

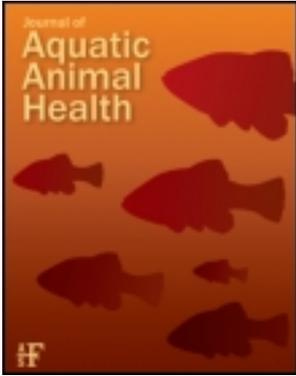
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Biologically Active Factors against the Monogenetic Trematode *Gyrodactylus stellatus* in the Serum and Mucus of Infected Juvenile English Soles

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Abstract.—Length of survival of the monogenetic trematode *Gyrodactylus stellatus* in serum and mucus collected from English soles *Pleuronectes vetulus* at different stages of a laboratory epizootic suggests that both the mucus and serum may be involved in resistance to the parasite. In general, trematode survival was shorter in the serum and mucus samples collected from English soles at the later, recovering stages of infection. A rabbit antiserum against English sole whole serum was used in a gel diffusion immunoassay to show that mucus from infested English soles contained proteins antigenically similar to English sole serum proteins. Precipitation reactions appeared strongest in mucus collected during later stages of infection. No precipitation reactions were detected in the mucus of uninfested fish, indicating that the precipitation bands that were observed were associated with *G. stellatus* infections.

English soles *Pleuronectes vetulus* utilize the Yaquina Bay, Oregon, estuary as a nursery ground for their first year of life. During this time, they

often have a low-level infection of the monogenetic trematode *Gyrodactylus stellatus* (Olson and Pratt 1973). Juvenile English soles captured and held in the laboratory have been observed to become much more heavily infected than those in the estuary, resulting in high mortality. In a previous study, English soles sampled from the estuary had an average infection level of 5.5 *G. stellatus* per fish, whereas the infection level in fish held in the laboratory underwent a logarithmic

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increase during the first 9 weeks of laboratory confinement, peaking at over 1,000 trematodes per fish, then decreasing rapidly over the following 3 weeks (Kamiso and Olson 1986).

Members of the genus *Gyrodactylus* are small (less than 1.0 mm long), live-bearing monogeneans found on the fins and skin of hosts, where they feed on the epidermis and epidermal secretions (Sproston 1946; Bychowsky 1957). The cutaneous mucus of fish provides a mechanical and chemical barrier against infection (Ingram 1980). In addition, because mucosal antibodies to various antigens have been induced, fish are thought to have a secretory immune system (Lobb and Clem 1981; Rombout et al. 1986; Wong et al. 1992).

In this study, the progress of a trematode infection in English soles was followed to determine if there were factors in the serum and in the mucus that had detrimental effects on live *G. stellatus* (i.e., possible resistance factors), and if so, to determine if there was a relationship between the presence of these factors and the level of infection. This was accomplished with bioassays that tested for differences in the length of survival of *G. stellatus* exposed to serum and mucus samples collected from English soles throughout a laboratory epizootic.

In lieu of specific anti-immunoglobulin reagents for the assessment of English sole antibody responses, a simple alternative immunoassay was developed to monitor the appearance of serumlike proteins in the mucus. The appearance of such proteins was correlated with anti-*G. stellatus* biological activity.

Methods

Collection and maintenance of fish.—Juvenile English soles (total length, mean \pm SE: 107.4 \pm 14.57 mm) were collected from Yaquina Bay, Oregon, with a 5-m otter trawl. The fish were transported to the Oregon State University Hatfield Marine Science Center, where they were held in 125-L, flow-through fiberglass tanks provided with filtered, ultraviolet-sterilized seawater from Yaquina Bay. Water temperature was ambient and was measured every other day. Fish were initially fed frozen krill, then they were gradually switched to a diet of commercial moist salmon feed over a period of 3 weeks.

Disinfection of fish.—Uninfected experimental fish were obtained by treating newly collected English soles with a 1:4,000 formalin solution for 1 h to remove trematodes (Puz and Hoffman 1963).

Formalin-disinfected English soles were held in the laboratory for 8 weeks before use in experiments.

Laboratory assessment of infection level.—Twenty-five naturally infected English soles were sampled on the day of capture and then every 2 weeks during a laboratory epizootic. Fish removed from tanks were not replaced. Each fish was observed under a dissecting microscope to subjectively determine the infection level and verify that the epizootic followed the course described by Kamiso and Olson (1986). In preliminary experiments, direct counts of trematodes were made on English soles at all levels of infection. The direct counts were used to establish a basis for the subjective determinations.

Collection of serum and mucus samples.—Fish were anesthetized in a 1:1,500 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 of the caudal fin or by cardiac puncture. Mucus was kept on ice during collection, refrigerated at 4°C overnight, and then centrifuged for 15 min at 1,500 \times gravity. The supernatant was stored at -70°C. Blood was allowed to clot for 1–2 h at room temperature, refrigerated at 4°C overnight, and centrifuged for 15 min at 1,500 \times gravity. Serum was also stored at -70°C.

Serum and mucus samples were obtained from the same fish examined to determine the level of trematode infection. Sera were pooled and mucus samples were pooled for each sampling period, except when fish began to recover from the infection (at 8 and 10 weeks after capture). At those times there was a marked difference in infection levels, and the serum and mucus samples from heavily infected fish were separated from those of lightly infested fish. In heavy infections, trematodes numbered in the thousands, and the fish had dense patches of trematodes on their fins and bodies. In light infections, trematodes numbered in the hundreds and appeared more evenly distributed and widely spaced over the fins and bodies of the fish. Serum and mucus samples were also collected from the uninfected English soles described above.

Artificial induction of immune response.—To artificially induce anti-*G. stellatus* activity in English sole serum and mucus, 15 uninfected English soles (total length, mean \pm SE: 172.8 \pm 23.4 mm) were anesthetized and injected intraperitoneally

with 0.1 mL of a 1:1 (volume : volume) suspension of whole, formalin-killed *G. stellatus* in Freund's complete adjuvant. Booster injections were administered in the same manner 2 weeks later. Four weeks after the booster, blood and mucus were collected.

Mucus bioassay.—A bioassay testing the length of survival of *G. stellatus* in English sole mucus samples was performed in 96-well, polystyrene, flat-bottomed plates held at 15°C. Mucus samples collected throughout a laboratory epizootic, as well as from uninfested and *G. stellatus*-injected fish, were tested.

Live *G. stellatus* were obtained by anesthetizing infected English soles with a 1:1,500 dilution of 2-phenoxyethanol for 30 s to 1 min (Lester and Adams 1974a). Trematodes were filtered out of the anesthetic on a 53- μ m Nitex screen, rinsed in seawater, and collected in small glass bowls.

One to two trematodes were placed in each well of a 96-well plate, and mucus was added to obtain a 5% concentration of mucus in seawater. Each sample was randomly assigned to a column of a 96-well plate, and most samples were run on three replicate plates. Seawater served as the negative control. Mucus from newly captured English soles served as the pretest control and represented the mucus of wild English soles in the estuary with typical, low-level *G. stellatus* infections. Trematodes were examined every 3–4 h with a dissecting microscope until all of the trematodes had died. Trematodes were considered dead when they no longer responded to stimulus (tapping the plate against the stage of the microscope). Dead trematodes frequently appeared swollen and their tegument appeared roughened.

Serum bioassay.—A serum bioassay was conducted in a manner similar to the mucus bioassay described above. The serum samples tested were those collected from English soles 8 and 10 weeks after capture. Serum samples were not tested at earlier sampling times because the mucus bioassay did not suggest the presence of a factor affecting *G. stellatus* survival. Sera collected from uninfested and *G. stellatus*-injected English soles were also tested. Serum from newly captured fish served as the pretest control. Trematode survival was monitored every 30 min for the first 2 h and then every 2 h until the worms in all serum samples were dead.

Statistical analyses of bioassay results.—Newborn worms could not be distinguished from adults. Therefore, wells in which trematode births occurred were excluded from the statistical anal-

yses (births per treatment, mean \pm SE: 2.5 ± 0.64 in the mucus bioassay, 2.6 ± 1.10 in the serum bioassay). The mean and standard error of survival times were calculated for worms in each treatment. A one-way analysis of variance was performed and the Tukey multiple-comparison test was used to compare treatment groups. Differences were considered significant at $P \leq 0.05$.

Rabbit antiserum.—Rabbit antiserum against English sole whole serum was obtained by injecting a 2–2.5-kg, female New Zealand White rabbit with English sole whole serum (collected from an uninfested, adult English sole) in Freund's complete adjuvant (1:1, volume : volume; 400 μ g protein/mL). The rabbit was injected with 0.1 mL of the antigen preparation intramuscularly in each leg, and with 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 μ g protein/mL) was given 2 weeks later following the same injection regime. Two weeks after the booster injections, 10 mL of blood was collected from the rabbit by cardiac puncture. The gel diffusion test described below was used to assess the presence of antibody against English sole serum proteins.

Immunoassay.—An agarose gel, double-diffusion precipitation test (Ouchterlony) was used to determine if the rabbit antiserum contained specific antibodies against English sole serum and mucus. The assay was performed in 5.0-cm-diameter Gelman plates holding 5 mL of 1% agarose in 0.01 M phosphate-buffered saline at pH 7.2. Six wells surrounding a central well were cut out of the gel; each well held approximately 25 μ L of sample.

The rabbit antiserum against English sole serum was placed in the center well, and mucus samples collected during a laboratory epizootic were placed in the surrounding wells. Mucus collected from uninfested and *G. stellatus*-injected English sole were also tested. English sole serum served as a positive control. After the samples were added, the plates were incubated in a humidity chamber at room temperature and read after 24 and 48 h (Anderson and Dixon 1981).

Results

Laboratory Conditions and Infection Levels

The average seawater temperature (\pm SE) during the laboratory epizootic was $15.0 \pm 1.13^\circ\text{C}$,

TABLE 1.—Effect of English sole mucus on the mean survival time (MST) of *Gyrodactylus stellatus* in bioassays. Fish were held in tanks until sampling, and fish sampled at 8 or 10 weeks after capture were characterized by the level of trematode infection (heavy or light). Means with no letter in common are significantly different (Tukey multiple comparison, $P \leq 0.05$).

Mucus sample from:	N	MST \pm SE (h)
Naturally infected fish		
Newly captured (pretest control)	20	16.2 \pm 1.28zy
Held 2 weeks	24	14.5 \pm 1.24zyxw
Held 4 weeks	28	16.6 \pm 1.00zy
Held 6 weeks	24	14.0 \pm 1.03zyxw
Held 8 weeks, heavy	21	15.2 \pm 1.17zyx
Held 8 weeks, light	31	10.1 \pm 0.50xw
Held 10 weeks, heavy	25	13.1 \pm 0.82zxw
Held 10 weeks, light	31	9.4 \pm 0.39w
Uninfected, held 8 weeks	28	18.9 \pm 1.09y
<i>G. stellatus</i> -injected fish	31	13.9 \pm 1.00zyxw
No fish, seawater only (negative control)	30	26.3 \pm 2.44v

which corresponded to the water temperature of Yaquina Bay. The average of the water temperatures measured every 2 weeks ($N = 5$) was 14.2 \pm 0.43°C.

Biweekly observations showed that the course of infection experienced by the naturally infected, laboratory-held English soles over 10 weeks was similar to that described by Kamiso and Olson (1986). Newly captured fish appeared to have very low levels of infection. Levels of infection increased dramatically during holding and appeared to reach their peak by 8 weeks, after which some fish began to show signs of recovery. Mortality began occurring at about 5 weeks and was highest between weeks 5 and 7. Mortality was 48% by the final sampling period at 10 weeks. At the end of the experiment, only 6 of the original 300 fish remained (150 fish had been sampled without replacement, and 144 fish died during the test).

Mucus Bioassay

The mean survival times (MSTs) of trematodes in mucus collected from lightly infected (recovering) English soles at 8 and 10 weeks after capture were significantly shorter than the MST in mucus from fish that were newly captured, in a tank for 4 weeks, or uninfected. The MST in seawater, the negative control, was significantly longer than in any mucus sample. Except in mucus from recovering fish with light infections, the MSTs of *G. stellatus* in all mucus samples were not signifi-

TABLE 2.—Effect of English sole sera on the mean survival time (MST) of *Gyrodactylus stellatus* in bioassays. Fish were held in tanks until sampling, and fish sampled at 8 or 10 weeks after capture were characterized by the level of trematode infection (heavy or light). Means with no letter in common are significantly different (Tukey multiple comparison, $P \leq 0.05$).

Serum sample from:	N	MST \pm SE (h)
Naturally infected fish		
Newly captured (pretest control)	26	6.7 \pm 0.27z
Held 8 weeks, heavy	19	11.5 \pm 0.57y
Held 8 weeks, light	22	5.0 \pm 0.17x
Held 10 weeks, heavy	27	4.6 \pm 0.21x
Held 10 weeks, light	25	2.8 \pm 0.09w
Uninfected, held 8 weeks	11	5.5 \pm 0.21zx
<i>G. stellatus</i> -injected fish	17	3.0 \pm 0.00w

cantly different from that in the pretest control (Table 1).

Serum Bioassay

Trematode MSTs in sera collected from English soles injected with *G. stellatus*, from those lightly infested after 8 weeks, and from those heavily or lightly infested after 10 weeks were significantly shorter than the MST in the pretest control. The MST in serum collected from heavily infected fish after 8 weeks was significantly longer than the MST in the pretest control. The trematode MST in serum collected from lightly infected fish was significantly shorter than the MST in serum collected from heavily infected fish at both 8 and 10 weeks postcapture (Table 2).

Rabbit Antiserum

An immunodiffusion precipitation reaction between English sole serum and the rabbit antiserum confirmed that the rabbit antiserum did contain antibodies against English sole serum factors (Figure 1, wells A1 and B1). Four to five bands of precipitation were formed at antiserum dilutions of 1:1 and 1:2, but none at higher dilutions (not shown).

Immunoassay

The immunoassay showed precipitation reactions between English sole mucus samples and the rabbit antiserum. The rabbit antiserum recognized serum factors in all of the mucus samples collected throughout the laboratory epizootic; the differences were in the relative strengths of the precipitation reactions (Figure 1). Precipitation reactions appeared stronger in mucus samples collected at later times during the trematode infection. The rabbit antiserum did not recognize any

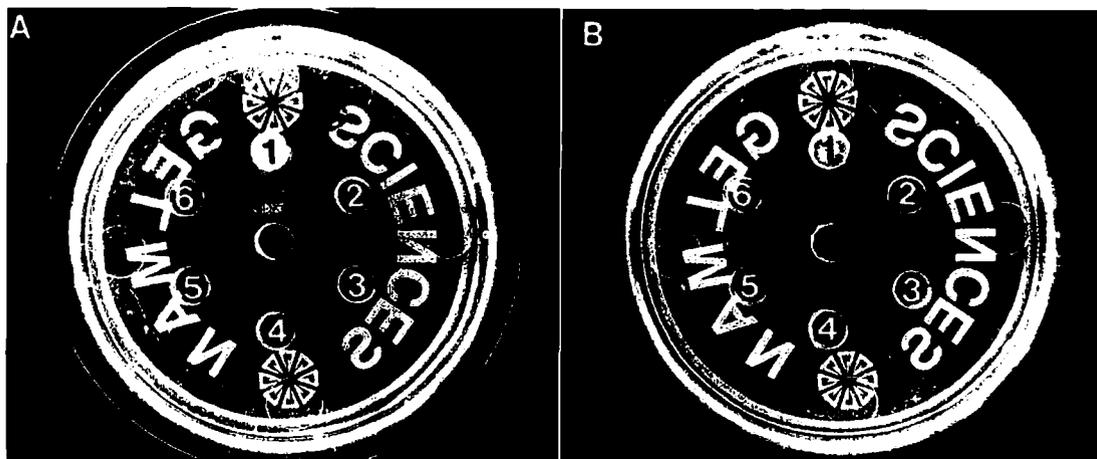


FIGURE 1.—Gel diffusion immunoassay to detect factors in English sole mucus that are antigenically similar to serum factors. The center wells in both assay plates contained rabbit antiserum against English sole serum. (A) The surrounding wells contained (1) English sole serum (positive control), (2) a sample not pertinent to this experiment, (3) mucus collected from fish upon capture, (4) mucus from fish at 2 weeks, (5) mucus from fish at 4 weeks, or (6) mucus from fish at 6 weeks after capture. (B) The surrounding wells contained (1) English sole serum, (2) mucus collected from English soles injected with *G. stellatus*, (3) mucus from heavily infected fish at 8 weeks, (4) mucus from lightly infected fish at 8 weeks, (5) mucus from heavily infected fish at 10 weeks, and (6) mucus from lightly infected fish at 10 weeks after capture.

serum factors in mucus from uninfected (not shown) or *G. stellatus*-infected English soles. The rabbit antiserum formed four to five bands of precipitation with English sole serum, the positive control.

Discussion

The results of this study demonstrate the changes in English sole resistance to *G. stellatus* during laboratory holding, and suggest that both the serum and the cutaneous mucus of infested English soles contain resistance factors against the trematode. Previous studies determined that, although water temperature, nutrition, and crowding were factors that influenced the host-parasite relationship between juvenile English soles and *G. stellatus*, they did not account for the high intensities of the infections that develop on laboratory-held English soles (Kamiso 1983). Stresses associated with handling, transport, and captivity are known to influence the disease susceptibility of fishes by suppressing immune responses (Miller and Tripp 1982; Ellsaesser and Clem 1986) and may explain the changes in English sole resistance to *G. stellatus*.

Nigrelli (1935a) studied the effects of marine fish mucus on the monogenetic trematode *Epibdella melleni* and found that trematodes exposed to mucus from fish with natural immunity to the

parasite had shorter survival times under experimental conditions. Hanson (1973) observed similar results when he exposed the monogenetic trematode *Diclidophora embiotoci* to serum and mucus from the striped seaperch *Embiotoca lateralis*, a fish with natural resistance to the parasite. He suggested that the results of hemagglutination tests indicated that specific antibodies in the mucus were involved in the resistance. The exact mechanisms operating in resistance to monogenetic trematode infections are not known, but it has been suggested that resistance to gyrodactylid infections may be associated with mucus secretions (Lester and Adams 1974b; Kamiso 1983; Scott and Robinson 1984; Evans and Gratzek 1989).

Proteins antigenically identical to serum proteins have been detected in the mucus of European bass *Morone labrax*, the sea catfish *Tachysurus australis*, and rainbow trout *Oncorhynchus mykiss* (O'Rourke 1961; Di Conza and Halliday 1971; Harrell et al. 1976; St. Louis-Cormier et al. 1984). In this study, antigenic similarity between English sole mucus and serum factors was demonstrated with a gel diffusion immunoassay with rabbit antiserum prepared against English sole whole serum. Generally, bands of precipitation indicating antigenic recognition were weakest in mucus collected from English soles during periods of in-

creasing infection intensity, and were strongest in mucus from English soles at later stages of infection, when resistance to the trematode may have been increasing. There was no precipitation reaction in the mucus of uninfected English soles, indicating that the factors involved in the precipitation reaction were either not present, or not at levels high enough to detect by the methods used in this study. The lack of a precipitation reaction in the mucus of uninfected fish suggests that the precipitating factors are associated with *G. stellatus* infections.

Khalil (1964) found that gray bichir *Polypterus senegalus* that recovered from infections of *Macrogryrodactylus polypteri* were not susceptible to reinfection as 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 *Gasterosteus aculeatus* that had become free of *G. alexanderi* were refractory to further infections for about 3 weeks and then were susceptible to reinfection. Our preliminary results suggested this was also the case with English soles and *G. stellatus*. The uninfected fish used in these studies, which were held in the laboratory for 8 weeks after disinfection with formalin, must have lost any resistance they might have had to *G. stellatus* during the 8-week holding period. This assumption is supported by the results of bioassay and immunoassay tests, which indicated no anti-*G. stellatus* activity or serum antigens in the mucus of these uninfected English soles.

In previous studies involving monogenetic trematodes, Lester (1972) found that intramuscular injections of whole *G. alexanderi* antigen conferred no protection in threespine sticklebacks. Nigrelli (1935b) observed similar results when pompano *Trachinotus carolinus* were injected with ground, dried and ground, fresh *E. melleni*. In our study, bioassays showed that the serum of *G. stellatus*-injected English soles had a significant effect on trematode survival but the mucus from these fish did not, and there was no precipitation reaction between the mucus of *G. stellatus*-injected fish and the rabbit antiserum in the immunoassay. In contrast, both the mucus and the serum of recovering, naturally infested, laboratory-held fish had significant effects on trematode survival, and a precipitation reaction did occur between the mucus and rabbit antiserum in the immunoassay. Fish are thought to have a secretory immune system that is separate from the systemic immune system (Fletcher and Grant 1969; Di Conza and Halliday

1971; Lobb and Clem 1981; Rombout et al. 1986; Wong et al. 1992). From our experiment with English soles receiving an injection of the trematode, the results—the lack of anti-*G. stellatus* activity and antigenically related factors in their mucus—are evidence that the route of administration of the antigen affects the development of the immune response. When administered by injection, the antigen seems to have bypassed the mucosal and epidermal defense systems.

Temperature was found to be an important factor in regulating the host's immune system against *Trypanoplasma bullocki* infections in juvenile summer flounder *Paralichthys dentatus* (Sypek and Burreson 1983). In our study, the changes in English sole resistance to *G. stellatus* were not due to changes in water temperature because the laboratory water temperature corresponded with the water temperature of Yaquina Bay, and remained relatively constant throughout the 10 weeks of the experiment.

A difficulty in interpreting the results of this research arises when the mucus samples from lightly infected (recovering) fish and from heavily infected fish are compared. In the immunoassay, the mucus samples from heavily infected English soles had a stronger precipitation reaction with the rabbit antiserum than the mucus samples from lightly infected fish. In comparison, the mucus bioassays showed that biological activity against *G. stellatus* was not significantly different between lightly and heavily infected English soles, though the mucus from only the lightly infected fish was significantly different from the pretest control. There are several possible explanations for these results. One explanation is that there may be no relationship between the biological activity against *G. stellatus* and the appearance of immunodiffusion bands. Alternatively, a high concentration of *G. stellatus* antigens on heavily infected fish may have effectively neutralized (by adsorption) the anti-*G. stellatus* activity in the mucus. In this case, the immunoreactive factors would still be present but they would be unable to bind or react with *G. stellatus* (to form antigen-antibody complexes) and thus would be unable to affect trematode survival. A third explanation is that the mucus taken from heavily infected English soles may have been contaminated with blood, resulting in serum proteins in the mucus that were recognized by the rabbit antiserum in the immunoassay. Contamination could have occurred through small areas of hemorrhaging that are often seen on the fins and skin of heavily infected English soles.

Although the biologically active factors that appear to be present in the serum and mucus of *G. stellatus*-infected English soles were not characterized, they probably play a role in host defense. Specific and nonspecific protective 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). Only precipitating antibodies resembling immunoglobulin M (plus complement) have so far been indicated in resistance to helminth infections (McVicar and Fletcher 1970; Harris 1972; Cottrell 1977; Evans and Gratzek 1989).

More work is needed to identify, quantify, and characterize the anti-*G. stellatus* factors that appear to be present in the serum and mucus of infected English soles. Further studies may result in a better understanding of the mechanisms involved in fish immunity to monogenetic trematodes.

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