Student's t-test, $P \leq 0.001$).

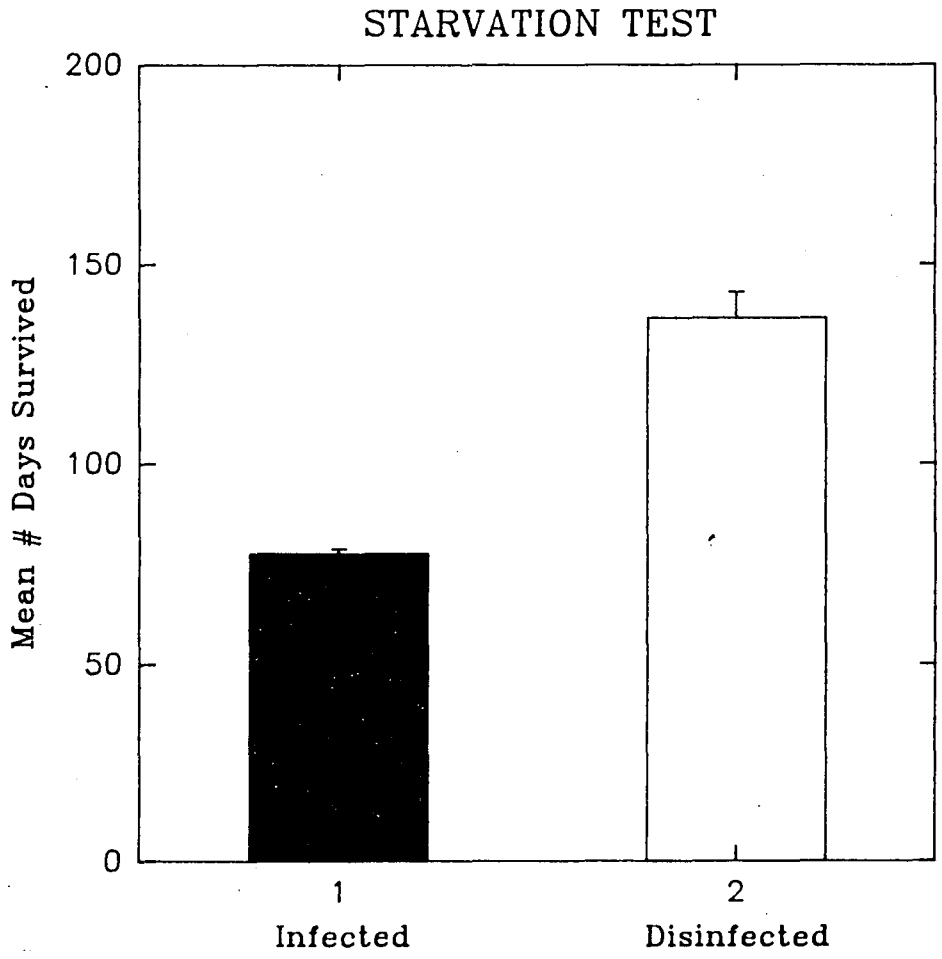
STARVATION TEST
Mean # Days Survived
200
150
100
50
0
1
Infected
2
Disinfected

Figure 6

Mucus Bioassays:

To assertain if English sole mucus contains components that are involved in resistance to monogenetic trematode infection, two bioassays testing the survival times of G. stellatus in English sole mucus collected from two groups of fish at different times during a laboratory infection were performed. In both bioassays, mucus from the newly captured fish in each group served as the treatment to which all other treatments were compared.

In the first bioassay, samples were collected from a group of sole at capture and every two weeks for six weeks. At six weeks, mucus samples collected from sole that carried heavy infections were tested separately from those collected from sole that carried light infections. In heavy infections, trematodes numbered in the thousands with the sole bearing dense patches of trematodes on the fins; in light infections, trematodes numbered in the hundreds and were more evenly and widely distributed over the fins of the sole than in heavy infections. Buffalo sculpin mucus and seawater served as negative controls.

In the first mucus bioassay test, trematodes survived significantly longer in seawater (20.5$\pm$4.42 S.E. hours) than they did in mucus from newly captured sole (8.7$\pm$1.45 S.E. hours) (Dunnett's test, $P \leq 0.05$). There was no significant difference between trematode survival

time in mucus from newly captured sole and any other mucus sample (Table 6 and Figure 7). Trematode survival time in mucus collected from heavily infected fish after six weeks (9.0±1.31 S.E. hours) was significantly longer than in mucus collected from lightly infected fish after six weeks (5.2±0.89 S.E. hours) (Student's t-test, $P \leq 0.001$).

Table 6: Mucus bioassay 1; the effect of English sole mucus on the mean survival time (MST) of *Gyrodactylus stellatus*. Mucus was collected from sole upon capture and every two weeks following for six weeks. Samples taken at six weeks were separated by infection level (heavy and light). Controls were Buffalo sculpin mucus and seawater.

Sample	N	# of Replicates	MST±S.E. (hours) (Range)
Newly captured	43	3	8.7±1.45 (7.3-10.2)
2 Weeks	49	3	11.0±1.12 (9.87-12.11)
4 Weeks	46	3	10.7±1.15 (4.4-6.1)
6 Weeks Heavy	49	3	9.0±1.31 (7.9-10.4)
6 Weeks light	42	3	5.2±0.89 (4.4-6.1)
Buffalo Sculpin	49	3	11.1±2.39 (8.6-13.3)
Seawater*	39	3	20.5±4.42 (15.7-24.3)

* Denotes samples significantly different from newly captured (Dunnett's test, $P \leq 0.05$).

Figure 7: Mucus bioassay 1; the effect of English sole mucus on the mean survival time of Gyrodactylus stellatus. Mucus was collected from sole upon capture and every two weeks following for six weeks. Samples taken at six weeks were separated by infection level (heavy and light). Controls were Buffalo sculpin mucus and seawater.

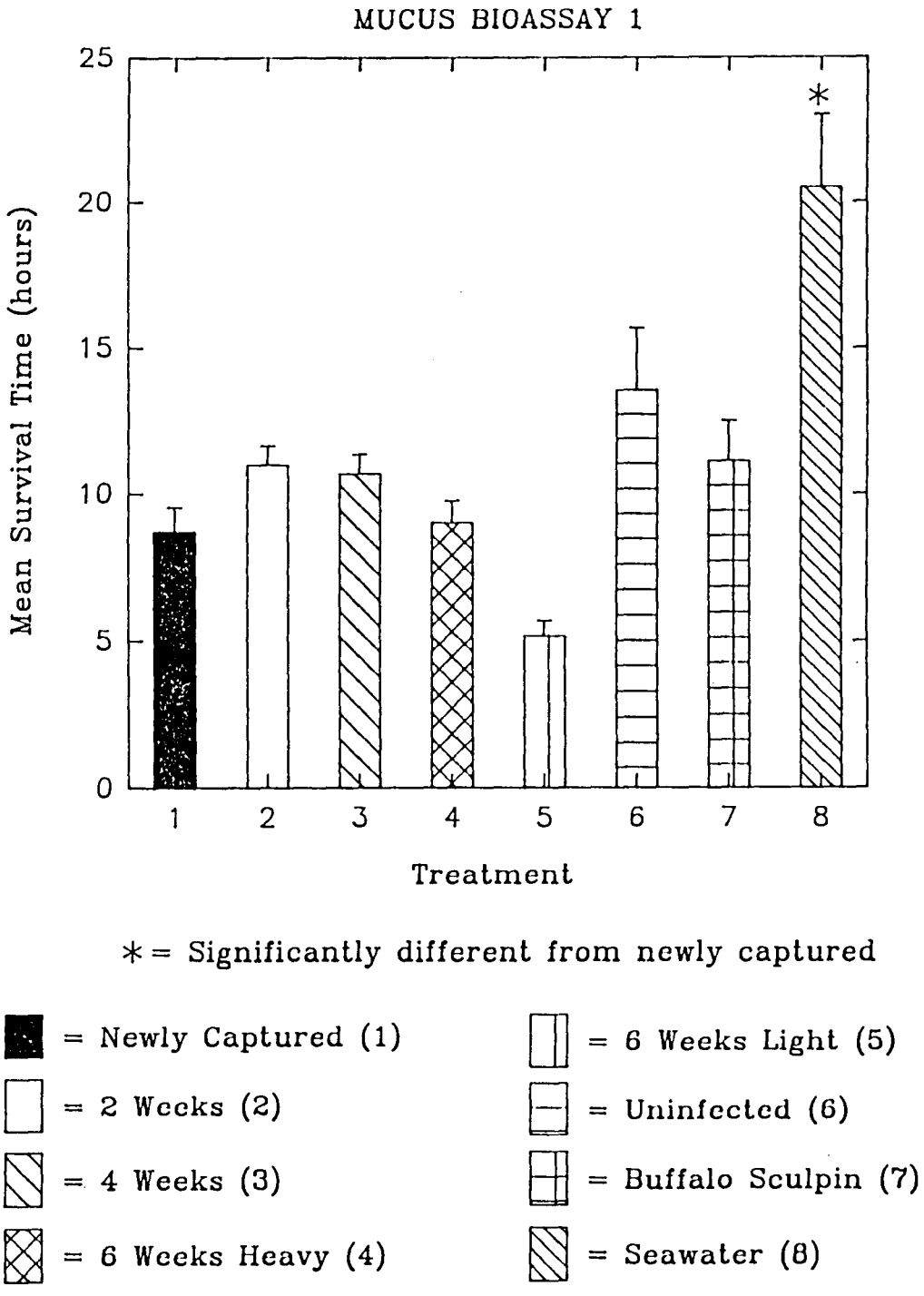
MUCUS BIOASSAY 1
Mean Survival Time (hours)
25
20
15
10
5
0
1
2
3
4
5
6
7
8
Treatment
* = Significantly different from newly captured
= Newly Captured (1)
= 2 Weeks (2)
= 4 Weeks (3)
= 6 Weeks Heavy (4)
= 6 Weeks Light (5)
= Uninfected (6)
= Buffalo Sculpin (7)
= Seawater (8)

Figure 7

In the second bioassay, mucus samples were collected from a group of sole at capture and every two weeks following for ten weeks. Mucus samples collected from heavily infected fish after eight weeks and 10 weeks were tested separately from those collected from lightly infected fish. Survival of G. stellatus was also tested in mucus from uninfected and G. stellatus injected sole. Survival of worms in Buffalo sculpin mucus and in seawater served as controls.

Trematode survival times in mucus from lightly infected (recovering) sole at eight weeks (10.2 $\pm$ 0.071 S.E. hours) and ten weeks (9.3 $\pm$ 0.94 S.E. hours) were significantly lower than was trematode survival time in mucus from newly captured sole (15.6 $\pm$ 3.40 S.E. hours) (Bonferroni t-test, $P \leq 0.05$). Trematode survival in seawater (26.0 $\pm$ 4.41 S.E. hours) was significantly higher than trematode survival in mucus from newly captured sole (Figure 8). The survival of G. stellatus in all other mucus samples was not significantly different from that in mucus from newly captured sole. Results from the second mucus bioassay are presented in Table 7. Trematode survival time in mucus from lightly infected sole was significantly shorter than that in mucus collected from heavily infected sole in both the eight and 10 weeks samples (Student's t-test, $P \leq 0.001$).

Table 7: Mucus bioassay 2; the effect of English sole mucus on the mean survival time (MST) of *Gyrodactylus stellatus*. Mucus was collected from sole upon capture and every two weeks following for ten weeks. Samples taken at eight and ten weeks were separated by infection level (heavy and light). Mucus samples from uninfected and immunized sole were also tested. Controls were Buffalo sculpin mucus and seawater.

Sample	N	# of Replicates	MST±S.E.(hours) (Range)
Newly captured	20	3	15.6±3.40 (11.0-18.6)
2 Weeks	24	3	14.4±0.88 (13.7-15.4)
4 Weeks	28	3	16.6±1.03 (15.5-17.6)
6 Weeks	24	3	14.2±2.76 (12.4-17.4)
8 Weeks Heavy	21	2	15.2±0.04 (15.2-15.2)
8 Weeks Light*	31	3	10.2±0.71 (9.5-10.9)
10 Weeks Heavy	25	3	13.4±2.33 (10.7-15.0)
10 Weeks Light*	31	3	9.3±0.94 (8.2-10.0)
Uninfected	28	3	18.8±0.86 (18.3-19.8)
Immunized	31	3	14.05±2.53 (11.6-16.6)
Buffalo Sculpin	28	3	18.7±0.84 (17.9-19.6)
Seawater*	22	4	26.0±4.41 (20.7-30.6)

* Denotes samples significantly different from newly captured (Dunnett's test, $P \leq 0.05$).

Figure 8: Mucus bioassay 2; the effect of English sole mucus on the mean survival time of Gyrodactylus stellatus. Mucus was collected from sole upon capture and every two weeks following for ten weeks. Samples taken at eight and ten weeks were separated by infection level (heavy and light). Mucus samples from uninfected and immunized sole were also tested. Controls were Buffalo sculpin mucus and seawater.

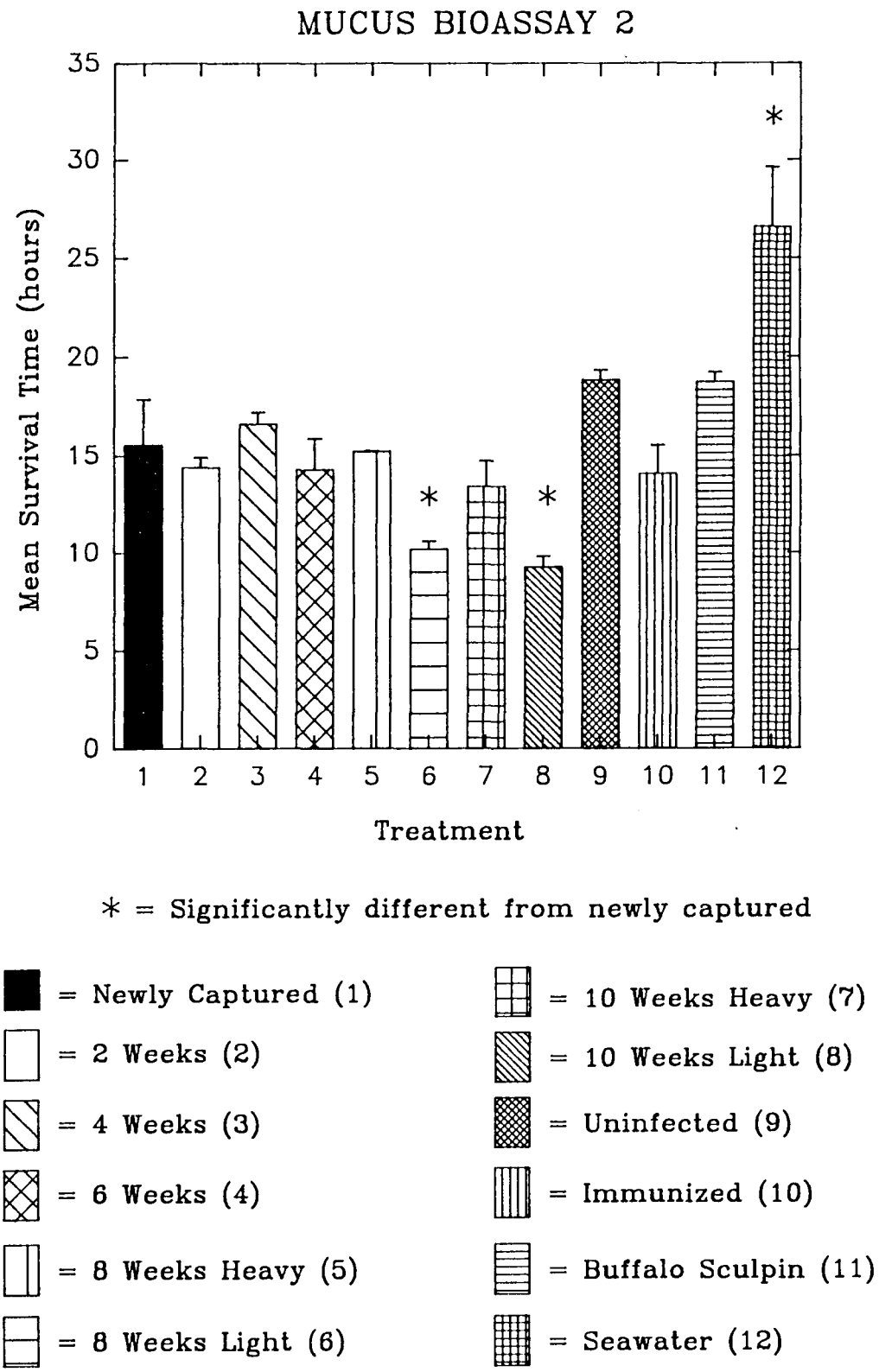
MUCUS BIOASSAY 2
Mean Survival Time (hours)
0
5
10
15
20
25
30
35
1
2
3
4
5
6
7
8
9
10
11
12
Treatment
*
*
*
* = Significantly different from newly captured
= Newly Captured (1)
= 2 Weeks (2)
= 4 Weeks (3)
= 6 Weeks (4)
= 8 Weeks Heavy (5)
= 8 Weeks Light (6)
= 10 Weeks Heavy (7)
= 10 Weeks Light (8)
= Uninfected (9)
= Immunized (10)
= Buffalo Sculpin (11)
= Seawater (12)

Figure 8

Serum Bioassays:

To assertain if English sole serum contained factors involved in resistance to trematodes, two bioassays testing the survival times of G. stellatus in English sole serum collected from two groups of fish at different times during a laboratory infection were performed. Serum samples were collected at the same time as were mucus samples for the mucus bioassay tests. The only serum samples that were tested were those from fish whose mucus produced a bioassay result suggesting the presence of a factor that affected G. stellatus survival. In both bioassays, serum from the newly captured fish in each group served as the treatment to which all other treatments were compared.

In the first bioassay, serum samples collected from a group of sole upon capture and after two weeks were tested. In the second bioassay, serum samples collected from a group of sole upon capture, after eight weeks and after 10 weeks were tested. The eight and ten week samples were separated according to trematode infection intensity as described above. Sera from uninfected and G. stellatus injected sole were also tested. Buffalo sculpin and chum salmon sera served as controls.

In the first group of sole, trematode survival times in the two week and uninfected sole sera did not differ significantly from newly captured sole. Trematode

survival times in buffalo sculpin serum (11.0 ± 1.21 S.E. hours) was significantly higher than survival in serum from newly captured sole (5.3 ± 0.37 S.E. hours) (Bonferroni t-test, $P \leq 0.05$). Results of the serum bioassay using serum from the first group of sole are given in Table 8 and Figure 9.

Table 8: Serum bioassay 1; the effect of English sole serum on the mean survival time (MST) of Gyrodactylus stellatus. Serum was collected from sole upon capture and after two weeks. Serum from immunized sole was also tested. Controls were Buffalo sculpin and chum salmon sera.

Sample	N	# of Replicates	MST±S.E.(hours) (Range)
Newly captured	24	3	5.3±0.37 (4.9-5.6)
2 Weeks	22	3	5.0±0.33 (4.6-5.3)
Uninfected	11	2	5.4±0.23 (5.3-5.6)
Immunized**	17	2	3.0±0.00
Buffalo Sculpin*	20	3	11.0±1.21 (9.8-12.3)
Chum Salmon**	28	3	0.5±0.00

* Denotes samples significantly different from newly captured (Dunnett's test, $P \leq 0.05$).

** The treatments couldn't be compared statisically because there was no variance.

Figure 9: Serum bioassay 1; the effect of English sole serum on the mean survival time of Gyrodactylus stellatus. Serum was collected from sole upon capture and after two weeks. Serum from immunized sole was also tested. Controls were Buffalo sculpin and chum salmon sera.

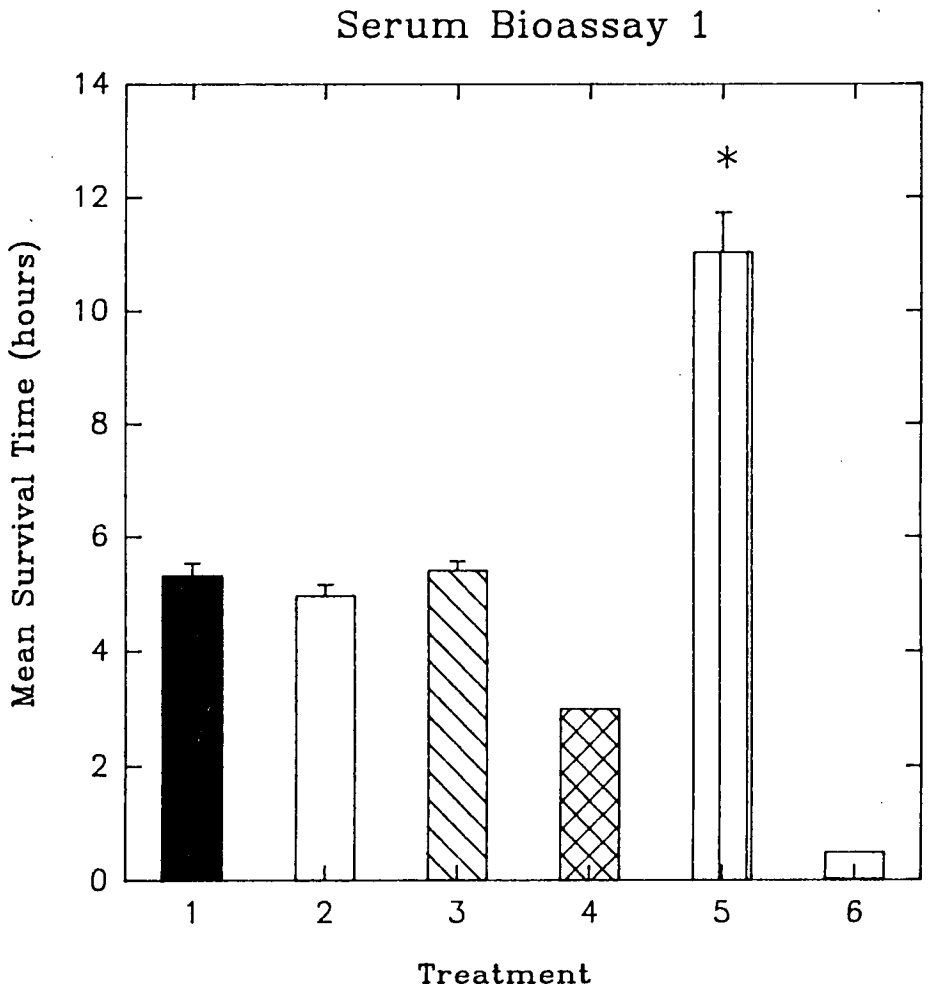
Serum Bioassay 1
Mean Survival Time (hours)
14
12
10
8
6
4
2
0
*
1
2
3
4
5
6
Treatment
* = Significantly different from newly captured
= Newly Captured (1)
= 2 Weeks (2)
= Uninfected (3)
= Immunized (4)
= Buffalo Sculpin (5)
= Chum Salmon (6)

Figure 9

When serum samples from the second group of sole were tested, trematode survival times in all serum samples, with the exception of uninfected sole, were significantly different from survival times in newly captured sole serum (Bonferroni t-test, $P \leq 0.05$). Trematode survival times in sera from lightly infected sole at eight weeks (5.0 $\pm$ 0.29 S.E. hours) and heavily (4.5 $\pm$ 1.12 S.E. hours) and lightly (1.7 $\pm$ 0.16 S.E. hours) infected sole at ten weeks were significantly shorter than in newly captured sole serum (6.8 $\pm$ 1.08 S.E. hours). Trematode survival time in sera from buffalo sculpin (11.0 $\pm$ 1.21 S.E. hours) and heavily infected sole at eight weeks (11.5 $\pm$ 0.73 S.E. hours) were significantly higher than in serum from newly captured sole. Results of the serum bioassay using serum from the second group of sole are given in Table 9 and Figure 10. Trematode survival time in serum collected from lightly infected sole was significantly shorter than that in serum collected from heavily infected sole in both the eight and 10 weeks samples (Student's t-test, $P \leq 0.001$).

Table 9: Serum bioassay 2; the effect of English sole serum on the mean survival time (MST) of Gyrodactylus stellatus. Serum tested was collected from sole upon capture and at eight weeks and 10 weeks. Eight and ten week samples were separated by infection levels (heavy and light). Serum from immunized sole was also tested. Controls were Buffalo sculpin and chum salmon sera.

Sample	N	# of Replicates	MST±S.E.(hours) (Range)
Newly captured	26	3	6.8±1.08 (5.7-7.9)
8 Weeks Heavy*	19	3	11.5±0.73 (10.7-12.0)
8 Weeks Light	22	3	5.0±0.29 (4.7-5.3)
10 Weeks Heavy*	27	3	4.5±1.12 (3.6-5.8)
10 Weeks Light*	25	3	2.7±0.16 (2.6-2.9)
Uninfected	11	2	5.4±0.23 (5.3-5.6)
Immunized**	17	2	3.0±0.00
Buffalo Sculpin*	20	3	11.0±1.21 (9.8-12.3)
Chum Salmon**	28	3	0.5±0.00

* Denotes samples significantly different from newly captured (Dunnett's test, $P \leq 0.05$).

** The treatment couldn't be compared statistically because there was no variance.

Figure 10: Serum bioassay 2; the effect of English sole serum on the mean survival time of Gyrodactylus stellatus. Serum tested was collected from sole upon capture and at eight weeks and 10 weeks. Eight and ten week samples were separated by infection levels (heavy and light). Serum from immunized sole was also tested. Controls were Buffalo sculpin and chum salmon sera.

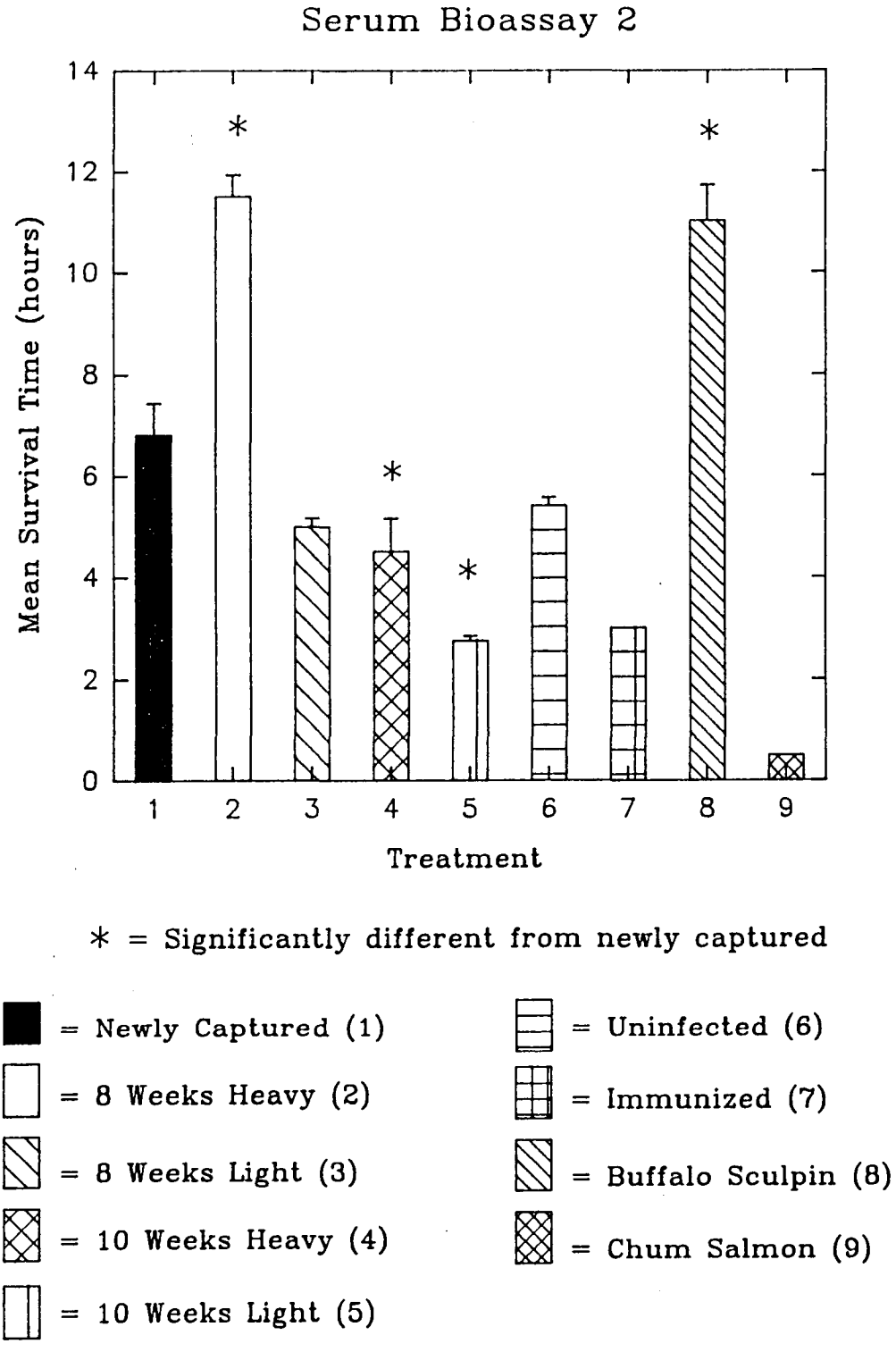
Serum Bioassay 2
Mean Survival Time (hours)
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9
Treatment
* = Significantly different from newly captured
= Newly Captured (1)
= 8 Weeks Heavy (2)
= 8 Weeks Light (3)
= 10 Weeks Heavy (4)
= 10 Weeks Light (5)
= Uninfected (6)
= Immunized (7)
= Buffalo Sculpin (8)
= Chum Salmon (9)

Figure 10

Gel Diffusion, Microtiter and Slide Agglutination Tests:

In an effort to determine if serum from English sole infected with G. stellatus contained precipitins (e.g. antibodies) against G. stellatus, gel diffusions of serum and mucus from infected sole were run against homogenized worms. No evidence of the presence of precipitins was found, possibly due to an inadequate (31 μg/ml) amount of soluble protein in the worm preparation.

Microtiter and slide agglutination tests to determine if agglutinating antibodies against G. stellatus could be detected in serum and mucus from trematode infected sole were attempted. Agglutinating antibodies were not detected by either method.

An effort was then made to determine if the mucus from G. stellatus infected English sole contained factors also found in the serum of infected sole. This was determined by Ouchterlony tests in which mucus samples were diffused with rabbit antiserum against English sole serum.

Rabbit antiserum was in the center well of the gel with undiluted samples of mucus described in the mucus bioassays in the surrounding wells. Sera from buffalo sculpin and chum salmon served as controls. English sole serum served as a positive control.

The rabbit antiserum recognized serum factors in most of the English sole mucus samples, with differences

in the strength of the precipitation reaction and/orthe number of precipitating bands (Figures 11, 12, and 13). Stronger precipitation reactions occurred in mucus samples collected at later times during the trematode infection. The rabbit antiserum did not recognize any serum factors in mucus from G. stellatus injected sole, uninfected sole, and buffalo sculpin and chum salmon sera. The rabbit antiserum formed four to five precipitating bands with the positive control, English sole serum. Results of the fish mucus, rabbit antiserum Ouchterlonys are given in Table 10.

To test for the effect of prozone, an Ouchterlony test was done with dilutions of 1:1, 1:2, 1:3, 1:4, 1:5 and 1:50 of mucus in PBS from uninfected sole in the surrounding wells and undiluted rabbit antiserum in the center well. As a control, mucus from lightly infected sole at 10 weeks was diluted in the same manner on a second Ouchterlony plate. No precipitation bands were seen in any of the dilutions of the mucus from uninfected sole, and a single band of precipitation was present in all dilutions except 1:50 in the mucus from lightly infected sole at 10 weeks. These results indicate that prozone was not inhibiting precipitation band formation, confirming that the mucus from uninfected sole did not contain factors antigenically related to those in English sole serum.

Table 10: Results of Ouchterlony tests of English sole mucus samples collected from two groups of sole (1) diffused with rabbit antiserum against English sole serum. Mucus from uninfected and immunized sole, and serum from buffalo sculpin and chum salmon were also tested. English sole serum served as a positive control.

Sample	# of Precipitation bands	Precipitation Relative to Newly Captured
Group 1:		
Newly captured	1	-
2 Weeks	1	Weaker
4 Weeks	1	Same
6 Weeks Heavy	1	Same
6 Weeks Light	2-3	Stronger
Group 2:		
Newly captured	1	-
2 Weeks	1	Weaker
4 Weeks	1	Weaker
6 Weeks	1	Same
8 Weeks Heavy	4-5	Stronger
8 Weeks Light	1	Stronger
10 Weeks Heavy	4-5	Stronger
10 Weeks Light	1	Stronger
Uninfected sole	0	-
Immunized sole	0	-
Sera:		
English Sole	4-5	Stronger
Buffalo Sculpin	0	-
Chum Salmon	0	-

1. Mucus was collected upon capture, and every two weeks following. Samples were separated by infection level (heavy or light) in some samples.

Figure 11: Plate 1; Ouchterlony test to detect English sole serum factors in English sole mucus. Mucus was collected from the first group of sole upon capture and every two weeks following for six weeks. Samples taken at six weeks were separated by infection level (heavy and light). The Center well contained rabbit antiserum against English sole serum. The first well contained English sole serum and served as a positive control (1). The remaining wells contained English sole mucus from: uninfected (2); newly captured (3); two weeks (4); four weeks (5); and six weeks (heavy infection) (6).

Figure 12: Plate 2; Ouchterlony test to detect English sole serum factors in English sole mucus. Mucus was collected from two groups of sole upon capture and every two weeks following. Samples were separated by infection level (heavy and light). The center well contained rabbit antiserum against English sole serum. The center well contained English sole serum and served as a positive control (1). The remaining wells contained English sole mucus from: six weeks (light infection), group 1 (2); and newly captured (3), two weeks (4), four weeks (5), and six weeks (6) from group 2 sole.

Figure 11

Figure 12

Figure 13: Plate 3; Ouchterlony test to detect English sole serum factors in English sole mucus. Mucus was collected from the second group of sole upon capture and every two weeks following for ten weeks. Samples were separated by infection level (heavy and light). The center well contained rabbit antiserum against English sole serum. The first well contained English sole serum and served as a positive control (1). The remaining wells contained English sole mucus from: immunized (2); eight weeks (heavy infection) (3); eight weeks (light infection) (4); ten weeks (heavy infection) (5); and ten weeks (light infection) (6).

Figure 13

IV. DISCUSSION

The purpose of this study was to explore the mechanisms of English sole resistance to G. stellatus by examining the basis for the transitory loss of resistance in the laboratory reported by Kamiso and Olson (1986). Previous studies determined that although water temperature, nutrition, and crowding were factors that influenced the host-parasite relationship between juvenile English sole and G. stellatus, they did not account for the high infection intensities that develop on laboratory held English sole (Handoyo 1983). Stresses associated with handling, transport, and captivity are known to influence the disease susceptibility of fishes by suppressing immune responses (Ellsaesser and Clem, 1986; Miller and Tripp, 1982) and may explain changes in English sole resistance to G. stellatus.

The three handling stress experiments in this study differed in length of acclimation, method of infection, and the extent of handling stress to which test fish were exposed to. In the first experiment, there was no difference in the infection intensity levels between treatments; in the second experiment, fish in the stressed treatment had significantly fewer trematodes than did fish in the unstressed treatment; and in the third ex-

periment, fish in the unstressed treatment had significantly fewer worms than fish in the stressed treatment.

Khalil (1964) found that Polypterus senegalus that recovered from infections by the Macrogyrodactylus polypteri were not susceptible to reinfection so long as they retained a few trematodes. If the fish remained without the parasite for a 'short while', reinfection was possible. Lester and Adams (1974b) observed that threespine sticklebacks that had lost their G. alexanderi infections were refractory to further infections for about three weeks and then were susceptible to reinfection. In the first two handling stress experiments, the infection intensity levels of the unstressed (control) treatments, were high. One explanation for this may be that the sole had lost resistance to the parasite during their trematode-free, pre-test acclimation period which exceeded three weeks in both experiments.

In the second experiment, trematode infection intensities were higher on the unstressed fish than on the stressed fish. The level of stress exposure in the second experiment was lower than the level of stress in the first and third experiments, and may not have been sufficient enough to cause a response. Fish in the second experiment were observed to secrete large quantities of mucus during exposure to the handling stress.

This in combination with a loss of trematode resistance in both the stressed and unstressed groups may have given the stressed fish a slight advantage over the unstressed fish when the parasite was reintroduced and could have resulted in lower infection levels on the fish that received the handling stress.

The third handling stress experiment was probably the most accurate re-creation of G. stellatus infection dynamics that occur when fish are captured and brought to the laboratory. The fish were not disinfected, went through a G. stellatus epizootic, recovered and had relatively low infection intensity levels at test initiation seven months after capture. Fish in the treatment group were exposed to the same handling stress as were fish in the first experiment, and infection intensity levels of stressed fish were significantly higher than those of unstressed individuals. Infection intensity levels of the unstressed group (control) remained close to the low levels typical for fish that had survived a laboratory epizootic (Kamiso and Olson 1986).
The results suggest that handling stress can influence the host-parasite relationship between English sole and G. stellatus, and that stresses associated with capture and laboratory holding may be the cause of the increased trematode intensity levels in laboratory held fish. Results of the first two experiments were probably in-

fluenced by manipulations that masked attempts to experimentally emulate handling stress.

In the estuary, juvenile English sole are found in areas with sand or mud substrate (Toole et al. 1987). In the experiment designed to assess the influence of substrate on G. stellatus infections of laboratory held English sole, fish in the tanks with substrate had significantly fewer worms than the fish in tanks without substrate. During the experiment, the fish in the tanks with substrate were observed to bury themselves, startle less easily and eat more readily than fish in tanks without substrate.

Although the reduced number of trematodes on fish in the substrate treatment may have been due to mechanical effects of the sand, the behavior of the fish in this treatment suggested they may have also been under less stress than fish in tanks without substrate. The difference in infection intensity levels between treatments was possibly due to reduced stress and accompanying nutritional advantages in the group with substrate.

A previous study reported a higher rate of G. stellatus increase on unfed English sole than on fed sole and also that fed fish dying with heavy trematode infections ceased feeding and became emaciated before succumbing (Kamiso and Olson, 1986). They suggested that death may have been due to the combined effects of starvation

and heavy parasitism, but they did not separate the effects of starvation and trematode infection on mortality.

An experiment designed to separate the effect of G. stellatus infection and starvation on mortality showed that the survival times of unfed, trematode infected sole were significantly less than survival times of unfed, disinfected sole so G. stellatus infection does accelerate the rate at which unfed fish die.

Humoral antibody responses appear to be the only mechanism yet described for helminth mediated immunity in fishes (Evans and Gratzek, 1989). Precipitating, IgM-like antibodies against digenetic (Cottrell 1977) and acanthocephalan (Harris 1972) parasites have been detected in the sera of infected fish.

Nigrelli (1935a) studied the effects of marine fish mucus on the monogenetic trematode E. melleni and found that mucus from fish with natural immunity to the parasite shortened trematode survival under experimental conditions. Hanson (1973) observed similiar results when he exposed the monogenetic trematode D. embiotoci to serum and mucus from the striped surfperch (Embiotoca lateralis), a fish with natural resistance to the parasite. He suggested that specific antibodies in the mucus were involved in the resistance based on results of hemagglutination tests. The exact mechanisms operating

in resistance to monogenetic trematode infections is not known, but it has been suggested that resistance in gyrodactylid infections may be associated with mucus secretions (Evans and Gratzek, 1989; Scott and Robinson, 1984; Lester and Adams, 1974a; Handoyo 1983).

The mucus bioassays performed in this study indicated that both the mucus and serum of English sole were involved in resistance to the G. stellatus. Generally, trematode survival was shorter in mucus and serum samples collected at later times in the laboratory infection, when sole were beginning to show signs of recovery; and trematode survival time in the mucus and serum from lightly infected (recovering) sole was always significantly shorter than trematode survival time in the mucus from heavily infected sole held in the laboratory for the same period of time. These results suggested that the factors causing resistance in the mucus of English sole were also present in the serum, and that these factors may result in recovery from the infection. Results of the gel diffusion tests gave evidence to support this.

Proteins antigenically related to serum factors have been detected in the mucus of bass, catfish, and rainbow trout (O'rourke, 1961; Di Conza and Halliday, 1971; Harrell et al., 1976). In this study, rabbit antiserum against English sole serum was used in gel diffusion tests to determine that factors in English sole skin

mucus were antigenically related to factors in English sole serum, but these factors were not characterized. These factors were detected in mucus collected from sole at all times during a laboratory infection. Generally, bands of precipitation that indicated antigenic recognition were weakest in mucus collected from sole during periods of increasing infection intensity, and were strongest in mucus from sole at later stages of infection. No precipitation reactions were detected in the mucus of uninfected sole, indicating that the precipitation bands that were observed were associated with G. stellatus infections.

Multiple serum factors were detected in Ouchterlony tests on mucus from sole still heavily infected at late stages of infection (eight and ten weeks), and a single band was detected in Ouchterlony tests on mucus from lightly infected sole at late stages of infection. The factor detected in mucus from the lightly infected sole may be associated with resistance to the parasite because the serum and mucus of lightly infected sole had the greatest effect on trematode survival times, and it was from fish that were recovering from the infection.

The reason for the differences in the number of precipitation bands detected in mucus at heavy and light levels of infection is not known. One explanation could be leakage of serum into the mucus of heavily infected

fish through small areas of hemorrhaging on the fins of heavily infected sole.

Di Conza and Halliday (1971) identified a protein in the mucus of catfish which shared antigenic determinants with catfish serum antibody, they determined that this protein was immunoglobulin, but because it did not have antibody activity found at the same time as in serum, they suggested that the immunoglobulin was locally synthesized rather than derived from the blood. In contrast, the mucus of plaice was shown to contain antibodies similiar to those found in serum (Fletcher and Grant, 1969). Although the results of the gel diffusion tests in this study do not allow us to conclude that the factors in the serum and mucus are identical it is likely that they are for the following reasons: The serum and mucus of English sole had similiar effects on trematode survival times; and gel diffusion tests detected factors in the mucus antigenically similiar to factors in the serum.

Lester (1972) found that intramuscular injections of whole G. alexanderi antigen conferred no protection in to threespine sticklebacks and Nigrelli (1935b) observed similiar results when pompano (Trachinotus carolinus) were injected with ground, dried and fresh E. melleni. Immunizing English sole with homogenized G. stellatus in FCA failed to illicit an antibody response detectable by

the methods used in this study. Neither precipitating or agglutinating antibodies were detected; but, although the results couldn't be analyzed statistically, serum from immunized sole did appear to have an effect on trematode survival times (Figures 9 and 10). Because of problems with the methodology, the failure to detect G. stellatus antibodies in English sole can not be interpreted to mean that antibodies weren't produced. Low levels of soluble protein in the trematode antigen preparation may be the reason for negative reactions in the gel diffusion tests, and neither the gel diffusion nor agglutination tests had a positive control, such as rabbit antiserum against G. stellatus.

In summary, the infection intensity of G. stellatus on laboratory held English sole was influenced by handling stress, substrate, and starvation. Trematode survival times were significantly reduced in the serum and mucus samples collected from sole at the later, recovering stages of infection, suggesting that both the serum and mucus of English sole are involved in resistance. The mucus of G. stellatus infected English sole contained factors antigenically similiar to factors in English sole serum. These factors were not present in the mucus of uninfected sole. Precipitation bands in the gel diffusions tests appeared to be the strongest in mucus samples from sole at later, recovering stages of infec-

tion. Results of the serum and mucus bioassays and the Ouchterlony tests suggest the possible presence of resistance factors in both the serum and the mucus at later stages of trematode infection, and that these factors result in recovery from the infection. The results also suggest that resistance factors in the mucus may originate from the serum.

Although the resistance factors were not characterized, they probably have an immunological function. Immunological factors that are found in both the serum and mucus of fish include: immunoglobulin (Fletcher and Grant, 1969; Harris 1972; Bradshaw et al., 1971); complement (Harrell et al., 1976); lysozyme (Fletcher and Grant, 1968); and C-reactive protein (Ramos and Smith, 1978). Thus far, only IgM-like, precipitating antibodies (plus complement) have been indicated in resistance to helminth infections (Evans and Gratzek, 1989; McVicar and Fletcher; 1970; Cottrell, 1977; Harris 1972).

Quantifying and characterizing the resistance factor that appears to be present in English sole serum and mucus may give us information that would allow us to better understand mechanisms involved in resistance to monogenetic trematodes. The factor could be quantified by obtaining a titer of the precipitins in each mucus sample, this would indicate if there were differences in

the amount of precipitins present at different stages of infection.

Separating the different components of the mucus by column chromatography and repeating the mucus bioassays on the fractions would be the first step in isolating and identifying the factor responsible for resistance. Once the fraction containing the resistance factor was isolated, the factor itself could be identified through biochemical analyses. Biochemical analyses would also indicate if the same factor was in the serum and the mucus.

If the factor was determined to be an immunoglobulin, then the presence of complement activity could be tested by repeating the bioassays with heat-treated mucus and serum. The Ouchterlonys should be repeated with the resistant fraction of the mucus to determine if the factors that precipitated in whole mucus samples were contained in the resistant fraction.

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