ANNUAL REPORT TO THE ALMOND BOARD OF CALIFORNIA

Project 79-S1

Title: Epidemiology and control of frost injury to almond incited by leaf surface ice nucleation active bacteria

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1. Objectives:

Earlier work on almond and work on other crops including potatoes, tomatoes, pears, and citrus have shown that these plants can avoid frost injury by avoiding bacterial ice nucleation. A new method of frost control was thus suggested for crops such as almond by avoiding bacterial populations which increased the sensitivity of almond to freezing injury. Although earlier work has shown that plants such as almond should be amenable to frost prevention to temperatures as low as -5 C or 24 F, by procedures which reduce bacterial ice nucleation on leaf or flower surfaces, several objectives needed to be undertaken to determine whether this was indeed practical. These objectives then included specifically: a) an investigation of the epidemiology of the colonization of almond leaves and flowers with ice nucleation active bacteria, including the assessment of sources of inoculum and the populations and species of epiphytic ice nucleation active bacteria which predominate on leaves and flowers of almond in periods of frost hazard; b) an investigation of cultural practices that may favor establishment of ice nucleation active bacteria on almond leaves and flowers and thereby increase the frost sensitivity of these plants by such procedures; c) determination of the most effective bactericides and ice nucleation inhibitors, i.e., chemicals which directly affect the nucleation activity of bacterial ice nuclei on leaves and flowers to control frost injury, and to determine the environmental parameters which influence the effectiveness of these two types of materials. Several more specific objectives included: 1) the evaluation of new bactericides and rates and frequencies of application of existing bactericides to control frost injury under standard field conditions using existing spraying techniques; 2) the quantitation of reductions in frost damage in relation to reductions in populations of oce nucleation active bacteria on leaves and flowers of almond as related to reductions in bacterial populations as influenced by bactericide application; 3) The examination of weeds and natural plants in the area of almond orchards as possible sources of ice nucleation active bacteria, and 4) quantitation of the numbers of ice nucleation active bacteria of all kinds on leaves and flowers of almond as a function of time during the period of frost hazard to these plants and this also correlated with environmental factors. 5) To examine the normal bacterial microflora on leaves and select from these microflora bacteria which produce compounds inhibitory to ice nucleation active bacteria. These bacteria will be evaluated in laboratory and greenhouse trials before application in 1980 field trials. These bacterial antagonists selected in theprevious step will be evaluated in field trials in 1980. 6) Evaluate chemicals shown to reduce the

nucleation activity of bacteria in laboratory tests - bacterial ice nucleation inhibitors. 7) Determine the most effective rates of these compounds in reducing frost injury and eliminating ice nuclei from leaf surfaces. 8) Determine the optimal time before frost for application of these types of materials. 9) Determine the persistence of reductions in frost sensitivity of almond following application of bacterial ice nucleation inhibitors.

II. Interpretative Summary

Work during 1979 has shown that the predominant bacteria found on leaf surfaces of almonds including flowers and young fruitlets is Pseudomonas syringae, the same bacterium which causes canker and blast diseases of almond. Also occasionally Erwinia herbicola, a yellow pigmented saprophytic bacteria, were found on leaf surfaces and occasionally isolates of Pseudomonas fluorescens, another saprophytic bacteria, were found. P. syringae was found in very high numbers on leaves and flowers of almond throughout msot of the period of high frost hazard to almond. Very high populations in excess of nearly 1 million per leaf or flower were found, accounting for the high frost sensitivity of these plants. Preliminary results suggest that frost injury to almond may be effectively controlled with existing bactericides. Important existing bactericidal compounds, including copper containing fungicides appear to be very promising in controlling both bacterial populations present on almond leaf surfaces and thus because of this the numbers of ice nuclei found on leaf surfaces. The reductions in frost sensitivity were found to be correlated with the extent of reductions of bacterial populations following bactericidal applications. Several antibiotics also were found to be very effective in reducing bacterial populations but are not registered for use in almond at this time. Procedures are being initiated to allow possible registration of antibiotics, including streptomycin and/or oxytetracycline on almond for frost control purposes. All of the immediate action bacterial ice nucleation inhibitors evaluated during 1979 showed promise as frost control agents. As a rule many of these materials may fall into an exempt category and will not require registration for use on almond, although this matter is being further investigated. In summary it was found that the bacteria had colonized almonds to very high populations and accounted for all of the bacterial ice nuclei found on these leaves, allowing reductions in frost sensitivity in these plants by either bactericides or bacterial ice nucleation inhibitors which reduced populations of these bacteria and/or the nucleation activity of these bacteria on leaves and flowers at the time of freezing conditions. Also, several existing bactericides and several commonly available chemicals offer promise for frost control based on this premise of enhancing the ability of these plants to avoid frost injury.

III. Experimental Procedure

Initial field trials were established in 1979 on mature trees which were donated by Tenneco West near Snelling, CA. This is a very cold, low lying area which would maximize our chance of frost injury. A randomized complete block design included three different bactericides, three bacterial ice nucleation inhibitors and one control. Bactericides included Kocide 101 applied at the maximum recommended rate for almond on about a 7-10 day spray schedule. All materials were applied with a speed sprayer at approximately 200 gallons per acre. The second bactericide

included a mixture of 100 ppm streptomycin sulfate and 50 ppm oxytetracycline. Bactericide number 3 was an experimental application of kasugamycin at 100 ppm. The three bacterial ice nucleation inhibitors were each applied just prior to anticipated frost which occurred only once this past year. These materials were applied within 24 hr of the first expected frost. One nucleation inhibitor consisted of a mixture of 0.5 M urea and 0.1 M sodium carbonate (Na_2CO_3). A second nucleation inhibitor consisted of a mixture of 0.5 M urea and 50 mM zinc sulfate ($ZnSO_4$). The third nucleation inhibitor was a mixture of 0.1% Triton XQS-20, an experimental cationic surfactant. Samples were taken from experimental trees in the plot area on a weekly basis to evaluate both the bacterial populations of various kinds on the leaves and flowers as well as directly determining the numbers of ice nuclei associated with these plant parts, as well as the frost sensitivity of the plants treated with either bactericides or bacterial nucleation inhibitors. Bacterial populations were measured by detaching leaves and flowers, washing the bacteria from the leaves and flowers in sterile buffer solution followed by dilution planting of the leaf washings onto suitable agar medium which was then followed by enumeration of the bacterial colonies on this medium, subsequently followed by a procedure known as a replica freezing technique which allows determination of that subset of the bacterial population which is active in ice nucleation. The numbers of ice nuclei associated with these plant materials were also determined by concentrating by centrifugation an aliquot of the leaf washing and determination of the ice nucleus concentration by a procedure known as the droplet freezing assay, in which a solution is divided into a large number of small droplets which are frozen at a given temperature, typically at -5 C. The numbers of ice nuclei associated with these droplets can be determined by a defined function from the number of droplets which remain unfrozen. A second technique involved freezing of large numbers of discs of leaf or flower material which were submerged in small droplets of water and cooled to various temperatures to determine the nucleation point of the plant tissue. Since no natural frost was observed during 1979, supplemental data on the frost sensitivity of these plant parts was determined by detaching fruit spurs, including leaves, flowers and young nutlets, bringing them to the laboratory and freezing in a controlled environmental chamber at given temperatures, typically 25 F.

IV. Results

Monitoring of the bacterial populations on unsprayed control trees throughout the 1979 frost season allowed us to determine the importance of various ice nucleation active bacteria in their role as determining frost sensitivity. Total numbers of bacterial populations increased from approximately 10^5 bacteria/g fresh wt. of leaf and flower material on about March 10 to in excess of 1 million and approximately 10 million bacteria/g fresh weight by mid-April falling again to about 10^5 bacteria/g by mid-May. Thus the seasonal fluctuation in bacterial populations active in nucleation seemed to reach a maximum at the time of maximum frost sensitivity of these plants. The populations of ice nuclei associated with the leaf material in unsprayed control trees also paralleled the increase and decrease in numbers of bacteria on these leaf surfaces, increasing from less than 10 nuclei/g fresh weight early in March to in excess of 1000 nuclei/g fresh weight in mid-April, again falling to less than 100 nuclei/g fresh weight in mid-May.

All three bactericides effectively reduced populations active in ice nucleation. Kocide 101 was nearly as effective as a mixture of Streptomycin and oxytetracycline in reducing bacterial populations. The experimental application of kasugamycin was not as effective as either Kocide 101 or the mixture of Streptomycin or oxytetracycline in reducing bacterial populations. Total numbers of bacteria present on leaves sprayed either with the Kocide or the Streptomycin/oxytetracycline mixture were typically less than about 10^6 cells/gm fresh weight throughout the growing season. More importantly, bacterial populations active in ice nucleation were lower than about 10^3 bacteria/gm fresh weight at all times during the growing season and thus were found to be more than one thousand-fold lower than unsprayed control trees. The numbers of ice nuclei associated with leaf surfaces was also reduced approximately one thousand-fold by application of either Kocide 101 or Streptomycin and terramycin. Neither of the three bacterial nucleation inhibitors reduced the populations of viable bacteria associated with leaf surfaces. Total bacterial populations ranged from in excess of 10^6 to nearly 10^7 bacteria/gm fresh weight throughout the growing season and the numbers of bacteria active in ice nucleation increased from 1000 cells/gm fresh weight early in March to in excess of 10^6 cells/gm fresh weight in mid-April, again falling to appriximately 10^3 cells/gm fresh weight by mid-May. However, consistent with the mechanism by which these materials act, the numbers of nuclei associated with these bacterial populations were reduced. Nucleation inhibitors were applied only once during 1977 in mid-March; ice nuclei populations were reduced compared to unsprayed control trees for two weeks following the single application of these three nucleation inhibitors. Thus as expected, bacterial ice nucleation inhibitors did not affect the numbers of bacteria associated with plants but did reduce the numbers of nuclei associated with these plants and reduced the potential for damaging ice formation. The populations of bacteria and numbers of ice nuclei are given in figures 1-6 according to the legend. Frost sensitivity of the treated plants was also evaluated during the same time as assays of bacterial populations. Injury was expressed as a fraction of fruiting spurs which sustained measurable frost injury at 24 - 25 F after exposure of 1 - 2 hours. Untreated control spurs typically sustained maximum amounts of frost injury throughout the growing season, typically in excess of 80% of the spurs being injured. Kocide 101 and the Streptomycin/oxytetracycline mixture were consistently most effective at reducing the frost sensitivity of these leaves. Frost damage was reduced to less than 20% injury at this temperature at many time points. Kasugamycin was intermediate in its effectiveness in reducing frost injury. Frost injury typically being approximately 50% or less. Bacterial nucleations were applied only once in about mid-March significantly reduced frost injury for about 10 days after that single application. Frost sensitivity should be directly compared with untreated control plants given by the circle in Figure 7. It can be seen that a mixture of urea and zinc

sulgate, a mixture of urea and sodium carbonate, and Triton XQS-20 all significantly reduced frost injury in the late-March freezing assay. Injury was reduced from approximately 90% to between 25 and 50%, a 2 to 4-fold reduction. Frost sensitivity increased most rapidly following application of nucleation inhibitors in those plants treated with urea and zinc sulfate, followed less rapidly by those of urea and sodium carbonate and Triton XQS-20 which significantly reduced frost injury at all time points following the single application in mid-March.

V. Discussion

These preliminary 1979 data indicate that Pseudomonas syringae is very effective at colonizing the leaves and flowers of almond. Because of this, frost sensitivity of almond can easily be understood. Several different control methods appear to be very effective in reducing the numbers of bacterial ice nuclei on leaf surfaces including both bactericides and bacterial ice nucleation inhibitors. Because frost sensitivity was reduced in the presence of either of these two types of materials, bacterial ice nuclei appeared to account for most if not all of the nuclei associated with the leaf surfaces of this plant. Because of the extremely high populations of primarily P. syringae on leaves and flowers, control methods must be very effective at reducing either the numbers of these bacteria and/or the nucleation activity of these bacteria on leaves to achieve significant frost control. The extent of frost control will be dependent on and appears to be directly correlated with the reductions of bacterial populations. It appears however, that commonly available bactericides when applied on an altered schedule compared with standard disease control, can offer significant frost control. Significant bacterial populations measured even early in March prior to significant bud growth, indicated that dormant applications of bactericides may be effective at reducing initial inoculum which may be important in determining large populations later in the season. For this reason, dormant applications will be evaluated in 1980 trials. Similarly, combinations of Streptomycin/ oxytetracycline, Kocide or other bactericides will be evaluated in conjunction with bacterial nucleation inhibitors in an attempt to prolong the effect seen by those nucleation inhibitors. Preliminary tests outside of the plot area indicate that lower rates of Kocide than the maximum rate used in this plot area also significantly reduced bacterial populations. Further work will be initiated in 1980 to determine effective rates of application and in addition other bacterial nucleation inhibitors and other experimental and commercially available bactericides will also be evaluated. 1979 also offered an opportunity to collect and analyze in laboratory and greenhouse trials various non-nucleating bacterial antagonists which may be effective in a form of biological control of frost injury. These bacterial antagonists were evaluated in laboratory trials for the ability to produce antibiotics inhibitory to ice nucleation active bacteria. These and other antagonists were also evaluated in greenhouse trials aimed at establishing these

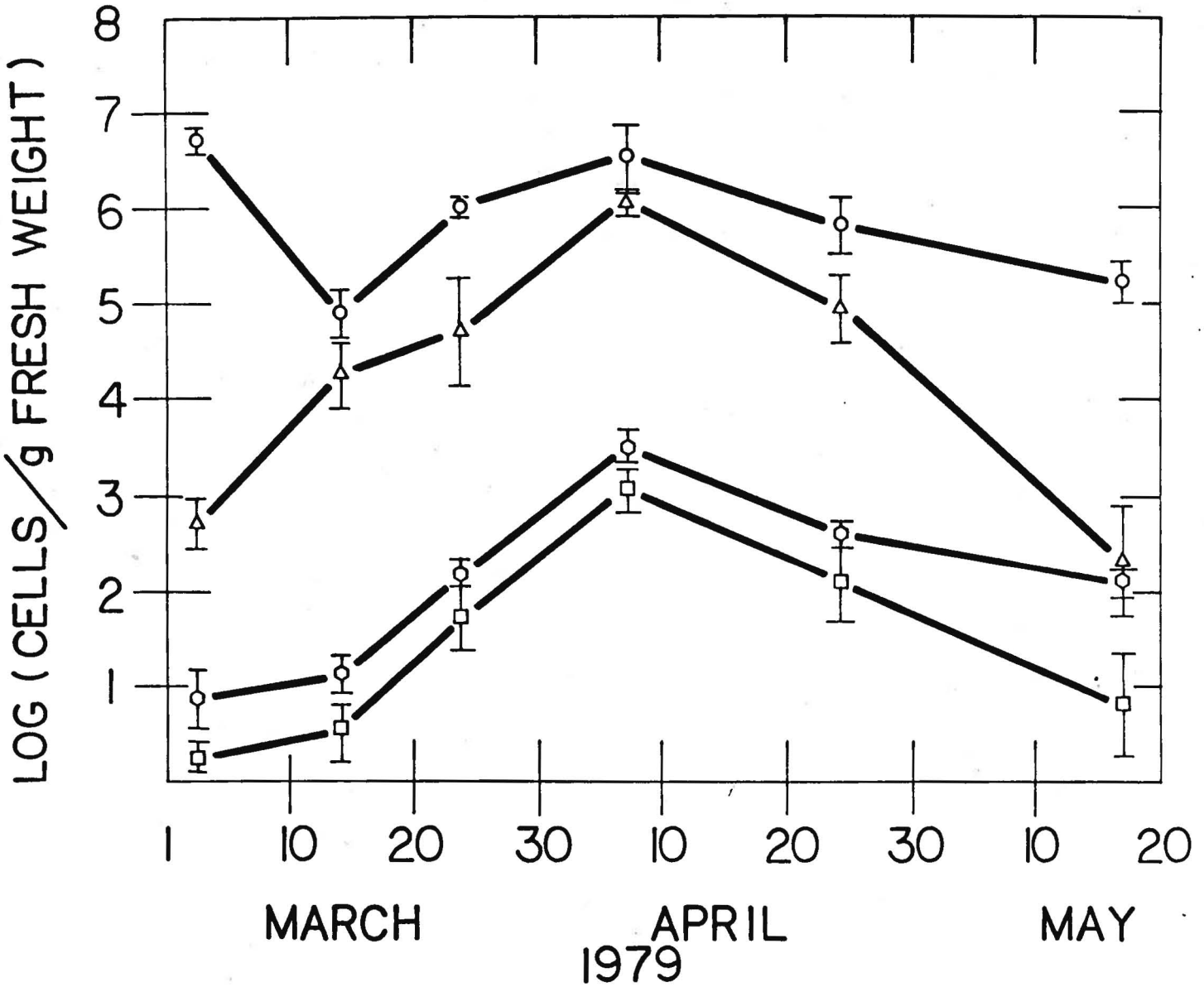
bacteria on leaf and flower surfaces prior to colonization by ice nucleation active bacteria. Bacterial antagonists found to be effective in either of these tests will be established on leaf surfaces in 1980 by foliar applications near bud break in an attempt to preclude colonization of almond by ice nucleation active bacteria by either prophylactic or active antagonistic mechanisms.

VI. Publications

None during 1979

Figures 1-6: Populations of total bacteria and ice nucleation active bacteria and numbers of ice nuclei associated with leaves and flowers of almond during 1979. Figure 1: unsprayed control plants; Figure 2: Kocide 101; Figure 3: Streptomycin plus terramycin; Figure 4: Kasugamycin; Figure 5: urea plus sodium carbonate; Figure 6: Triton XQS-20. In all figures top curve open circles represent total numbers of bacteria recovered on the given dates. The triangles represent total numbers of ice nucleation active bacteria including populations of P. syringae and Erwinia herbicola recovered on all dates. The hexagon represents the numbers of bacterial ice nuclei active at -9 C per gram fresh weight found associated with plant material at each date. The lower curve (the square) represents ice nuclei active at -5 C associated with plant material at each date.

Figure 7: Frost sensitivity of fruiting spurs of almond detached and frozen at -4 to -5 C at various dates during 1979.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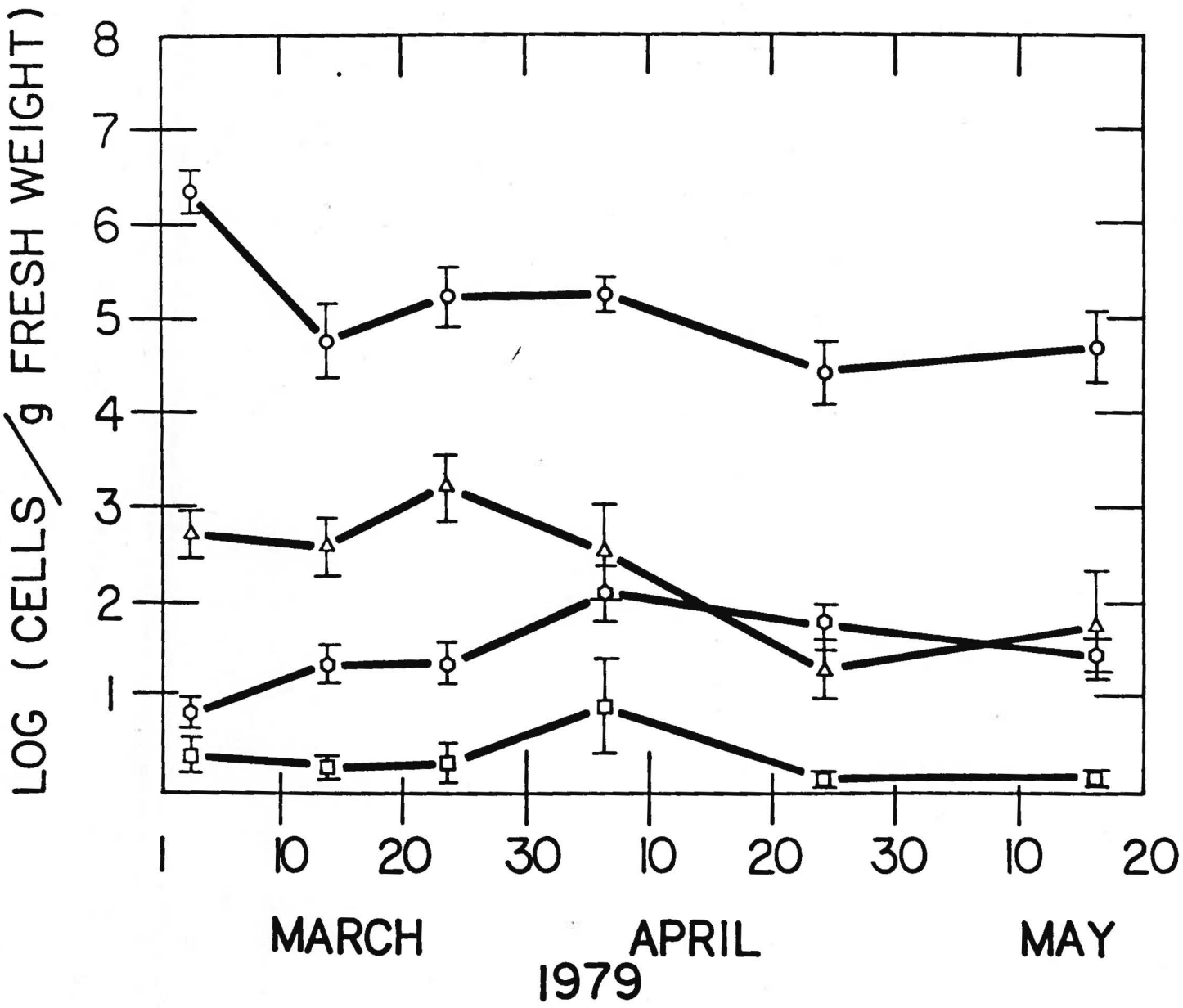
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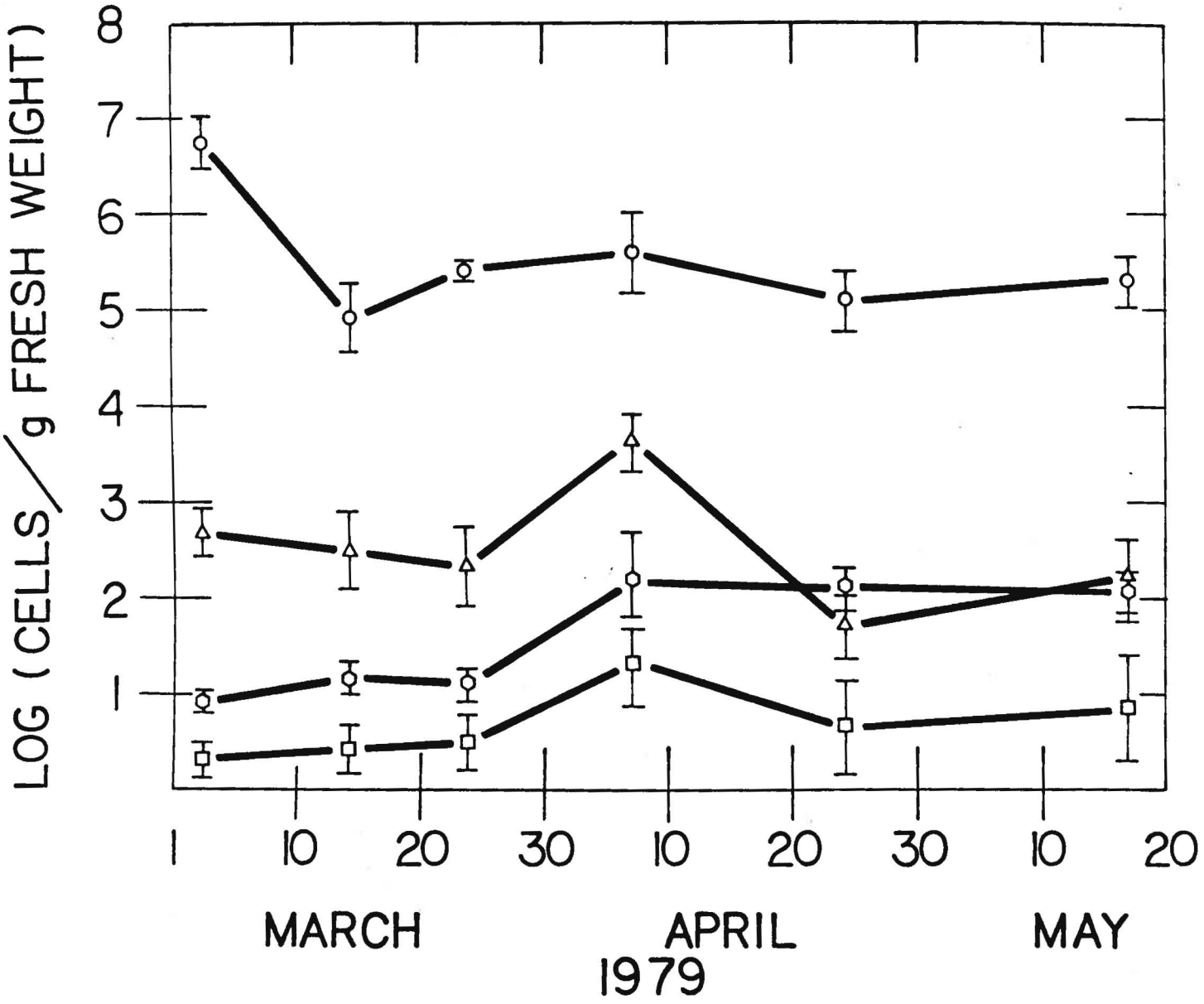
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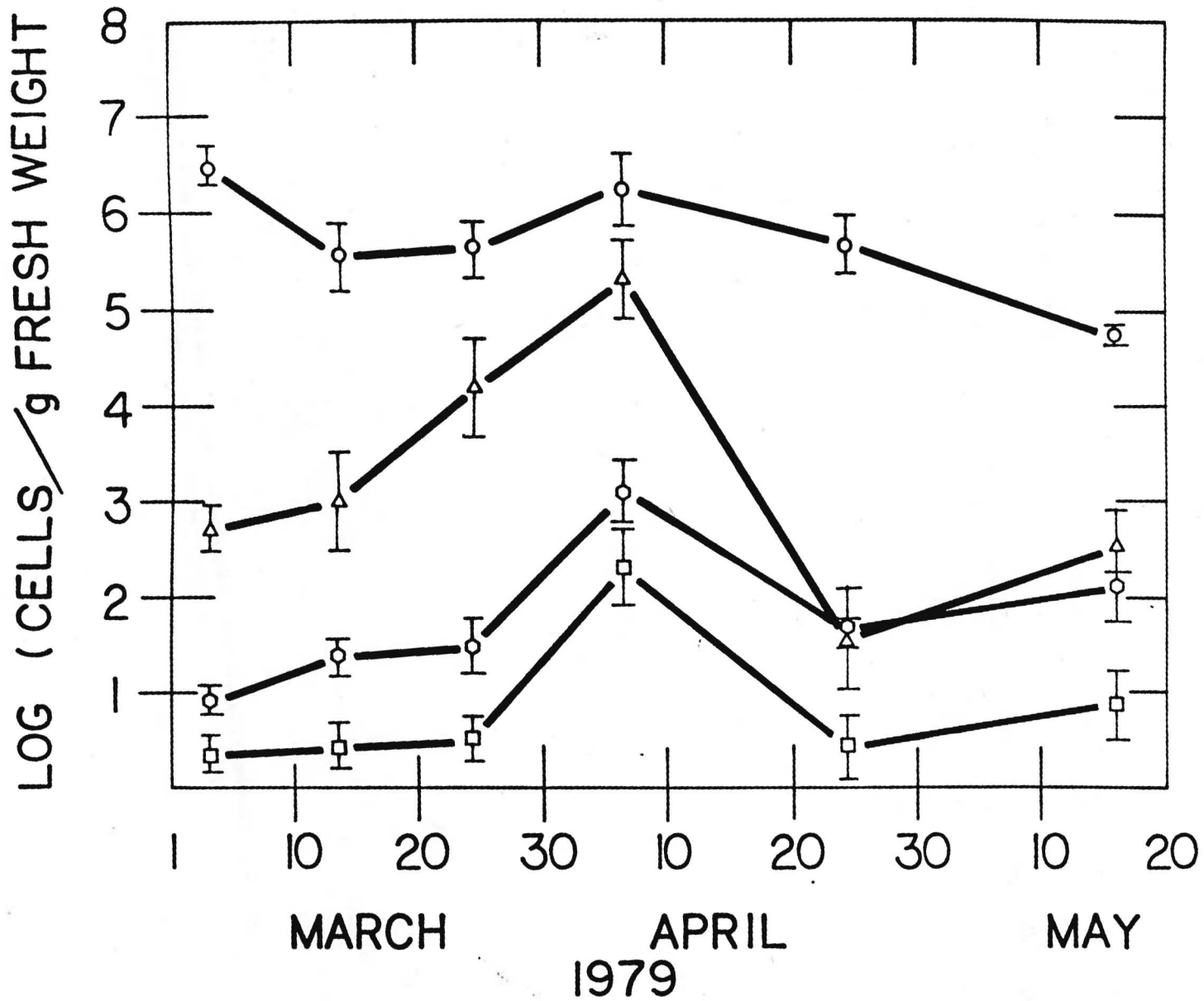
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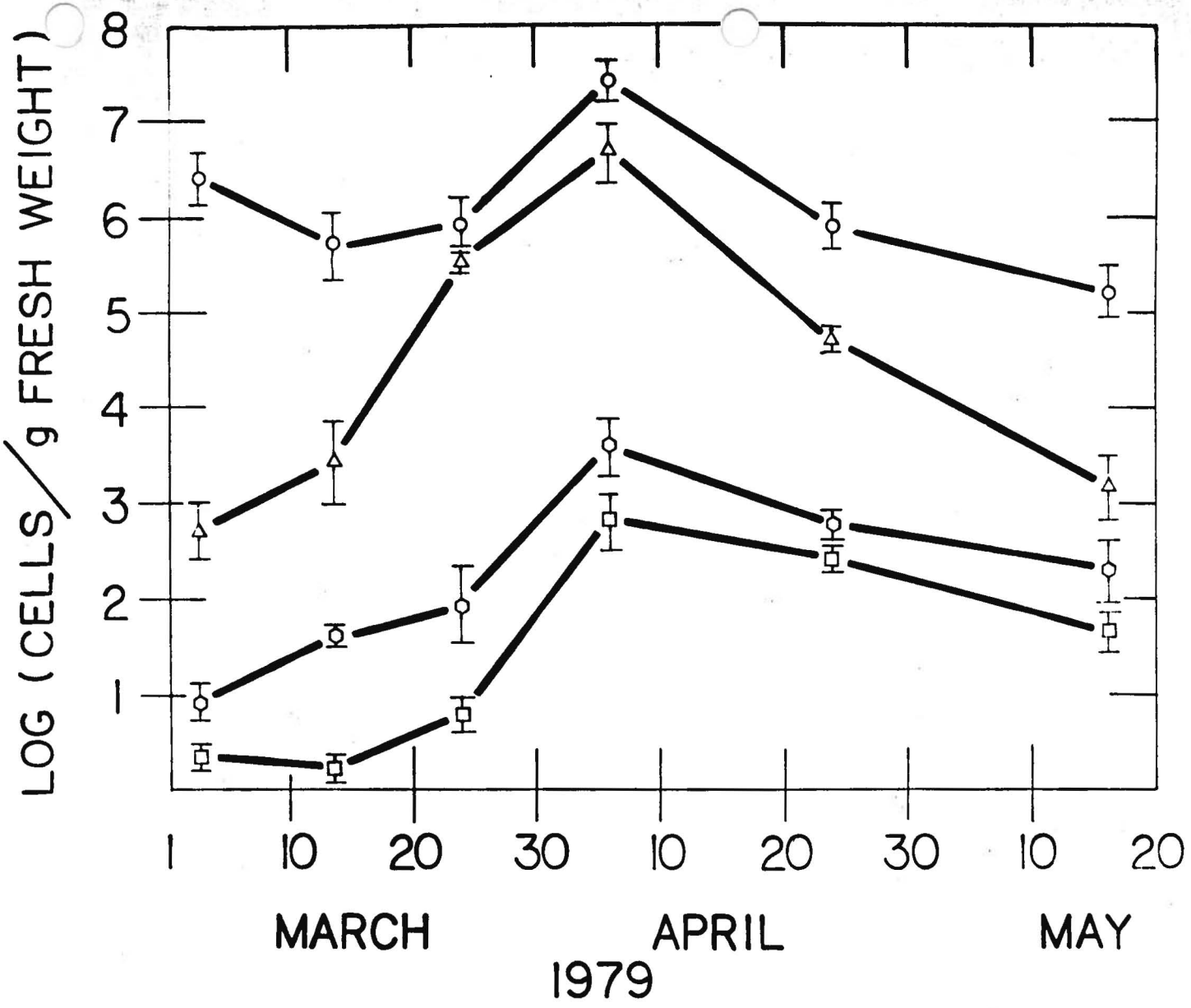
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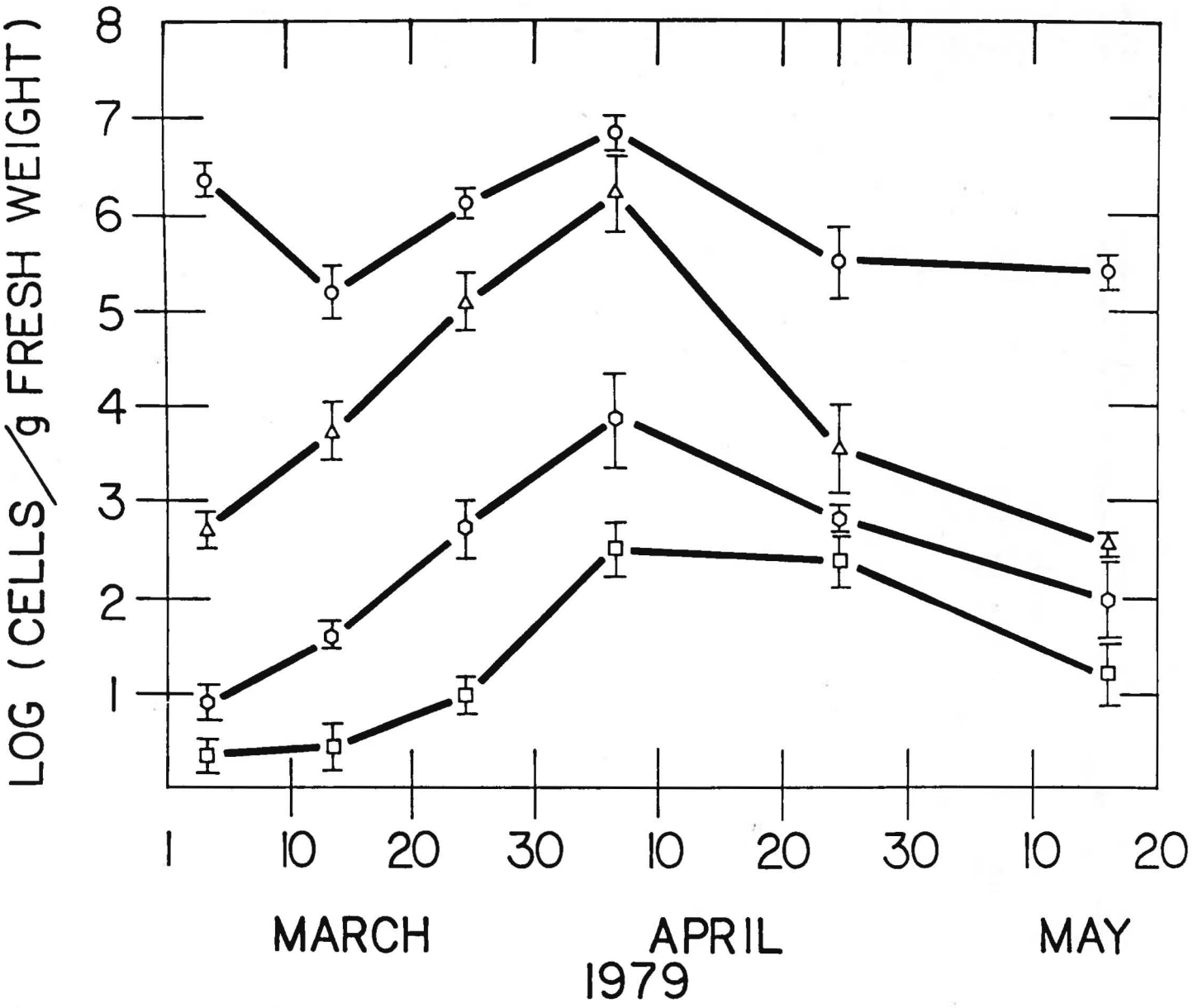
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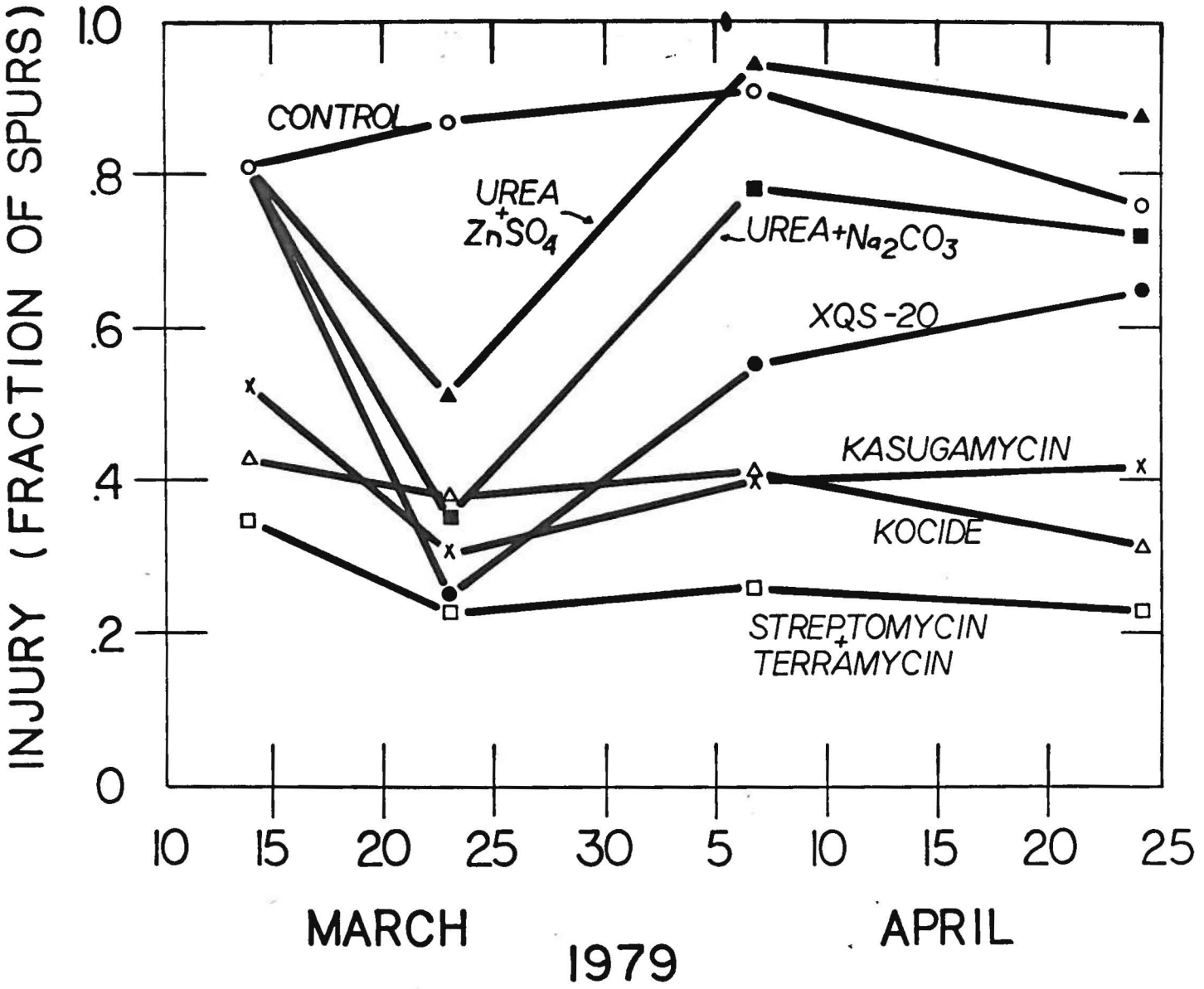
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