SEASONAL IMMUNE RESPONSE IN JUVENILE SUMMER FLOUNDER, *PARALICHTHYS DENTATUS* TO THE HEMOFLAGELLATE *TRYPANOPLASMA BULLOCKI* IN THE LOWER CHESAPEAKE BAY

A Thesis

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

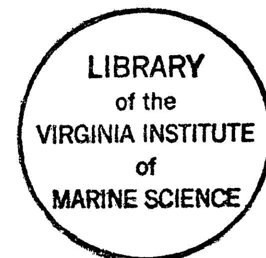
of the Requirement for the Degree of

Master of Arts

by

Linda J. Frizzell

1985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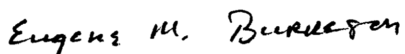

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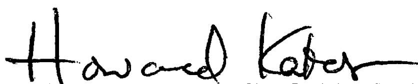

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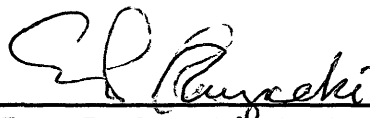

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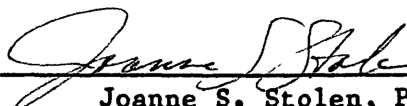

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ABSTRACT

The hemoflagellate *Trypanoplasma bullocki* (Strout) infects juvenile summer flounder *Paralichthys dentatus* (Linnaeus) from the lower Chesapeake Bay in fall and winter, but is not detectable in summer flounder in the spring when water temperature increases. To determine if the host's humoral immune system is responsible for this disappearance, an immuno-blot enzyme assay was used to measure specific antibody production of immunized, ethanol-treated, and non-immunized summer flounder maintained at 20°C and ambient environmental temperature (2°-25°C). At 20°C immunized and ethanol-treated fish had significantly higher antibody titers than control (non-immunized) fish, but all fish were able to eliminate the infection within eight weeks. At ambient temperatures, fish that survived did not eliminate the flagellate until 26-34 weeks post-challenge with live flagellates. Mortality was 60% in immunized fish, and 50% in ethanol-treated and control fish, with the highest occurrence of mortalities in January and February when water temperature was below 5°C.

Attempted suppression of the immune response via ethanol injection was not successful. Protective immunity of fish through immunization of formalin-killed *Trypanoplasma bullocki* did not occur; however, protective immunity was established in fish kept at 20°C that recovered from *T. bullocki* infections. Rechallenged summer flounder exhibited typical secondary immune response with titers 5 times greater than titers of the primary immune response.

Naturally infected summer flounder collected from the York River exhibited similar patterns and magnitudes of antibody titer and flagellate intensity as control fish maintained at ambient temperature. The prevalence of *Trypanoplasma bullocki* in flounder from the York River was highest from December to January, and gradually decreased in the spring. By July no infected summer flounder were found. After the initial growth phase of the flagellate, antibody titer varied directly with temperature and titer was inversely related to flagellate intensity in both experimentally and naturally infected summer flounder. Therefore, the immune system of summer flounder appears to be responsible for the spring disappearance of *T. bullocki*.

THE SEASONAL IMMUNE RESPONSE IN JUVENILE SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) TO THE HEMOFLAGELLATE *TRYPANOPLASMA BULLOCKI* IN THE LOWER CHESAPEAKE BAY.

INTRODUCTION

The hemoflagellate *Trypanoplasma bullocki* (Strout) infects a wide variety of estuarine and marine fishes along the Atlantic coast of North America from the Gulf of Maine to the Gulf of Mexico (Strout, 1965; Laird and Bullock, 1969; Becker and Overstreet, 1979). Most *T. bullocki* infections occur in juvenile fishes inhabiting inshore brackish waters (Becker and Overstreet, 1979). In the Chesapeake Bay, Burreson and Zwerner (1982) observed *T. bullocki* in at least thirteen species of teleost fishes, and they observed the flagellate to be most prevalent in the hogchoker, *Trinectes maculatus* (Bloch and Schneider), and the summer flounder, *Paralichthys dentatus* (Linnaeus).

Burreson (1982) demonstrated that *Trypanoplasma bullocki* is transmitted by an intermediate host (vector), the leech *Calliobdella vivida* (Verrill). *Calliobdella vivida* is present in the Chesapeake Bay from September to April when average water temperature is below 22°C (Sawyer and Hammond; 1973, Burreson and Zwerner, 1982). The vector phase of the life cycle of *T. bullocki* is similar to that of other marine trypanoplasms (Burreson, 1978; Lom, 1979) where ingested flagellates divide and multiply in the crop and posteca of the leech. The infective elongated flagellates develop within ten to twelve days, and then migrate to the proboscis sheath of the leech. The flagellates are then transmitted to the fish host while the leech is feeding.

The observed cycle of *Trypanoplasma bullocki* in the fish host begins with a short lag phase where the flagellate is absent from the peripheral blood (Burreson, 1982). The next phase is an appearance of flagellates in the circulatory system, followed by an increase in parasitemia which may result in mortality of the host (Burreson, 1982).

Summer flounder is an important commercial species on the east coast of the United States. Its geographic distribution encompasses the estuarine and continental shelf waters from Florida to Maine (Bigelow and Schroeder, 1953) but it is most abundant from Cape Cod, Massachusetts to Cape Lookout, North Carolina (Smith and Daiber, 1977). Juvenile summer flounder use estuaries as nursery grounds from the time of their recruitment in winter to late summer of their second year (Powell and Swartz, 1977).

In the Chesapeake Bay the greatest number of yearlings are taken from below the Potomac River (Hildebrand and Shroeder, 1928). During the winter, juvenile summer flounder migrate toward the mouth of the Chesapeake Bay and adjoining nearshore coastal waters where higher saline conditions help to increase growth efficiency (Powell and Swartz, 1977). Some juvenile summer flounder overwinter in the lower reaches of the York and James Rivers, the two seaward most tributaries of the Chesapeake Bay. During the late-fall through early spring, juvenile summer flounder are exposed to *Trypanoplasma bullocki* infection (Burreson and Zwerner, 1982). Adult summer flounder migrate offshore in early fall and are generally not exposed to *T. bullocki*.

Summer flounder become infected soon after the leeches have begun to hatch from their cocoons in November (Burreson and Zwerner, 1982).

Trypanoplasma bullocki infected flounder are found throughout the winter and early spring. As water temperature increases in the spring, the number of infected fish and the intensity of infection decreases (Sypek and Burreson, 1982). No infected flounder were found after mean water temperatures reached 22°C during June (Sypek and Burreson, 1982). However, the hogchoker, a resident species of the Chesapeake Bay, is infected year round. Burreson and Zwerner (1982) attributed the seasonality infection pattern in flounder to be "the result of host-mediated effects rather than of parasite temperature tolerance." The host-mediated effects are considered to be the immune response that is responsible for the elimination of the flagellate (Sypek and Burreson, 1983; Burreson and Zwerner, 1984).

Since the early 1900's, evidence has indicated that the immune response in poikliothermic vertebrates is temperature dependent (Lom, 1969; Avtalion, 1981; MacArthur, Fletcher, and Thomson, 1983). It is also known that fish produce antibodies which are part of the humoral immune system (Anderson, 1974). Avtalion (1981) and others (Barrow, 1954, 1955; Stolen et al., 1982) have observed a complete or severe reduction of antibody titers in cold-blooded vertebrates maintained at low temperatures (8-12°C or below). Other investigators observed only an extended latent period in the appearance of circulating antibodies in fish kept at low temperatures, but observed no reduction in antibody titer (Tait, 1969; Rijkers, 1980, 1981; Stolen et al., 1984). The immune response in cold-blooded vertebrates is not solely affected by temperature, but is also affected by the type of infection (viral, bacterial or parasitic) (Lom, 1969), the antigen dose (Wehnert and

Woo, 1980; Woo, 1981), and the immunological competence of the individual (Cottrell, 1977; Manning and Mughal, 1985).

Temperature is thought to be one of the most important factors in controlling the immune response in summer flounder against trypanoplasm infection. Sypek and Burreson (1982) observed an increase in trypanoplasmacidal lysis activity when water temperatures were elevated, and also observed elimination of *Trypanoplasma bullocki* from summer flounder in experimental infections at 24°C and natural infections at 22°C. They hypothesized that the humoral immune response of summer flounder was enhanced in the spring, which resulted in elimination of trypanoplasm infection (Sypek and Burreson, 1982).

The hypothesis tested in this study was that the production of specific antibodies (part of the humoral immune system) by summer flounder, *Paralichthys dentatus*, against the hemoflagellate *Trypanoplasma bullocki* is directly related to the ambient environmental temperature, and that the elimination of *T. bullocki* in the spring from the summer flounders' peripheral circulatory system is the direct result of temperature enhanced antibody production . Antibody titers and *T. bullocki* intensity from individual summer flounder experimentally infected were followed over a temperature and time regime simulating the natural infection period. Seasonal variation of *T. bullocki* infections and antibody production in naturally infected summer flounder collected from the York River was also examined. In addition, the effect of immunization and ethanol injection on the humoral immune response in experimentally infected summer flounder was studied. Stolen et al. (1985) found that an injection of 95% ethanol before immunization suppressed the humoral immune response of summer

flounder against the bacteria *Escherichia coli*.Summer flounder were immunized with either an intraperitoneal or intramuscular injection of formalin-killed *T. bullocki*.

The enzyme - linked immunoblot assay, a version of the enzyme - linked immunosorbent assay (ELISA) was used to measure the amount of specific antibody being produced (Voller et al., 1979). The immunoblot assay uses a nitrocellulose membrane as opposed to a polystyrene titer plate (ELISA) for the solid phase. A series of antigen-antibody reactions are bound to the solid phase, and then measured by an enzyme substrate reaction (Hawkes et al., 1982). Immunoblot is an efficient tool in detecting circulating antibodies in naturally and experimentally diseased fish. Advantages of the enzyme immunoassay include sensitivity, reproducibility, and stability of reagents (Yolken, 1982).

MATERIALS AND METHODS

LABORATORY EXPERIMENT

Collection and Maintenance

Sixty-eight juvenile summer flounder (150-230 mm) were collected by otter trawl from the lower York River during the summer of 1984. Fish were divided into three groups of 20 fish and tagged with numbered tags. Group 1 fish were ethanol-treated (ETOH-treated), Group 2 fish were immunized, and Group 3 fish were used as the control (non-immunized). Fifteen fish from each group were maintained in one indoor rectangular tank (0.6 X 3.2 m) with flowing York River water at ambient temperature. The remaining five fish from each group were placed in an aerated, filtered water system, and water temperature was kept at 20 $\pm$ 1°C. A fourth group containing eight non-immunized fish was also kept at 20 + 1° C. Fish were fed live shrimp (*Paleomonetes pugio*, *Crangon septemspinosa*), or frozen squid daily.

Immunization and Infection

Each fish from Group 1 (ethanol-treated fish) was injected with 0.05 ml of 95% ethanol intraperitoneally on October 10, 1984. One week later, all fish (Groups 1, 2, and 3) were bled and checked for trypanoplasm infection. At the same time, fish from Group 1 and Group 2 (immunized fish) were injected either intraperitoneally (IP) or intramuscularly (IM) respectively with 1.50 - 1.52 x 10^{6} formalin preserved *Trypanoplasma bullocki* in phosphate buffer saline, PBS

(Appendix I) or a 1:1 ratio of PBS to Freund's complete adjuvant. These fish were injected five days later with the same amount of fixed flagellates IP or IM. Fish from Group 3 (control fish) were injected with 0.2 ml of PBS. Three weeks after the first immunization, fish were challenged with live flagellates which were either injected IP (1.3 X 10^5 *T. bullocki* in 0.1 ml of minimum essential medium (MEM)/20% fetal calf serum (FCS) culture) or fish were fed on by infected leeches. After infection , fish were bled on a weekly schedule through December, then monthly through March, and bimonthly through June.

Fish from Group 4 were injected IP with 1.3 x 10^5 live flagellates. Fish were bled weekly for 11 weeks. During week 12, four fish were rechallenged with 1.3 X 10^5 IP injections of live flagellates. Fish from Group 4 were then bled bimonthly.

Preperation of Sera

One blood sample was collected at each sampling period from the caudal hemal arch of each fish with a sterile needle and syringe . A drop of blood was put in a 0.6% saline solution on a slide and observed under low power for *Trypanoplasma bullocki* infection. Intensity was scored as light, moderate, or heavy and converted to number of flagellates/mm^3 as described by Burreson and Zwerner (1984). The remaining blood was placed in a glass test tube, allowed to clot for 1 hour at room temperature and then refrigerated for 24 hours. Blood samples were then centrifuged for 5 minutes, sera were removed and placed in 0.5 ml polypropylene centrifugation tubes, and stored at -18°C until analyzed.

Immuno-blot

Antibody titers were measured by immuno-blot EIA (BioRad). Preliminary assays indicated that the best results were obtained by using the following dilutions: an initial 1:100 dilution of summer flounder serum in a 1% gelatin-tris buffer solution (antibody buffer), a 1:2000 dilution of rabbit anti-summer flounder serum in antibody buffer solution (RASF), a 1:2000 dilution of rabbit anti-*T. bullocki* serum (RATB) in antibody buffer solution, and a 1:3000 dilution of goat anti-rabbit - horseradish peroxidase (GAR - HRP, BioRad) conjugate in antibody buffer solution.

Summer flounder immunoglobin M (IgM) was purified on a sepharose 4B gel filtration column by J.S. Stolen, National Marine Fisheries Service, Sandy Hook, New Jersey. RASF was prepared by D.P. Anderson, National Fish Health Research Laboratory, Kearneysville, West Virginia. RATB was prepared by giving a rabbit 3 IM injections of 1.45×10^7 live flagellates resuspended in 1.0 ml of PBS on alternate days into a rabbit. The first two injections were given with an equal volume of Freund's complete adjuvant. The third injection was without Freunds. Twelve days after the last injection, the rabbit was injected subcutaneously with 1.45×10^7 PBS washed flagellates. The rabbit was bled 7 days later via cardiac pucture.

Assay

All experiments were run at room temperature (22-24°C). Square (5 mm) nitrocellulose membranes were numbered, soaked in tris buffered saline solution (TBS, pH 7.5) for 5 minutes, and air dried. A 3 ul drop of *T. bullocki* culture ($2.5 - 3.5 \times 10^6$ flagellates/ml) was applied to each square and air dried. The membranes were placed in 40

ml antigen blocking solution (3% gelatin-TBS) and agitated on a laboratory rotator for 45 minutes. Each membrane was transferred to the appropriate vial containing 3.0 ml of the first antibody solution (summer flounder serum in antibody buffer) and incubated for 2 hours with gentle agitation. The membranes were individually rinsed in deionized water and washed 2 times for 10 minutes each, in 50 ml of a 0.05% Tween -20 TBS solution (TTBS). The membranes were transferred to a petri dish containing 30 ml of a second antibody solution (1:2000 dilution RASF-antibody buffer solution) and incubated for 1 hour. Membranes were rinsed, washed, and then placed in a petri dish containing 30 ml of a 1:3000 dilution GAR-HRP/antibody buffer solution and incubated 1 hour with gentle agitation. Membranes were rinsed and washed 2 times before being placed in HRP substrate color development for 20 minutes (30 ul of 30% ice cold hydrogen peroxide in 50 ml of TBS added to 30 mg 3,3 -diamino benzidine tetrahydro-chloride in 10 ml of ice cold methanol). Purple dots where antigen was applied indicated a positive reaction. Two-fold dilutions were made on each summer flounder serum that initially tested positive. Antibody titers are equal to the reciprocal of the last dilution that tested positively.

Positive and negative controls were run with each assay. The positive control was rabbit anti-*Trypanoplasma bullocki* serum. The negative control was pre-injection serum collected from the same rabbit (Appendix II).

Significant differences of antibody titers and flagellate intensity between treatments was tested by ANOVA. Differences within a certain group were tested by Student's t-test.

FIELD EXPERIMENT

Juvenile summer flounder were collected by otter trawl monthly from November to July of 1983-1984 and 1984-1985; from the York River, James River and lower Chesapeake Bay. Fish were bled within 2-3 days of capture and examined for presence of *T. bullocki* infections. Serum was analyzed for the presence of antibodies by the method discussed above.

RESULTS

LABORATORY EXPERIMENT

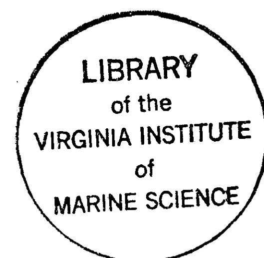
Fish that were challenged with live flagellates using lightly infected leech vectors never became infected and were excluded from data analysis. Therefore, sample sizes of fish maintained at ambient temperature were reduced from fifteen fish in each group to 8 fish in the ethanol-treated group, 10 fish in the immunized group, and 10 fish in the control group. The sample size of fish kept at 20°C remained at 5 fish per group 1, 2, and 3, and 8 fish for group 4. No differences in antibody titer values was noted between fish immunized IM with Freunds and those immunized without Freunds. Data from those two treatments were combined for analysis.

20°C Water Temperature

Immunized and ethanol-treateded juvenile summer flounder produced low antibody titers 3 weeks post-immunization with formalin-killed *T. bullocki* (Figure 1). A significant increase in titer in both the immunized and ethanol-treated fish occurred 2 weeks after challenge with live flagellates and was significantly greater ($P < 0.05$) than titers produced by the control fish. There was no significant difference between titers of the immunized and ethanol-treated fish.

All fish became infected after challenge with live flagellates (Figure 2), but parasitemia remained below 600 flagellates/mm^3. Most flagellate infections were eliminated by the fifth week post-challenge

Figure 1. Mean antibody titer of control, immunized, and ethanol-treated summer flounder maintained at 20°C. Closed arrow indicates time of immunization and open arrow indicates time of challenge with live flagellates. (N=5 for each group at each sample period.)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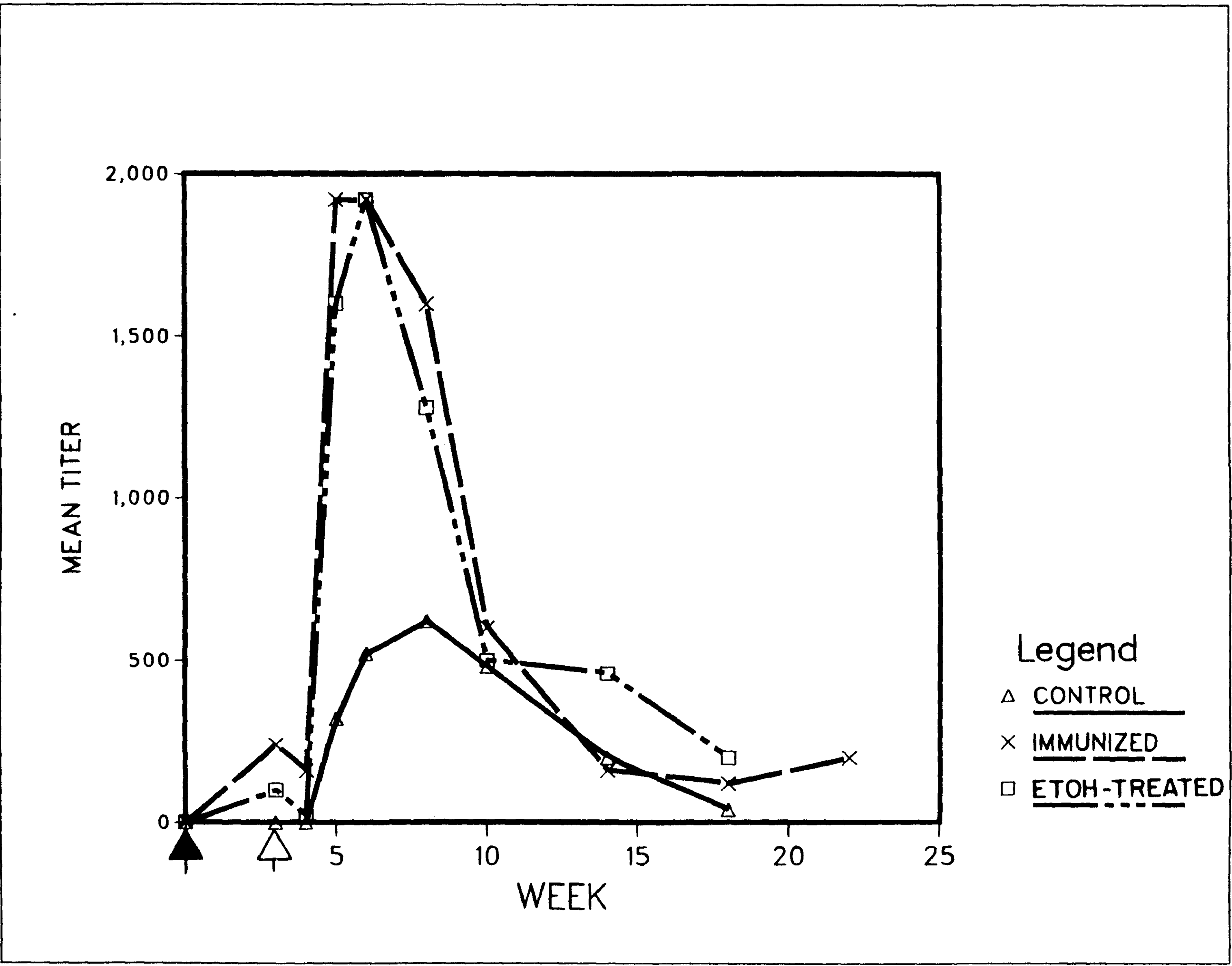
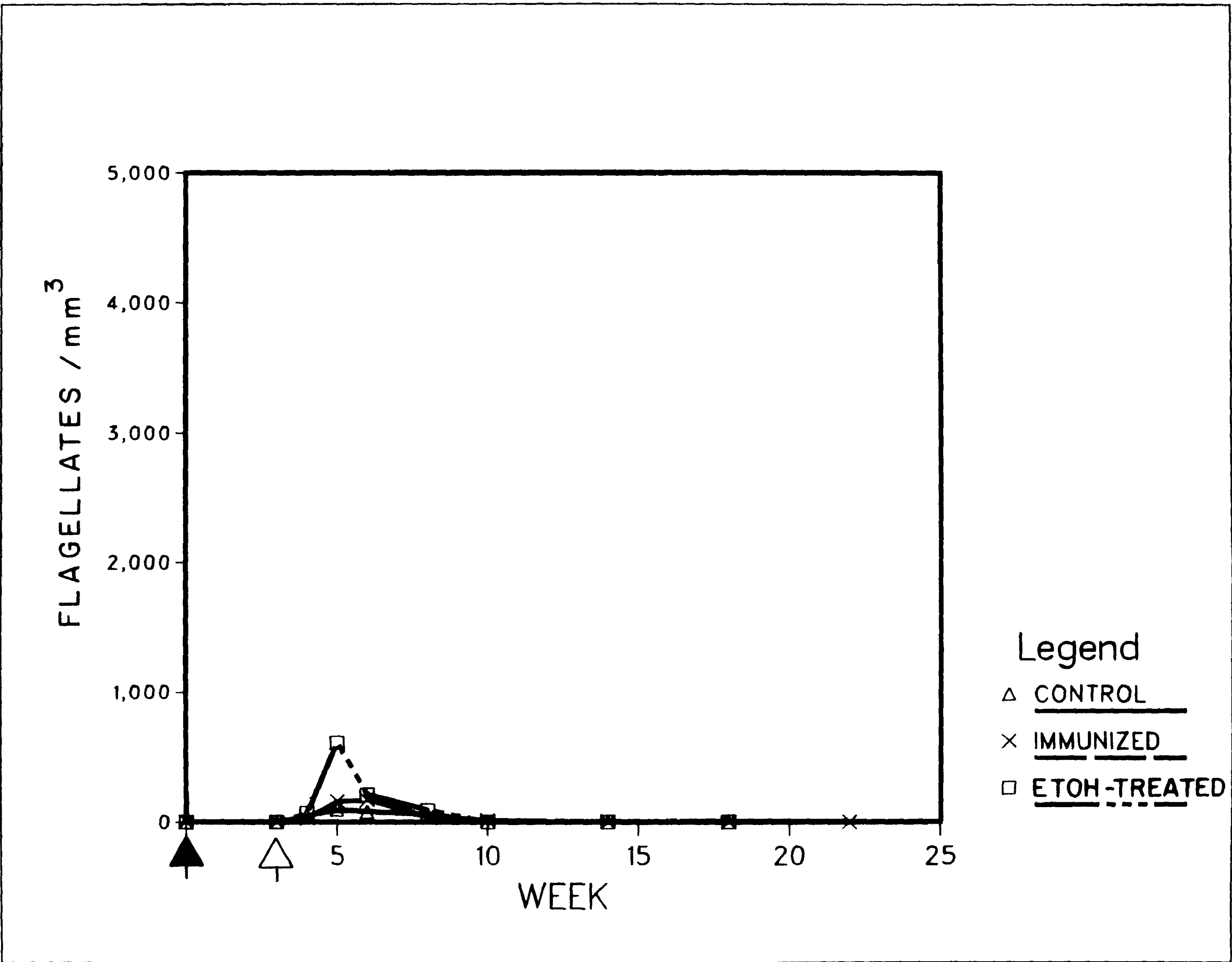

MEAN TITER
2,000
1,500
1,000
500
0
5
10
15
20
25
WEEK
Legend
CONTROL
IMMUNIZED
ETOH-TREATED

Figure 2. Mean *Trypanoplasma bullocki* intensity of control, immunized, and ethanol-treated summer flounder maintained at 20°C. Closed arrow indicates time of immunization and open arrow indicates time of challenge with live flagellates. (N=5 for each group at each sample period.)


FLAGELLATES / mm3
5,000
4,000
3,000
2,000
1,000
0
5
10
15
20
25
WEEK
Legend
CONTROL
IMMUNIZED
ETOH-TREATED

(week 8), and by the eighth week post-challenge (week 11) all fish had eliminated the parasite. Subsequently, all antibody titers decreased.

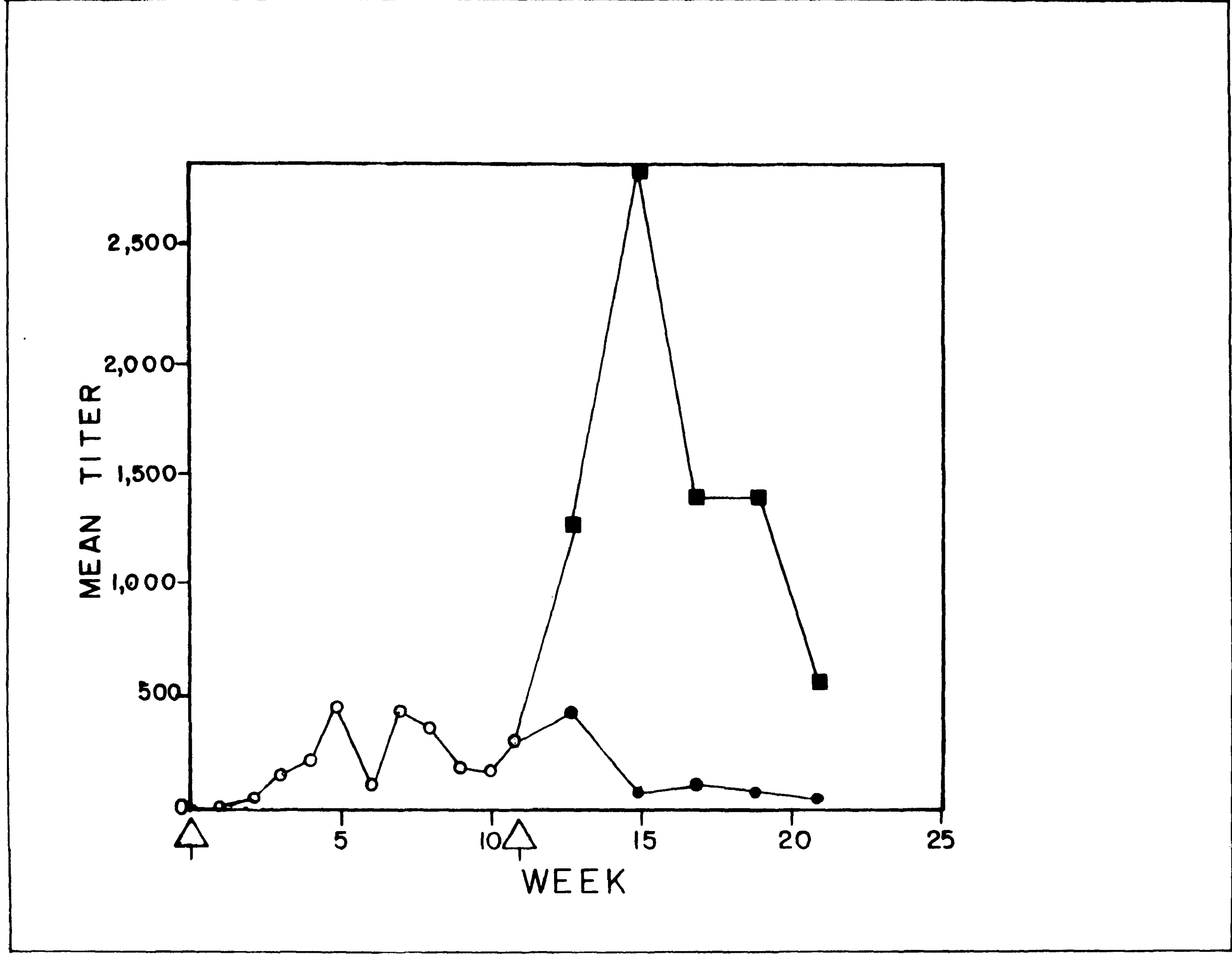
Antibody titers and flagellate intensities of fish from Group 4 showed no differences from the control group (Figures 3 and 4). Group 4 fish also eliminated infections 8 weeks post-challenge. The four fish reinjected with live flagellates exhibited a typical secondary response, i.e. titers were 5 times greater than titers during the primary response. Fish that were rechallenged did not become reinfected.

Ambient Water Temperature

Antibody titers and parasite intensities from each group were analyzed in two categories: mortalities and survivors. Mortality was 50% in ethanol-treated and control fish, and 60% in immunized fish (Table 1). Moribund fish began to show anemia and ascites during the month of December. Of the eventual mortalities 80 % expressed either anemia or ascites, with over half of the total mortalities having both symptoms. Mortality of fish began in late January with the highest number of mortalities occurring in February concurrent with the lowest ambient water temperatures (Figure 5).

No significant difference ($P < 0.05$) between titers of fish that died (Table 2 and Figure 6) and those that survived (Figure 7) was found from October through February. The lowest titers during the infection period occurred in early February when the water temperature was below $5^{o}C$. The fish that survived this period showed an increase in antibody production when water temperatures increased. Maximum titer values of survivors in all three groups occurred in April when water temperature was greater than $12^{o}C$.

Figure 3. Mean antibody titer of summer flounder from Group 4 kept at $20^{\circ}C$. Open arrows indicate time of challenges with live flagellates. Open circles = titer of 8 fish after first challenge. Closed circles = titer of 4 fish challenged once. Closed squares = titer of 4 fish challenged twice.



MEAN TITER
2,500
2,000
1,500
1,000
500
0
5
10
15
20
25
WEEK

Figure 4. Mean *Trypanoplasma bullocki* intensity of summer flounder from Group 4 kept at 20°C. Open arrows indicate time of challenges with live flagellates. Open circles = flagellate intensities in 8 fish after first challenge. Closed circles = flagellate intensities in 4 fish challenged once. Closed squares = flagellate intensities in 4 fish after second challenge.

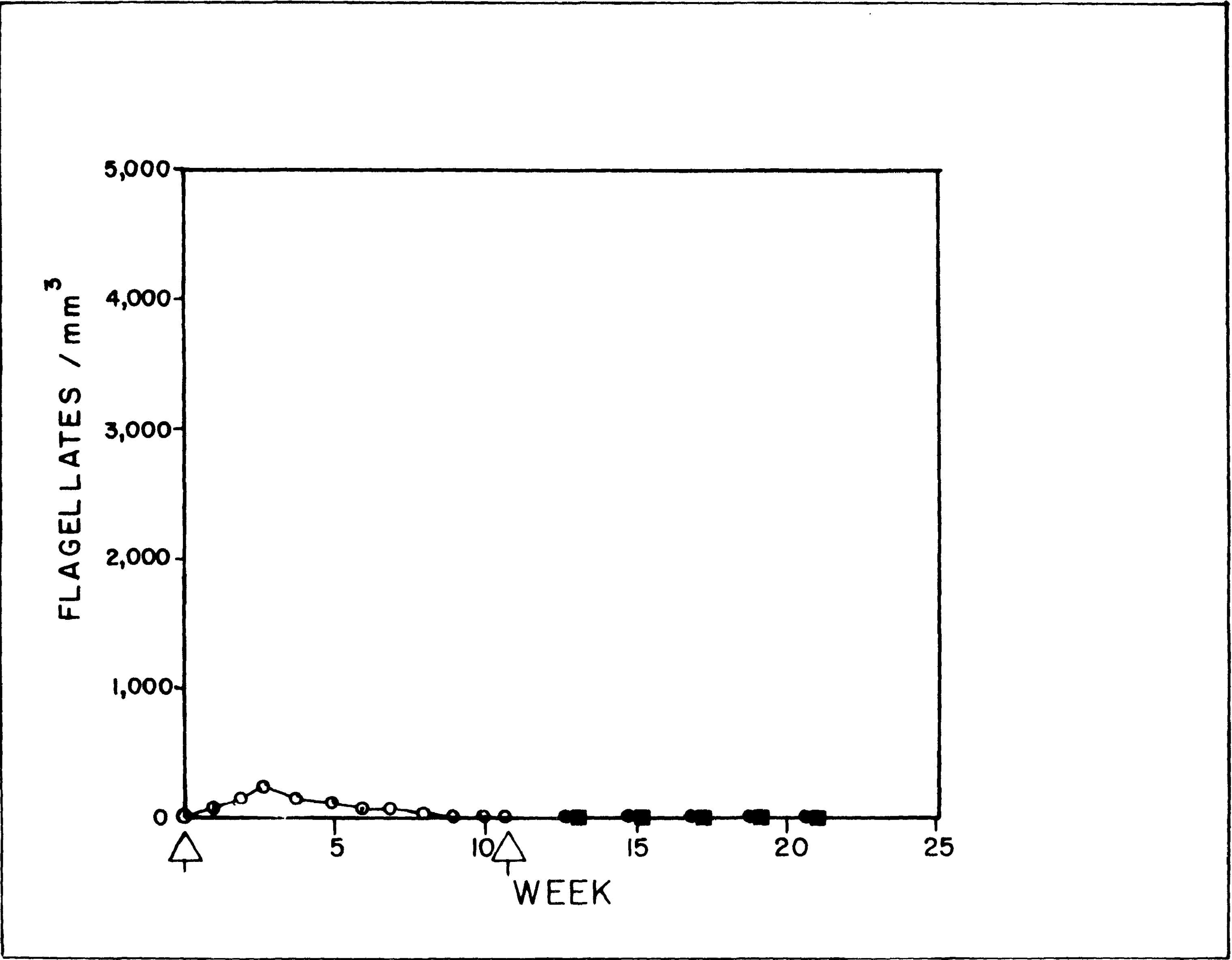

FLAGELLATES / mm3
5,000
4,000
3,000
2,000
1,000
0
5
10
15
20
25
WEEK

Table 1. Number of surviving summer flounder each month of 1984-1985 laboratory experiment.

Treatment	NOV	DEC	JAN	FEB	MAR	APR	JUN	JUL
Ethanol-treated	8/8	8/8	8/8	5/8	4/8	4/8	4/8	4/8
Immunized	10/10	10/10	7/10	5/10	4/10	4/10	4/10	4/10
Control	10/10	10/10	10/10	5/10	5/10	5/10	5/10	5/10

a/b where a = number of fish alive
b = number of fish originally

Figure 5. Average weekly ambient water temperature from November 1984 - July 1985. Closed arrow indicates time of immunization and open arrow indicates time of challenge with live flagellates.

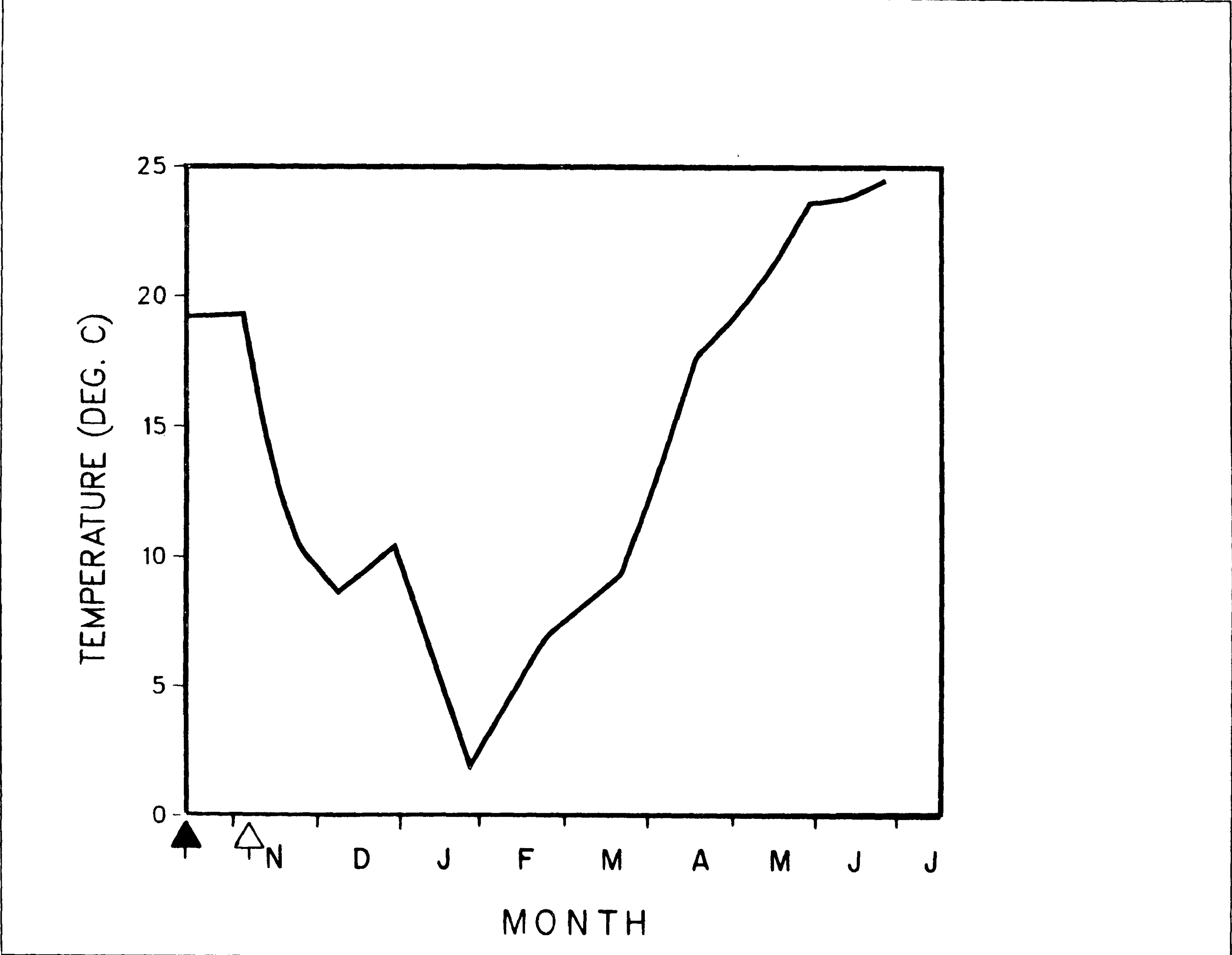

TEMPERATURE (DEG. C)
0
5
10
15
20
25
N
D
J
F
M
A
M
J
J
MONTH

Table 2. Number of eventual mortalities sampled per month for data presented in Figures 6 and 8.

Treatment	NOV	DEC	JAN	FEB	MAR	APR
Ethanol-treated	4	4	4	1	0	0
Immunized	6	6	3	1	0	0
Control	5	5	5	0	0	0

Figure 6. Mean antibody titer of control, immunized, and ethanol-treated summer flounder maintained at ambient water temperature and that eventually died. Closed arrow indicates time of immunization and open arrow indicates time of challenge with live flagellates.

MEAN TITER

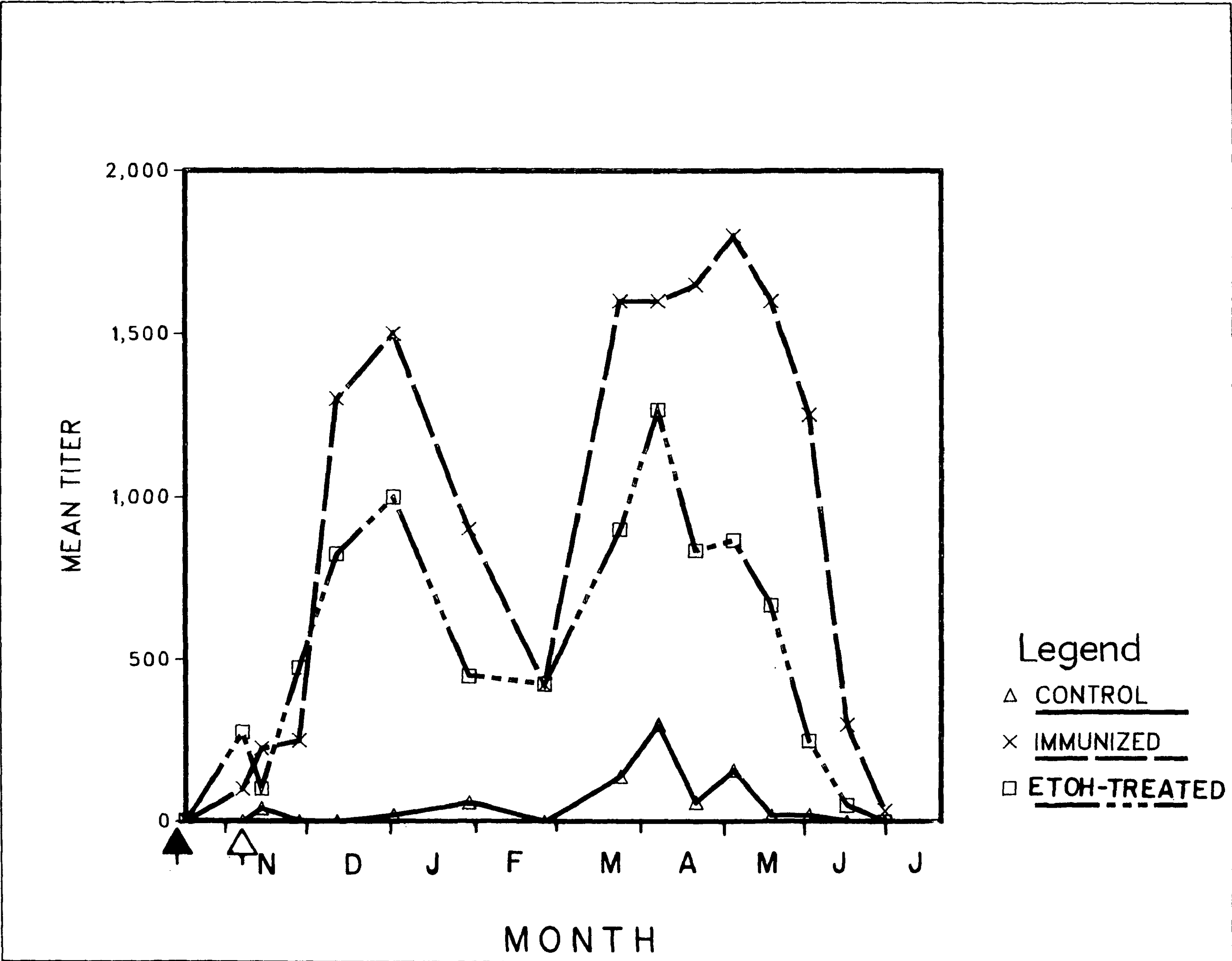
2,000
1,500
1,000
500
0

N D J F M A M J J

MONTH

Legend
△ CONTROL
× IMMUNIZED
□ ETOH-TREATED

Figure 7. Mean antibody titer of control, immunized, and ethanol-treated summer flounder maintained at ambient temperature and that survived. Closed arrow indicates time of immunization and open arrow indicates time of challenge with live flagellates. Control group N=5, immunized group N=4, and ehtanol-treated group N=4.



MEAN TITER
2,000
1,500
1,000
500
0
N
D
J
F
M
A
M
J
J
MONTH
Legend
CONTROL
IMMUNIZED
ETOH-TREATED

Flagellate intensity was significantly greater ($P < 0.05$) in immunized and ethanol-treated fish which succumbed than in fish that survived (Figures 8 and 9). Initial peak intensity in December was greater than 2000 flagellates/mm^3 in control fish and 4500 flagellates/mm^3 in immunized and ethanol-treated fish that eventually died compared to 1500 flagellates/mm^3 in all three survivor groups. Parasite intensity declined in January in both moribund and survivor fish when titers reached their initial peak. Once temperature and antibody titers decreased in late January, flagellate infections in the eventual mortalities rose above 3000 flagellates/mm^3 compared to less than 2000 flagellates/mm^3 in fish that surivived.

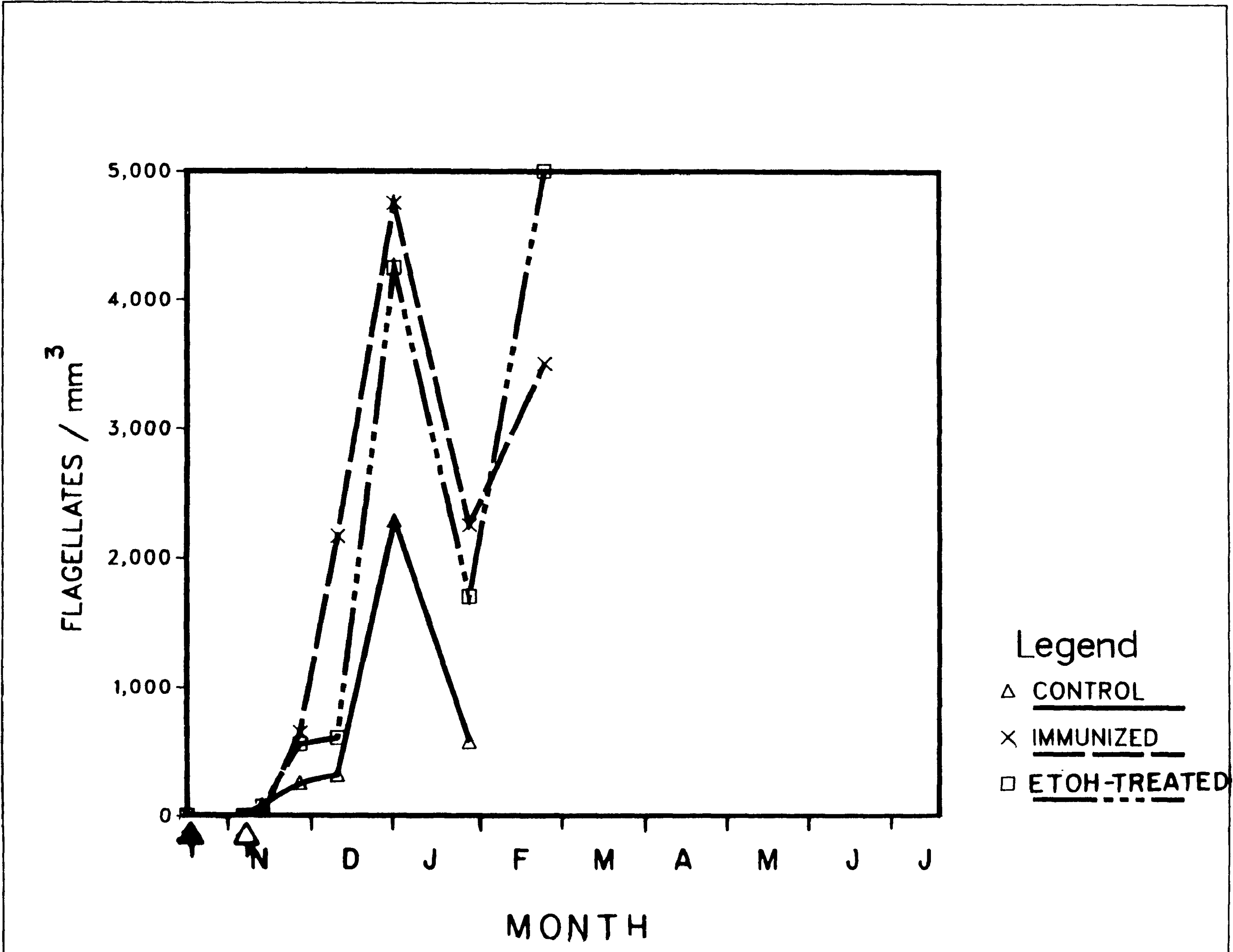
Those fish that survived the winter months were able to eliminate the flagellate. Time of flagellate elimination varied in individuals from the same group. The first occurrence of flagellate elimination was in late April when temperature reached 18°C. The majority of infections in all three groups was resolved during late May and early June. No fish was infected in July. After the initial log growth phase of the flagellate, flagellate intensity was inversely related to antibody titer; and antibody titer was directly related to ambient temperature in both the mortality and survivor groups.

FIELD EXPERIMENT

Juvenile summer flounder were extremely rare during the 1984-1985 infection period and too few fish were collected to permit meaningful analysis. Therefore, field data includes only those fish caught from the lower York River during the 1983-1984 season.

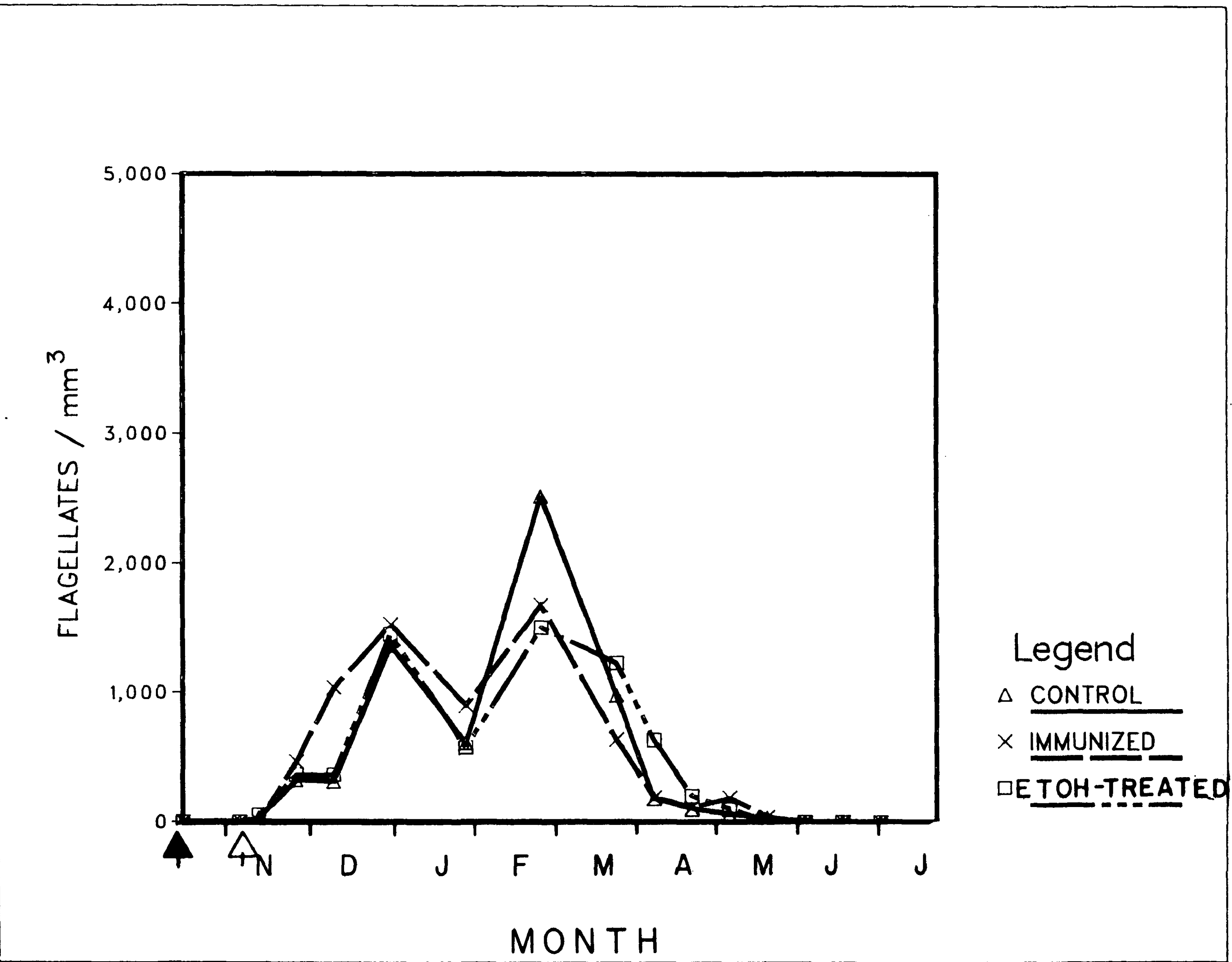
The prevalence in _Trypanoplasma bullocki_ infections increased from October to February (Figure 10). In March and April approximately

Figure 8. Mean *Trypanoplasma bullocki* intensity of eventual summer flounder mortalities maintained at ambient water temperature. Closed arrow indicates time of immunization and open arrow indicates time of challenge with live flagellates.



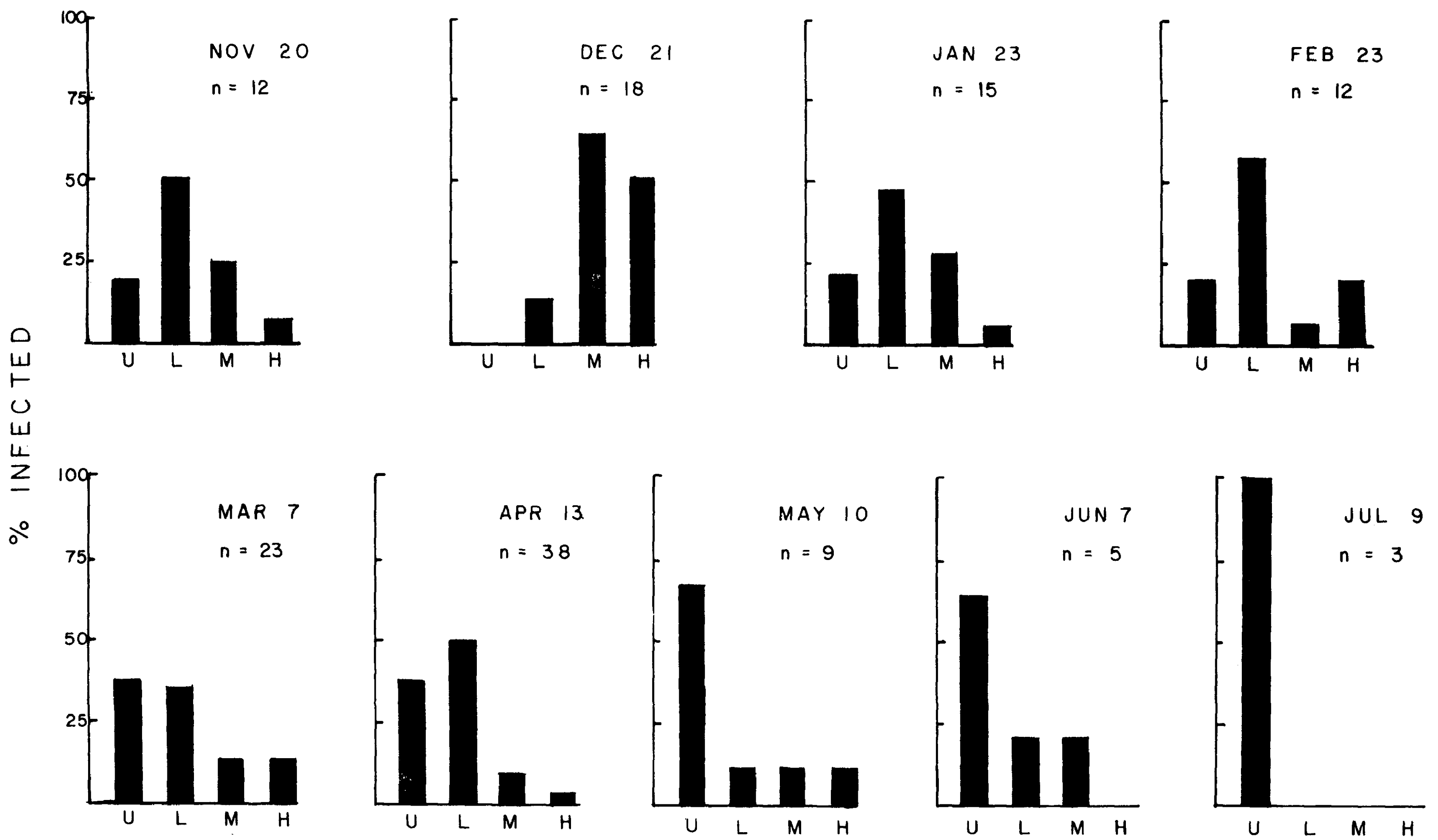
FLAGELLATES / mm3
5,000
4,000
3,000
2,000
1,000
0
N
D
J
F
M
A
M
J
J
MONTH
Legend
CONTROL
IMMUNIZED
ETOH-TREATED

Figure 9. Mean *Trypanoplasma bullocki* intensity of summer flounder survivors maintained at ambient water temperature. Closed arrow indicates time of immunization and open arrow indicates time of challenge with live flagellates.



FLAGELLATES / mm3
5,000
4,000
3,000
2,000
1,000
0
N
D
J
F
M
A
M
J
J
MONTH
Legend
CONTROL
IMMUNIZED
ETOH-TREATED

Figure 10. Prevalence of *Trypanoplasma bullocki* in naturally-infected summer flounder from the York River during 1983-1984 infection period. n =sample size, U = uninfected, L = low infection, M = moderate infection, and H = heavy infection.


NOV 20
n = 12
DEC 21
n = 18
JAN 23
n = 15
FEB 23
n = 12
MAR 7
n = 23
APR 13
n = 38
MAY 10
n = 9
JUN 7
n = 5
JUL 9
n = 3
100
75
50
25
U
L
M
H
% INFECTED
RELATIVE INTENSITY

equal numbers of infected to uninfected fish were present. By May the prevalence of *T. bullocki*-infected summer flounder decreased, and in July no fish sampled were infected.

Antibody titers and flagellate intensities of summer flounder caught from the lower York River exhibited similar patterns and magnitudes to those titers and intensities of control fish maintained at ambient temperatures (Figures 11 and 12). The lowest titer value occurred in January and February when water temperature was below $5^{o}C$ (Figure 13). Titers increased after water temperatures increased above $10^{o}C$ in the spring.

The mean intensity of *Trypanoplasma bullocki* infection was highest during November and December and reached similar values to intensities found in fish that died in the laboratory experiment.

Figure 11. Mean antibody titer of naturally-infected summer flounder during 1983-1984. See Figure 10 for sample size.

MEAN TITER

2,000
1,500
1,000
500
0

N D J F M A M J J

MONTH

Figure 12. Mean *Trypanoplasma bullocki* intensity of naturally-infected summer flounder during 1983-1984. See Figure 10 for sample size.

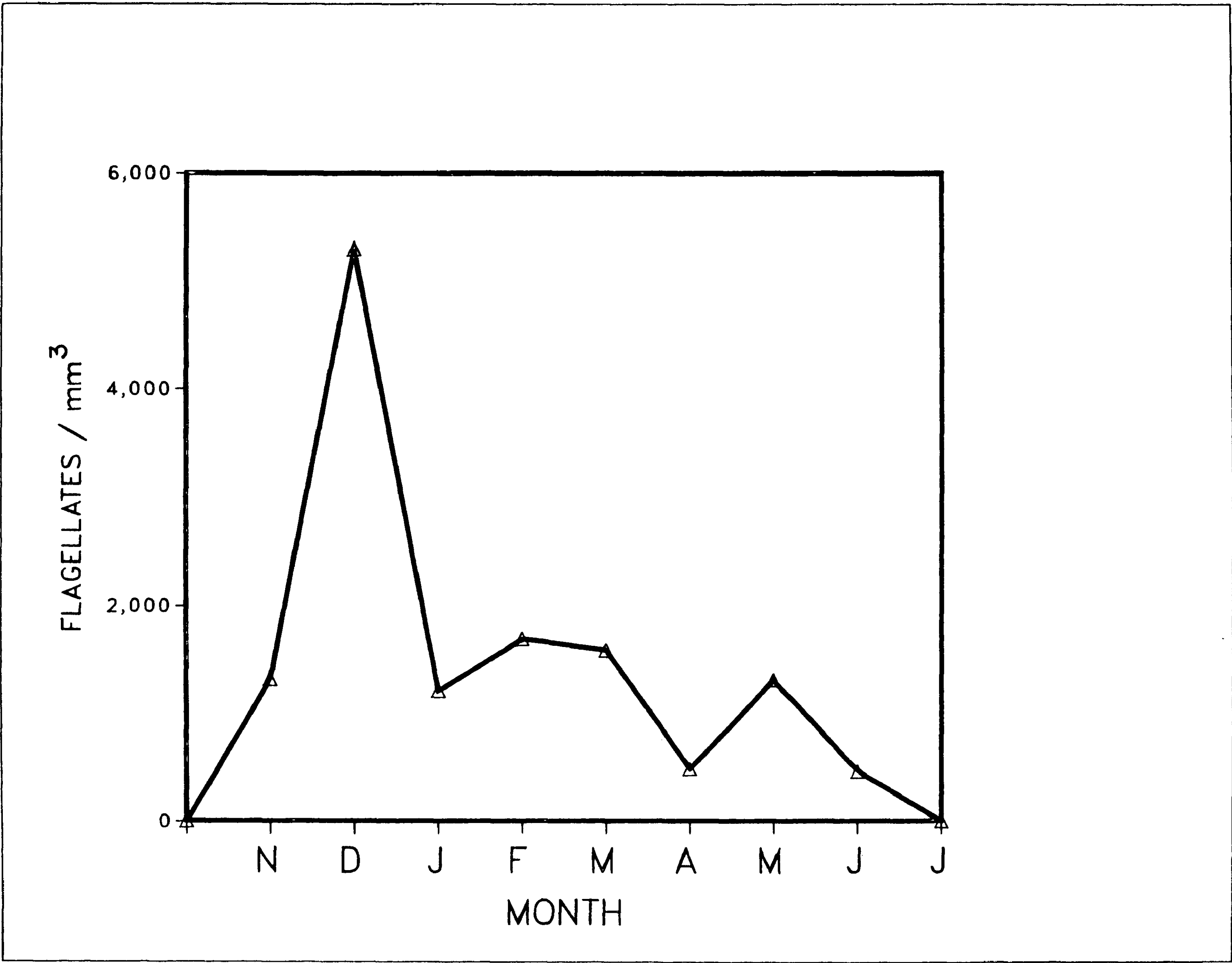
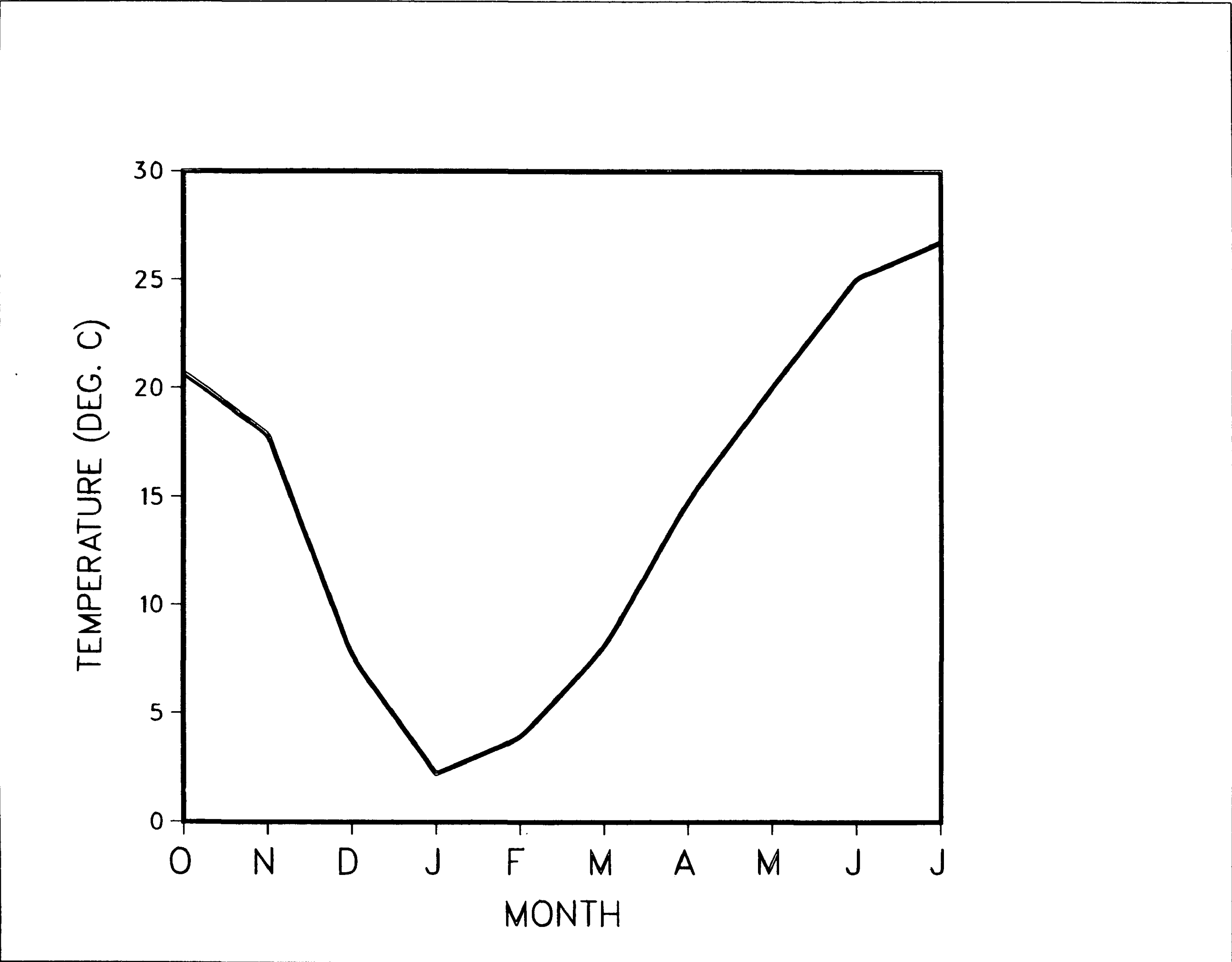

FLAGELLATES / mm3
6,000
4,000
2,000
0
N D J F M A M J J
MONTH

Figure 13. Average monthly York River water temperature during 1983-
1984.


TEMPERATURE (DEG. C)
30
25
20
15
10
5
0
O
N
D
J
F
M
A
M
J
J
MONTH

DISCUSSION

The kinetics of the acquired immune response in naturally and experimentally *Trypanoplasma bullocki*-infected summer flounder were temperature dependent. Summer flounder maintained at 20°C were able to establish a significant response which enabled them to eliminate the flagellate within eight weeks. Fish maintained at ambient temperature and those collected from the field were unable to elicit an immune response significant enough to eliminate the hemoflagellate until twenty-eight weeks post-infection. During this twenty-eight week period, temperatures remained less than 5°C for eight weeks. Ethanol-treated summer flounder were able to eliminate the flagellate in the same time period as immunized flounder. Also, ethanol-treated fish exhibited a similar antibody response as that found in immunized fish. This result was not expected because previous work by Stolen et al. (1985) showed that the humoral immune response of summer flounder was suppressed when the fish were pretreated with an injection of 95% ethanol.

Seasonal variation in flagellate intensities, as well as specific antibody production was apparent in field and ambient-temperature experimental fish. All experimental fish which became infected were able to produce specific antibodies against *Trypanoplasma bullocki*. Antibody response was variable in individual fish of the same treatment, and may be explained by differences in the genetic capabilities

in summer flounder populations (Robohm and Sparrow, 1981). It appeared that fish which had a high antibody response were able to survive the infection. When temperatures decreased to below 5°C, the production of specific antibody was drastically reduced, and when temperatures began to increase in the spring, so did antibody production. In fish held at ambient temperature, antibody titers of the magnitude attained by fish kept at 20°C occurred only in the late spring (18 weeks later) . This agrees with work by Stolen and coworkers (1982) that showed a delay in the appearance of circulating antibodies, but no marked suppression in titer of summer flounder held at low temperatures. Immunological memory was established because experimental infections occurred before temperatures decreased below 15°C, . According to Avtalion (1969) immunological memory can be established within four days if it is allowed to develop at high temperatures.

Control fish held at ambient temperature and fish from the York River exhibited similar antibody patterns, with maximum titers occurring in the late spring. This may suggest that most initial infections of _Trypanoplasma bullocki_ in wild summer flounder occur when water temperatures are above 10°C. Although flagellate intensity patterns were similar in the field and experimental samples, initial peak intensity in field fish was over 5000 flagellates/mm^3. The high intensity may be explained by the fact that fish in the wild may be exposed to higher initial infective doses. Another possible explaination could be that temperatures in December, 1983 decreased from 10°to 3°C, and samples were collected near the latter part of the month, whereas in the experimental study, temperatures stayed close to 10°C for the month of December.

Seasonal variations in the levels of hemoflagellates in fish over the course of one year has been noted in other reports. Cottrell (1977) found that a high prevalence of *Trypanosoma plattessae* Lebailly in wild plaice occured in February and March when water temperatures were at their lowest, and a low prevalence of infection occurred during the summer. He attributed this seasonal pattern to be the result of temperature-controlled immunity to these parasites. Barrow (1954) showed nine European freshwater fish suffered their highest trypanosome infections just after the winter season, which he attributed to a decrease in lytic activity at low temperatures .

The question arises as to which phase of the immune response is temperature dependent and which is responsible for the elimination of the flagellate. It may well be that non-specific components of the humoral immune system, such as complement, complement-reactive protein, and lysozyme, may be the key factor in defense against some diseases in fish (Fletcher, 1982). Bower and Woo (1977) showed that one mechanism for lysing hemoflagellates is the alternate pathway of complement activation which does not require the presence of specific antibodies. Sypek and Burreson (1983) used the in vitro plasma incubation test to measure the amount of lytic activity in experimentally-infected summer flounder. Their results showed no lytic activity in fish kept at 5°C, as well as an increase in flagellate intensity. They did find that lytic activity occurred in fish held at 24°C, and that flagellates were eliminated within 6 weeks. This may explain why summer flounder from the wild, infected in the fall when temperatures begin to fall below 12°C, are unable to eliminate the flagellate even though antibodies are present. The cellular immune response may also

play an important role in the elimination of the hemoflagellate. Fish kept at 5°C contained macrophages with engulfed trypanoplasms (Sypek and Burreson, 1983). Further studies on the role, if any, that macrophages play in the elimination of *T. bullocki* are needed.

This study found that moribund fish exhibited symptoms of anemia and ascites. Burreson and Zwerner (1984) observed that ascitic fish do not survive winter temperatures (5°C or less) from December through February. They also found the period of highest mortality in experimentally *Trypanoplasma bullocki*-infected fish occurred at temperatures of 0° to 1.5°C .

Different types of immunization routes and different forms of killed antigens have been tested for their ability to protect fish against a varied list of diseases. Mortality rates are used to establish the differences in the degree of protection between groups of different treatments (Anderson, 1974). Some immunological treatments have proved successful (Klontz, 1966, Fujihara and Nakatani, 1979), whereas others have not (Krantz et al. 1964). Ross and Klontz (1965) found oral administration of phenol-killed redmouth enteric organisms to yearling rainbow trout provided effective protection with 10% mortality in immunized and 90% mortality in non-immunized fish. Lom (1979) reported that goldfish inoculated with irradiated *Trypanoplasma borelli* Laveran and Mesnil and then subsequently challenged with live flagellates were not protected. Sniesko and Friddle (1949) were not able to show any significant differences in mortality patterns between immunized and control eastern brook trout against *Aeromonas salmoncida*. The use of formalin-killed antigens has had contradictory results. Avtalion (1981) found that mortalities in carp immunized

intraperitoneally with formalin-killed *Aeromonas liquefaciens* was 1 out of 29 as opposed to 9 out of 10 in non-immunized fish. Krantz et al. (1964) were not very successful in protecting trout immunized with formalin-killed *Aeromonas salmoncida* without adjuvant. From this study, with 50-60% mortality in immunized and non-immunized summer flounder, it appears that immunizing summer flounder with formalin-killed *Trypanoplasma bullocki* does not provide protective immunity. Formalin may have been responsible for altering the antigenic binding sites.

Cottrell (1977) believes that the use of living attenuated strains of pathogens might be more effective in providing protection than using killed organisms. Summer flounder held at 20°C developed protective immunity once they eliminated the initial infection. This was demonstrated by the fact that the fish did not become reinfected. Lom (1979) and Woo (1981) showed that goldfish which had survived experimental infections of *Trypanosoma danielewskyi* Laveran and Mesnil could not be reinfected.

In conclusion, summer flounder produced specific antibodies against the flagellate *Trypanoplasma bullocki*. Immunization with formalin-killed flagellates did stimulate the production of specific antibodies, i.e., fish mounted a high secondary response, but did not provide protective immunity against trypanoplasm infection. Experimental fish which survived a moderate infection, did not become reinfected. Future studies on field fish may demonstrate the same phenomenon. It is apparent from this study, that temperature plays an important role in affecting the immune response. At this time it would be premature to single out the production of specific antibodies as

the main part of the immune response affected by temperature. Further research is needed in the areas of non-specific and cellular defense mechanisms .

APPENDIX I. Cultures and Stock solutions.

Trypanoplasma bullocki sterile culture

78 ml Minimum essential medium with 25 mM HEPES buffer and Hanks' salts, without L-glutamine

28 ml Fetal calf serum, heat inactivated at 56°C for 30 minutes

1 ml L-glutamine

1 ml Penicillin-Strptomyocin

0.33 g Dextrose

Phosphate Buffer Saline (PBS) pH 7.2

7.20 g NaCl

1.48 g Na_2HPO_4 (anhydrous, dibasic)

0.43 g KH_2PO_4 (anhydrous, monobasic)

Bring to 1 liter with distilled, deionized H_2O

pH adjusted to 7.2 with HCl

Tris Buffer Saline (TBS) pH 7.5

2.42 g Tris

29.24 g NaCl

Bring to 1 liter with distilled, deionized H_2O

pH adjusted to 7.5 with HCl

Antigen Blocking Solution

100 ml Tris Buffer Saline

3 g Gelatin

Antibody Buffer Solution

200 ml Tris Buffer Saline

2 g Gelatin

Color Development Solution

a 30 mg HRP Color Development (BIORAD)

10 ml Ice Cold Methanol

Make fresh daily and protect from light

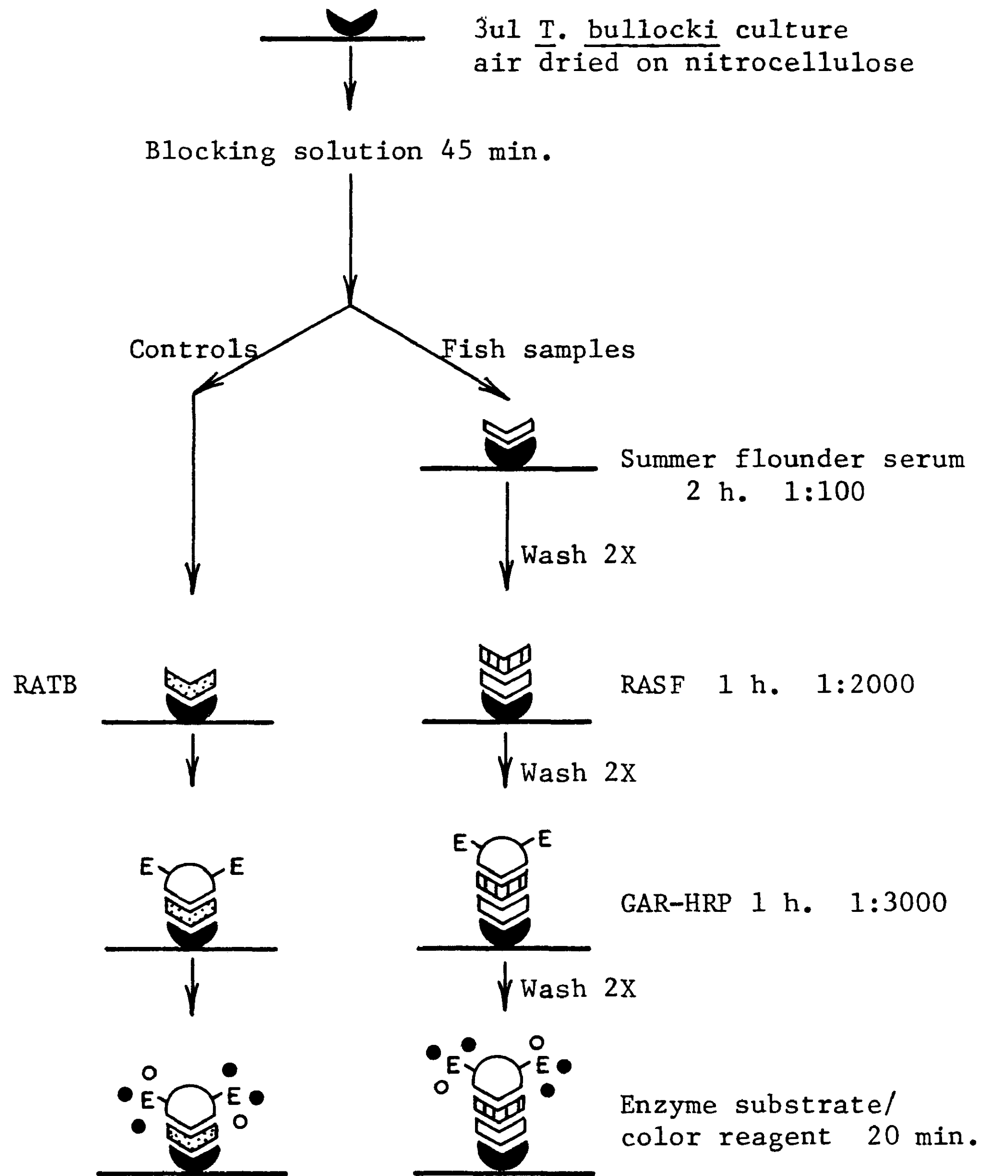
b Make immediately prior to use

30 ul Ice cold 30% H_2O_2

50 ul Tris Buffer Saline

Mix with (a) at room temperature

APPENDIX II. IMMUNO BLOT ASSAY FLOW CHART



Key: RATB - Rabbit anti-*Trypanoplasma bullocki* serum
RASF - Rabbit anti-summer flounder immunoglobulin
GAR-HRP - Goat anti-rabbit immunoglobulin/horseradish peroxidase conjugate

APPENDIX III. Observations of experimentally infected juvenile summer flounder

KEY: TAG = Tag number of fish

TR = Treatment 1 - Group 4, 20°C
2 - Control, 20°C
3 - Control, ambient
4 - Immunized, 20°C
5 - Immunized, ambient
6 - ETOH-treated, 20°C
7 - ETOH-treated, ambient

DATE = Date flounder blood was sampled

WK = Week

INTEN = Flagellate intensity (flagellates/mm^3)

TITER = Anitbody titer

TAG	TR	DATE	WK	INTEN	TITER
029	2	101884	00	00000	0000
034	2	101884	00	00000	0000
038	2	101884	00	00000	0000
040	2	101884	00	00000	0000
047	2	101884	00	00000	0000
028	3	101884	00	00000	0000
030	3	101884	00	00000	0000
031	3	101884	00	00000	0000
032	3	101884	00	00000	0000
033	3	101884	00	00000	0000
037	3	101884	00	00000	0000
039	3	101884	00	00000	0000
041	3	101884	00	00000	0000
045	3	101884	00	00000	0000
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142	4	101884	00	00000	0000
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132	5	101884	00	00000	0000
133	5	101884	00	00000	0000
134	5	101884	00	00000	0000
136	5	101884	00	00000	0000
138	5	101884	00	00000	0000
139	5	101884	00	00000	0000
140	5	101884	00	00000	0000
145	5	101884	00	00000	0000
104	6	101884	00	00000	0000
105	6	101884	00	00000	0000
107	6	101884	00	00000	0000
111	6	101884	00	00000	0000
116	6	101884	00	00000	0000
101	7	101884	00	00000	0000
103	7	101884	00	00000	0000
108	7	101884	00	00000	0000
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034	2	110784	03	00000	0000
038	2	110784	03	00000	0000
040	2	110784	03	00000	0000
047	2	110784	03	00000	0000
028	3	110784	03	00000	0000

TAG	TR	DATE	WK	INTEN	TITER
030	3	110784	03	00000	0000
031	3	110784	03	00000	0000
032	3	110784	03	00000	0000
033	3	110784	03	00000	0000
037	3	110784	03	00000	0000
039	3	110784	03	00000	0000
041	3	110784	03	00000	0000
045	3	110784	03	00000	0000
048	3	110784	03	00000	0000
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128	4	110784	03	00000	0800
130	4	110784	03	00000	0400
137	4	110784	03	00000	0000
142	4	110784	03	00000	0000
126	5	110784	03	00000	0100
129	5	110784	03	00000	0100
131	5	110784	03	00000	0100
132	5	110784	03	00000	0100
133	5	110784	03	00000	0000
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031	3	111484	04	00150	0100
032	3	111484	04	00150	0000
033	3	111484	04	00050	0100
037	3	111484	04	00050	0000
039	3	111484	04	00000	0100

TAG	TR	DATE	WK	INTEN	TITER
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045	3	111484	04	00000	0100
048	3	111484	04	00050	0000
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128	4	111484	04	00050	0400
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142	4	111484	04	00000	0100
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129	5	111484	04	00000	0400
131	5	111484	04	00000	0000
132	5	111484	04	00000	0400
133	5	111484	04	00000	0400
134	5	111484	04	00050	0200
136	5	111484	04	00000	0100
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105	6	111484	04	00150	0000
107	6	111484	04	00000	0000
111	6	111484	04	00050	0000
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112	7	111484	04	00000	0000
113	7	111484	04	00150	0000
118	7	111484	04	00050	0200
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034	2	112184	05	00150	0800
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040	2	112184	05	00050	0200
047	2	112184	05	00150	0200
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039	3	112184	05	99999	9999
041	3	112184	05	99999	9999
045	3	112184	05	99999	9999
048	3	112184	05	99999	9999
127	4	112184	05	00150	1600
128	4	112184	05	00150	1600
130	4	112184	05	00050	1600

TAG	TR	DATE	WK	INTEN	TITER
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142	4	112184	05	00150	1600
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129	5	112184	05	99999	9999
131	5	112184	05	99999	9999
132	5	112184	05	99999	9999
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134	5	112184	05	99999	9999
136	5	112184	05	99999	9999
138	5	112184	05	99999	9999
139	5	112184	05	99999	9999
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145	5	112184	05	99999	9999
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107	6	112184	05	00300	1600
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101	7	112184	05	99999	9999
103	7	112184	05	99999	9999
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118	7	112184	05	99999	9999
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034	2	112884	06	00050	1600
038	2	112884	06	00000	0200
040	2	112884	06	00050	0200
047	2	112884	06	00150	0400
028	3	112884	06	00300	0000
030	3	112884	06	00700	0000
031	3	112884	06	00300	0100
032	3	112884	06	00150	0000
033	3	112884	06	00300	0000
037	3	112884	06	00300	0000
039	3	112884	06	00300	0000
041	3	112884	06	00300	0000
045	3	112884	06	00150	0000
048	3	112884	06	00150	0000
127	4	112884	06	00300	1600
128	4	112884	06	00050	1600
130	4	112884	06	00150	1600
137	4	112884	06	00300	3200
142	4	112884	06	00050	1600
126	5	112884	06	00150	0100
129	5	112884	06	00700	0400
131	5	112884	06	00050	0000
132	5	112884	06	00300	0400

TAG	TR	DATE	WK	INTEN	TITER
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134	5	112884	06	02000	0800
136	5	112884	06	00300	0100
138	5	112884	06	00300	0400
139	5	112884	06	00700	0000
140	5	112884	06	00150	0200
145	5	112884	06	00700	0000
104	6	112884	06	00050	1600
105	6	112884	06	00300	1600
107	6	112884	06	00200	3200
111	6	112884	06	00300	1600
116	6	112884	06	00200	1600
101	7	112884	06	00700	0200
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028	3	121284	08	00200	0000
030	3	121284	08	01000	0000
031	3	121284	08	00700	0000
032	3	121284	08	00150	0000
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037	3	121284	08	00200	0000
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048	3	121284	08	00150	0000
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128	4	121284	08	00000	1600
130	4	121284	08	00050	1600
137	4	121284	08	00100	3200
142	4	121284	08	00000	0800
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129	5	121284	08	01000	1600
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132	5	121284	08	01000	0400
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134	5	121284	08	02000	1600
136	5	121284	08	01000	0100
138	5	121284	08	01000	0400
139	5	121284	08	01000	0000
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TAG	TR	DATE	WK	INTEN	TITER
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105	6	121284	08	00150	1600
107	6	121284	08	00000	1600
111	6	121284	08	00150	0800
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103	7	052285	30	00000	0800
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APPENDIX IV. Observations of Trypanoplasma bullocki infections in summer flounder from the York River 1983-1984.

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022384	188	00300	0000
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041384	146	00000	0100

030784 183 00000 0000

041384 158 00150 0400

DATE	LENGTH	INT.	TITER
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060784	194	00000	0800
060784	177	00000	0100
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070984	180	00000	0200
070984	200	00000	0100

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