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GROWTH AND SEXUAL MATURATION OF LABORATORY-CULTURED MONTEREY *BOTRYLLUS SCHLOSSERI*

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ABSTRACT

Growth and sexual maturity were compared among Monterey *Botryllus schlosseri* colonies cultured in the laboratory and in Monterey Marina in two separate studies: Phase I—April to July 1984, a time when *Botryllus* growth is rapid in the field, and Phase II—December 1984 to March 1985, a time when growth is minimal in the field. Laboratory colonies were kept in tanks of standing, aerated filtered seawater at 15°C, 20°C, and 24°C; the seawater was changed daily or on alternate days. Food types were two concentrations of an algal mixture and five concentrations of Liquifry, a commercially available food for filter-feeding marine invertebrates. All algae-fed colonies did poorly. During Phase I, the colonies kept in Monterey Marina grew much faster than those in the laboratory, but they took longer to reach sexual maturity than the Liquifry-fed colonies grown at 20°C and had a lower survivorship. In Phase II, *Botryllus* growth rates and survivorships in seven of the eight laboratory treatments were better than those of marina colonies. These laboratory treatments yielded a substantial proportion of sexually mature colonies, whereas none of the marina colonies produced eggs during Phase II.

INTRODUCTION

The colonial tunicate *Botryllus* has become increasingly important as an experimental animal. It has been the subject of research in developmental biology (Mukai and Watanabe, 1976), genetics (Sabbadin, 1964; Scofield *et al.*, 1982), immunology (Taneda and Watanabe, 1982; Scofield and Nagashima, 1983; Schlumpberger *et al.*, 1984), ecology (Grosberg, 1981, 1982), and physiology (Mackie and Singla, 1983). Research endeavors would be enhanced by the existence of genetically defined, sexually reproductive stocks of *Botryllus* colonies maintained in the laboratory.

Laboratory cultures of Monterey *Botryllus schlosseri* will allow circumvention of seasonal variation in colony availability, growth rate, and sexual reproduction, and will permit the production of defined genetic stock colonies. We attempted to identify optimal culture conditions by comparing the growth rate and onset of egg production among colonies raised at different temperatures and fed different diets. Treatment conditions were partially based on earlier methods applied to laboratory culture of colonial ascidians in the northeastern United States (Berrill, 1937; Milkman, 1967), Italy (Sabbadin, 1960), and Japan (Nakauchi *et al.*, 1979).

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MATERIALS AND METHODS

Procedural differences between Phases I (April–July 1984) and II (December 1984–March 1985) were primarily due to experiences and results of Phase I.

Animals

Phase I. Monterey *Botryllus sp.* has been classified as *B. schlosseri* by T. Newberry (University of California, Santa Cruz), in collaboration with Y. Saito and H. Boyd (Hopkins Marine Station of Stanford University). Wild-fertilized *Botryllus* colonies were collected from algal substrates attached to floating docks in Monterey Marina (Monterey, California). Each of five colonies containing mature embryos was placed in filtered (5–10 microns) seawater in a transparent 266 ml plastic cup that had been wrapped with black tape. A clear plastic petri dish, covered with a black plastic circle nearly the diameter of the top of the cup, served as a loose-fitting lid. This cup was placed in a light-tight box (66 × 33 × 42 cm), containing a Vita-Lite (Duro-Lite, 15WT8) and equipped with an externally mounted cooling fan (Archer, 10.5 cm diameter) and exhaust port. The box was kept in a 15°C temperature-controlled room. The Vita-Lite and fan were controlled by a timer which was set to approximate the natural daylength. When the cup was placed under the Vita-Lite inside the box, its lid created a shadow on the water surface, while still allowing some light to reach the colony. Upon hatching from the colony, *Botryllus* tadpoles are positively phototactic and negatively geotactic (Grave, 1937), and thus many tadpoles swam upward and metamorphosed, inverted, at the water's surface. When their tails were almost completely resorbed, the oozooids were transferred with a Pasteur pipette to a 7.6 × 5.1 cm glass slide; two oozooids were placed about 2 cm apart in the center of each slide. Progeny from the 5 colonies were distributed equally among the different treatments, with a total of 14 oozooids per treatment. On 4/23/84 (considered as day 0 for Phase I), these colonies were 18–27 days old, depending upon the date of hatching from their respective maternal colony.

Phase II. Tailwrap embryos were dissected from each of nine wild-fertilized Monterey *Botryllus* colonies and placed in 10 ml filtered seawater, containing 40 μl of Penicillin (10,000 U/ml)-Streptomycin (10 mg/ml) (P/S), in individual Poly-Con dishes (Cole-Parmer Instrument Co.; 2 cm high × 4 cm diameter). The dishes were kept closed, at 15°C or at room temperature, with a daily change of filtered seawater-P/S, until hatching occurred. Newly metamorphosed oozooids were carefully peeled from the sides and bottom of the dish with the aid of forceps and dissecting microscope. Each oozooid was placed in the center of a 7.6 × 5.1 cm glass slide. Progeny from the 9 colonies were distributed equally among the different treatments, with a total of 14 oozooids per treatment. On 12/3/84 (day 0 of Phase II), colonies were 18–58 days old, depending upon the date of hatching from their respective maternal colony.

Physical set-up

Phase I. Slides containing colonies to be maintained in the laboratory were placed vertically in adjacent slots of glass staining racks and kept in eight individual glass tanks containing 4 liters of standing filtered seawater and aerated with an airstone. Typically, this allowed for at least 10 ml of filtered seawater per zooid during the experiment (Milkman, 1967). The seawater was changed daily in each tank. The tanks were kept in the 15°C room. Half of the tanks each contained a 50 watt aquarium

heater to maintain the water temperature at 20°C. The colonies in all tanks were exposed to 14 hours of indirect light (1.5 microeinsteins/m^2/s), in order to approximate the daylength during the season when most colonies in the Monterey Marina contain eggs and fertilized embryos.

A cohort of colonies on slides was kept in a wooden rack in the Monterey Marina about 60 cm below the water surface, near where wild colonies are found. These slides were placed horizontally in the rack, with colonies on the undersides of the slides.

Phase II. In the 15°C room, slides containing laboratory-maintained colonies were placed in alternate slots of glass staining racks in eight individual glass tanks containing 17 liters of standing filtered seawater and aerated with an airstone. The seawater was changed daily in one of the tanks, but was only changed on alternate days in the remaining seven tanks. Using individual 50 watt aquarium heaters, the water temperature in five of the tanks was maintained at 20°C, and at 24°C for the other three tanks. Lighting conditions were as in Phase I.

A set of colonies was kept in the marina under the same conditions as described for Phase I.

Food

Phase I. Half of the laboratory-maintained colonies were fed once daily with a mixture of the green alga *Dunaliella tertiolecta* (obtained from the University of Texas Algae Collection, Austin, Texas) and the diatom *Phaeodactylum tricornatum* (obtained from Moss Landing Marine Laboratory, Moss Landing, California). *Dunaliella* was grown in Provasoli's Enriched Seawater and *Phaeodactylum* was grown in the same medium without iron (Stein, 1973). Algae suspensions were dispensed to each tank by pipette. Cell concentrations for "1× algae" in the tanks were 2.5×10^4 cells/ml for each algal species. Choices of algae and their concentrations were based on studies by Milkman (1967), Sabbadin (1960), and Nakauchi *et al.* (1979).

The remaining half of the laboratory-maintained colonies were fed once daily with Liquifry marine (Interpet Ltd., Dorking, England), a liquid food for filter-feeding marine invertebrates. Liquifry contains dextrin, pea flour, whole egg, yeast, spinach, and sulphurous acid (preservative). It consists of ≥3.5% crude protein, ≥1.6% crude fat, and ≤1.0% crude fiber. The designation "1× Liquifry" was equivalent to 37.5 μl per liter of a 1:10 dilution of Liquifry in filtered seawater. The Liquifry solution was delivered to each tank by pipette.

Phase II. All laboratory tanks received either 2×, 4×, 8×, or 16× Liquifry once daily (see Phase I).

Growth and colony condition

In Phase I, the initial number of zooids per colony was recorded on 4/23/84 (day 0); the experiment began on 4/26/84 and ended on 7/10/84 (day 78). Experimental conditions for Phase II began on 12/3/84 (day 0) and the initial number of zooids per colony was recorded on this date. Phase II was terminated on 3/14/85 (day 101). All colonies in Phase II were cleaned at least once each week with a small paintbrush. Growth was measured by counting the number of zooids per colony once each week (and once every two weeks at the end of Phase II). In addition, during Phase II growth observations, colony condition was recorded based on morphology of zooids and ampullae and integrity of tunic. A colony's condition was rated as "satisfactory" if the majority of its zooids and ampullae were well-formed (*i.e.*, not shrunken), had vigorous blood circulation, and a firm tunic.

Sexual maturity

In Phases I and II sexual maturity was determined by the presence of newly metamorphosed oozooids, unfertilized eggs, or embryos developing inside the zooids (the latter observation sometimes involved dissection of zooids in Phase I). This is a conservative definition of sexual maturity, because *Botryllus* colonies develop testes before they produce eggs (Milkman, 1967; and pers. obs.). However, we decided to focus on the presence of eggs or embryos because of the need for breeding colonies to establish defined genetic stocks. Once a colony was observed to have eggs or embryos, it was automatically scored as being sexually mature in subsequent weeks; this has been our general observation with Monterey *Botryllus*.

Data analysis

Treatment means and standard errors for the number of zooids per colony were computed for each weekly observation, excluding dead colonies. The growth data in both Phases I and II were analyzed nonparametrically according to Steel and Torrie (1960) or Sokal and Rohlf (1981). Nonparametric tests were used because the heterogeneity of treatment variances could not be alleviated by standard transformations of the data, and the data had distinctly non-normal distributions. (Since tests on the same colonies at different dates in the time series are not strictly independent, the probabilities in such tests should be interpreted with some caution.) Where the data consisted of blocks determined by the maternal parent, the Wilcoxon signed rank test, which is a nonparametric equivalent of the *t*-test for paired data, was used for comparison of two treatments. The Friedman's test, which is analogous to a randomized block analysis of variance, was used for the comparison of more than two treatments. With significant Friedman's tests ($P < 0.05$), treatment means were compared with Wilcoxon-Wilcox tests.

In Phase II, four additional variables were analyzed as above: (1) Maximum size (number of zooids per colony) was used as an index of growth in a treatment; (2) The number of days in the experiment required to reach 50% of maximum size was used as an index of growth rate; (3) The number of days in the experiment required to reach sexual maturity was used to investigate how rapidly reproductive colonies could be obtained; (4) Colony size at sexual maturity was analyzed to determine which treatments yielded the largest colonies at the onset of sexual maturity. Where colony mortality, or the failure of some colonies to achieve sexual maturity, resulted in a non-balanced experimental design, comparisons involving two treatments were done with the Wilcoxon rank sum test, and comparisons involving more than two treatments were made with Kruskal-Wallis tests. When the Kruskal-Wallis test was significant ($P < 0.05$), further comparisons between pairs of treatments were made with the Wilcoxon rank sum test. The four variables were also analyzed to compare young colonies (18–32 days old at day 0) to old colonies (46–58 days old at day 0) in Phase II to determine if colony age affected the responses to the treatments.

Experimental design

The experimental designs of Phases I and II are shown in Table I.

RESULTS

Phase I: Growth of Liquifry-fed colonies was superior to that of colonies fed algae

The *Botryllus* colonies that were fed a mixture of *Dunaliella* and *Phaeodactylum* at 15°C and 20°C had a low growth rate (Fig. 1A). *Dunaliella* tended to stick to, and

TABLE I

Experimental designs

PHASE I (April–July 1984)		
Laboratory treatments:	*15°C*	*20°C*
	1× algae[a]	1× algae
	2× algae	2× algae
	1× Liquifry[a]	1× Liquifry
	2× Liquifry	2× Liquifry
Field treatment: Monterey Marina		
PHASE II (December 1984–March 1985)[b]		
Laboratory treatments:	*20°C*	*24°C*
	2× Liquifry (daily)[c]	—
	2× Liquifry (alternate)[c]	—
	4× Liquifry	4× Liquifry
	8× Liquifry	8× Liquifry
	16× Liquifry	16× Liquifry
Field treatment: Monterey Marina		

[a] For details of algae and Liquifry concentrations, see Food section (above).

[b] In the text, Phase II treatments will be referred to as follows: 2×,20°C (daily); 2×,20°C (alternate); 4×,20°C; . . . 4×,24°C; and so forth.

[c] Filtered seawater was changed daily in the first 20°C treatment in Phase II, thus following the same procedure as in Phase I. Filtered seawater was changed on alternate days in the remaining seven laboratory treatments in Phase II.

presumably grow on, the animals. The algal treatments were terminated on day 57, when survivorship of colonies in the 1× algae,20°C treatment was about 70%, and survivorships for the other three treatments were below 30%. By this date, the surviving colonies had small poorly developed zooids and sluggish blood circulation.

The animals fed Liquifry at 15°C and 20°C grew larger and were healthier than those fed algae (Fig. 1B). During the first 20 days, all colonies remained small, including those in the marina. By day 50, the colonies grown at 20°C were significantly larger, as a group, than the colonies grown at 15°C (Friedman's test: Chi-square = 35.9, d.f. = 3, $P < 0.01$). However, in the final stages of the study, the growth curves for animals kept at 20°C tended to level off. By day 78, only the 1× and 2× Liquifry treatments at 15°C were significantly different from one another (Wilcoxon signed rank test: $Z = 3.2$, $P < 0.01$). The animals fed 2× Liquifry at 15°C had been growing rapidly in this final phase and by day 78 the mean colony size for this treatment was not significantly different from that for animals fed 1× Liquifry at 20°C (Wilcoxon signed rank test: $Z = 0.3$, $P = 0.40$). On day 78, survivorships for all four Liquifry treatments ranged from 93% to 100%.

From day 22 to the end of the study, the mean size of colonies growing in Monterey Marina was larger than those of the colonies grown in the laboratory (Figs. 1A, B). The marina colonies approximately doubled in size weekly from day 22 to day 50. Survivorship in the marina was about 80% on day 78.

Phase I: Sexual maturity occurred in laboratory-maintained colonies at 20°C

Sexual maturity occurred in colonies fed Liquifry at 20°C or grown in the marina (Fig. 2). Eggs and/or embryos were first observed in these Liquifry treatments on day

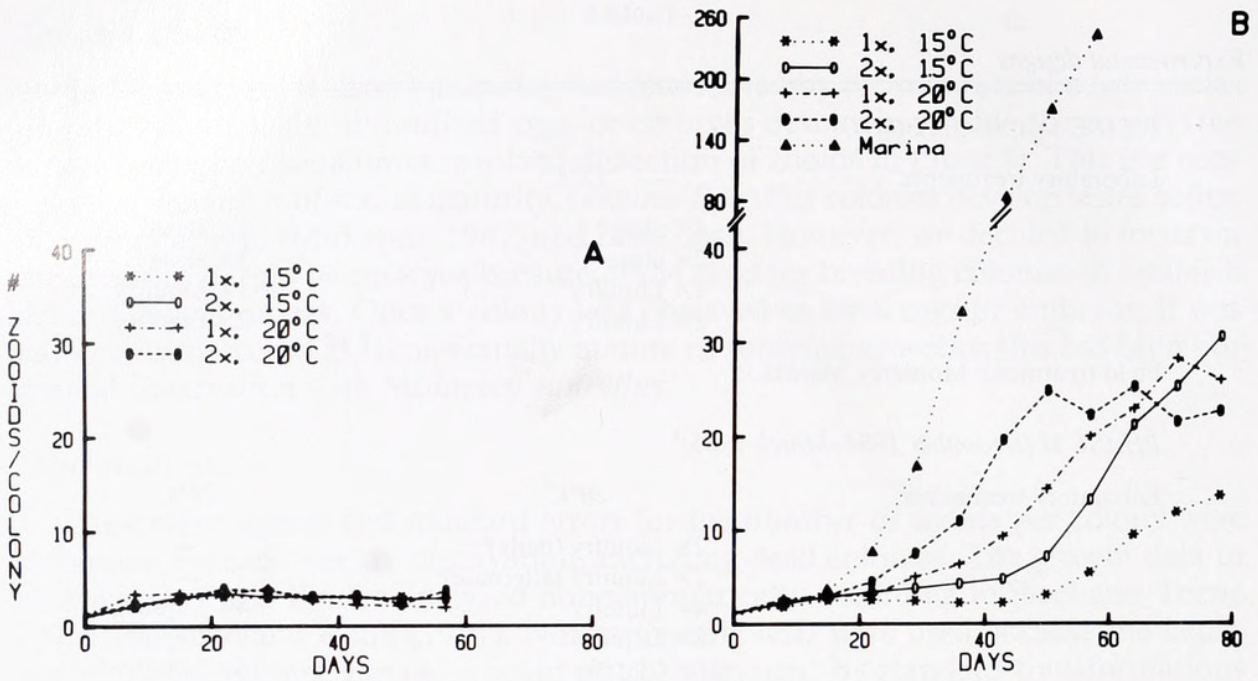

FIGURE 1. *Botryllus* growth curves for Phase I. The ordinate represents mean number of zooids per colony. Day 0 = 4/23/84. Note that 1×, 2×, etc. refer to the food concentrations, and not the frequency of daily feeding. (Zooid counts were not continued for marina colonies after day 57 because the colonies were very large, such that in some cases the growing edges of two colonies on a slide had made contact, sometimes resulting in vascular fusion.) A: Colonies fed 1× or 2× algae at 15°C and 20°C. B: Colonies fed 1× or 2× Liquifry at 15°C and 20°C, or grown in Monterey Marina.

57; by day 78, approximately 70% of the 1× Liquifry colonies and nearly 60% of the 2× Liquifry colonies were sexually mature. In the marina, eggs and/or embryos were first observed on day 50; by day 78, only about 40% of the marina colonies were sexually mature.

Phase II: Healthy, rapidly growing Botryllus *colonies were raised under longterm controlled laboratory conditions*

Although *Botryllus* colonies grow well in Monterey Marina from April to July (Phase I), we suspected that this would not be the case during winter months. To investigate this further, we initiated Phase II culture treatments in December, and chose higher temperature and food levels for the laboratory conditions based on the results from the Phase I experiment. Our major finding was that *Botryllus* colonies raised on a Liquifry diet at 20°C and 24°C in the laboratory grew faster than colonies grown in the marina in winter. In addition to the initial growth increase experienced by all colonies, those grown at the higher food levels at 20°C underwent a second growth rise. As in Phase I, the survivorship of colonies in the marina treatment was lower than those for all laboratory treatments in Phase II.

Before the details of Phase II culture are discussed, the high degree of variability among *Botryllus* colonies with respect to growth should be noted. Individual growth curves for the colonies in each treatment in Phase II are depicted in the Appendix figure. Although the distribution of colonies is matched across treatments with respect to age and maternal origin, it is clear that, even under specified environmental conditions, *Botryllus* colonies as a group do not exhibit readily defined growth patterns. The high variability among colonies within treatments necessitated the use of nonparametric statistics for data anlaysis.

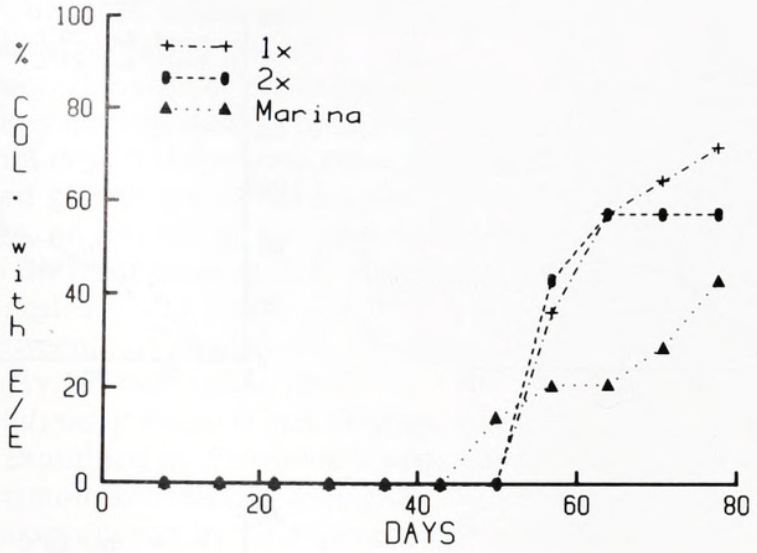

FIGURE 2. Sexual maturity of *Botryllus* colonies in Phase I. The ordinate represents cumulative percentage of colonies with eggs and/or embryos. Day 0 = 4/23/84. Data are for colonies fed 1× or 2× Liquifry at 20°C, and for colonies grown in Monterey Marina.

The effect of frequency of seawater change on growth. In Phase I, filtered seawater was changed daily in all of the laboratory treatments. In Phase II, daily and alternate day seawater change were compared under treatment conditions that yielded the best colony growth in Phase I. The growth curves of colonies fed 2× Liquifry at 20°C receiving daily filtered seawater change [2×,20°C (daily)] and those receiving alternate day filtered seawater change [2×,20°C (alternate)] were similar (Fig. 3A), although the mean sizes for colonies receiving alternate day changes of seawater were significantly higher from day 30 to day 51 of the experiment (Wilcoxon signed rank tests, $P < 0.05$). The survivorships at day 101 for these two treatments were greater than 90%.

The effect of varying food concentration on growth at 20°C. Figure 3B depicts the growth of colonies at 20°C. During the first month of the experiment, the growth rates of all four Liquifry treatments were similar. Notably, a second growth rise occurred initially in the 16× and then in the 8× Liquifry treatment; the 2× (alternate) and 4× treatments did not exhibit this second increase in growth rate (also see Appendix Figs. B–E). There were no significant differences among the four Liquifry treatment means after day 58 due to the high degree of variability among colonies. Survivorships at day 101 for these Liquifry treatments at 20°C were greater than 90%.

The effect of varying food concentration on growth at 24°C. Growth data for the 24°C laboratory colonies are plotted in Figure 3C. By visual inspection, 8× Liquifry gave the best growth, 4× Liquifry yielded the next best results, and the 16× Liquifry treatment was clearly unacceptable for *Botryllus* culture at 24°C; the 16× Liquifry treatment was terminated on day 58, when more than one-third of the colonies had died, and the remaining colonies were in poor condition. During the first month of Phase II, the 4× and 8× Liquifry treatments had similar growth curves; subsequently the curves separated, with 8× Liquifry maintaining a larger mean colony size than 4× Liquifry throughout the remainder of the experiment. However, the weekly 4× and 8× treatment means were never significantly different from one another due to the high variability among colonies, particularly within the 8× treatment (Wilcoxon signed rank tests, $P > 0.05$). At the end of the experiment, the survivorship was 100% for the 4×,24°C treatment and about 80% for the 8×,24°C treatment.

The effect of varying temperature on growth. Mean colony sizes were compared between the 20°C and 24°C treatments having the same food concentration. Mean

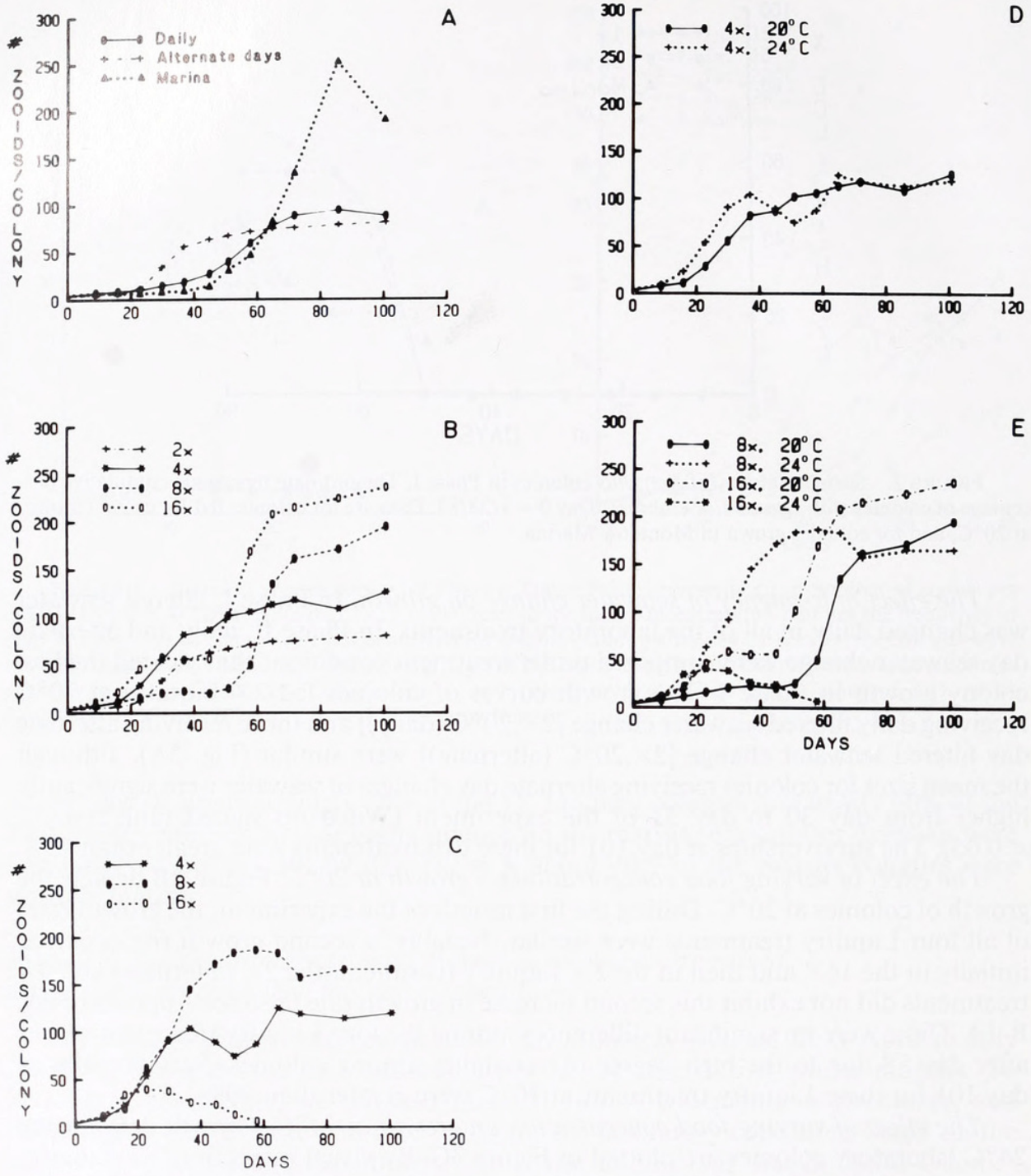

FIGURE 3. *Botryllus* growth curves for Phase II. The ordinate represents mean number of zooids per colony. Day 0 = 12/3/84. (Except as noted in A, laboratory treatments received alternate day seawater change.) A: Comparison of daily seawater change with alternate day seawater change for colonies fed 2× Liquifry at 20°C, and with the marina treatment. B: Colonies fed 2×, 4×, 8×, or 16× Liquifry at 20°C. C: Colonies fed 4×, 8×, or 16× Liquifry at 24°C. D: Colonies fed 4× Liquifry at 20°C and at 24°C. E: Colonies fed 8× or 16× Liquifry at 20°C and 24 °C.

colony sizes for the 4× Liquifry treatments at these two temperatures were fairly similar throughout Phase II (Fig. 3D), with differences observed only from day 16 to day 30 (Wilcoxon signed rank tests, $P < 0.05$). However, at the higher food levels, more prolonged differences were seen (Fig. 3E). Colonies fed 8× Liquifry grew much faster at 24°C than the 8×,20°C within the first two months of the experiment. Mean colony sizes were significantly different from days 23 to 58 (Wilcoxon signed rank tests, $P < 0.05$). On the other hand, colonies fed 16× Liquifry at 20°C grew much

better than their 16×,24°C cohorts, which began a steady decline in growth after about three weeks in the observation period.

During the second month of this experiment the marina treatment's mean colony size (Fig. 3A) was generally smaller than those of most of the laboratory treatments. However, at about day 58 the marina curve began a sharp climb, similar to that seen during the second growth rise in the 8× and 16× Liquifry treatments at 20°C (Fig. 3B). Nevertheless, on day 86, at the peak of the marina curve, the mean size of its colonies was no different from that of colonies in any of the laboratory treatments (Kruskal-Wallis test: H = 11.1, d.f. = 7, $P > 0.10$). Because the two largest marina colonies died between days 86 and 101, this growth curve started to decline abruptly; survivorship at day 101 was about 70%.

Statistical analyses of Phase II growth data—maximum size. Two variables related to growth were examined by Friedman's tests and Wilcoxon-Wilcox multiple comparisons: (1) maximum size reached during the observation period; and (2) time elapsed to reach 50% maximum size in the experiment. Significant differences were observed among the treatments with respect to maximum size attained by individual colonies during the experiment. The Friedman's test result and the treatment rankings are shown in Table II. The order of treatments resulting from this statistical test is based on rank sums, and, due to variability among colonies, does not necessarily correspond to the numerical order determined by arithmetic means of the treatments. Some revised means were calculated which exclude anomalously large animals, and these means seem more appropriate for the ranks given (see footnote "c" in Table II). In practical terms, the results suggest that 16× Liquifry at 20°C is the best treatment for obtaining large colonies, whereas the 2×,20°C (daily) and the 16×,24°C are significantly worse.

Statistical analyses—growth rates. Growth rates were determined by recording days to reach 50% of each individual colony's maximum size in the experiment. Due to the poor growth of colonies in the 16×,24°C treatment, they were excluded from this analysis. Significant differences were observed among treatments for this parameter. The Friedman's test result and the treatment rankings are shown in Table III. Although the 16×,20°C colonies attained the largest maximum size during Phase II (Table II), this treatment was in the lowest range for days to reach 50% maximum size. On the

TABLE II

Results of Friedman's test and Wilcoxon-Wilcox multiple comparisons for maximum size (# zooids/colony) in Phase II

Friedman's test: Chi-square = 27.9 d.f. = 8 $P = 0.0005$

Treatment[a]	Mean ± S.E.	Group[b]
16×,20°C	262.4 ± 39.1	A
4×,24°C	147.8 ± 22.8	A B
8×,24°C	214.6 ± 76.3[c]	A B
Marina	299.6 ± 111.3[c]	A B C
4×,20°C	134.5 ± 28.2	A B C
8×,20°C	197.6 ± 73.9[c]	A B C
2×,20°C (alternate)	82.4 ± 19.5	A B C
2×,20°C (daily)	99.4 ± 28.8	B C
16×,24°C	47.6 ± 9.7	C

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16× for the laboratory treatments. See Materials and Methods for details.

[b] Treatment means which do not share a group letter are significantly different ($P < 0.05$).

[c] Mean ± S.E. when the few anomalously large animals were excluded (see Appendix) are as follows: 8×,24°C = 109.1 ± 19.6 (n = 12); Marina = 161.8 ± 93.5 (n = 12); 8×,20°C = 69.2 ± 11.4 (n = 10).

TABLE III

Results of Friedman's test and Wilcoxon-Wilcox multiple comparisons for days to reach 50% maximum size (# zooids/colony) in Phase II

Friedman's test: Chi-square = 72.4 d.f. = 7 $P = 0.0000$

Treatment[a]	Mean ± S.E.	Group[b]
4×,24°C	34.0 ± 5.0	A
8×,24°C	35.5 ± 2.3	A B
2×,20°C (alternate)	39.2 ± 2.6	A B C
4×,20°C	42.4 ± 4.3	A B C
2×,20°C (daily)	56.1 ± 1.9	B C D
16×,20°C	57.5 ± 2.5	C D
8×,20°C	67.7 ± 1.8	D
Marina	71.4 ± 8.7	D

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16× for the laboratory treatments. See Materials and Methods for details. The 16×,24°C treatment was excluded from this analysis because its colonies grew poorly and this treatment was terminated early.

[b] Treatment means which do not share a group letter are significantly different ($P < 0.05$).

other hand, the 4× and 8× treatments at 24°C yielded large colonies in a relatively short period of time (Tables II, III). When the daily *versus* alternate day seawater change treatments (2×,20°C) were compared in a separate Wilcoxon signed rank test, it was seen that colonies in the 2×,20°C (alternate) treatment reached 50% maximum size significantly faster than those in the 2×,20°C (daily) treatment ($Z = 3.2$, $P < 0.01$). Based on the rankings in Table III, the marina group grew more slowly than any of the laboratory treatment groups.

Since the colonies used in Phase II had a wide range of ages at the start of the experiment, additional Friedman's and multiple comparison tests were run separately for young and old colonies (age at day 0 = 18–32 days, n = 5 young colonies; age at day 0 = 46–58 days, n = 9 old colonies). With respect to maximum size attained by colonies during the experiment, the greatest ranking difference between young and old colonies occurred with the marina treatment, which was ranked as second for old colonies whereas it was ranked as seventh for young colonies. However, based on the distribution of the statistical ranges, we do not consider this difference to be significant. Statistical tests on data for days to reach 50% maximum size gave similar treatment rankings for young and old colonies.

Asexual generation time, which is reported to be influenced by temperature (Grosberg, 1982), was measured for three treatments in Phase II. Asexual generation time is the time interval which begins when a given zooid generation starts to function as filter-feeding adults and ends when this generation of zooids is completely resorbed and replaced by the new generation which arose by budding. The mean asexual generation times for the 4× Liquifry treatments were 5.5 days (standard error = 0.2 days, n = 8) at 20°C, and 4.1 days (standard error = 0.1 days, n = 14) at 24°C. For colonies grown in the marina, mean asexual generation time was 13.0 days (standard error = 1.0 days, n = 3).

Phase II: Colonies achieve sexual maturity under laboratory culture conditions faster than in the marina

Not only do certain culture conditions provide superior growth rates and sizes of *Botryllus* colonies, but a few of these treatments, notably 4× and 8× Liquifry at 24°C,

allow the very early attainment of sexual maturity. In fact, all but one (16×,24°C) of the eight laboratory treatments in Phase II gave rise to a large proportion of sexually mature colonies during the observation period, with colonies grown at 24°C generally reaching sexual maturity earlier than those kept at 20°C. In contrast to the eventual attainment of sexual maturity by some marina colonies in Phase I (Fig. 2), the marina colonies did not produce eggs during Phase II.

The time course of appearance of eggs and/or embryos in colonies during Phase II is shown in Figure 4. In the 2×,20°C treatments, the initiation of sexual maturity in colonies receiving alternate day seawater changes preceded the production of eggs in daily seawater change colonies by two weeks; the percentage of sexually mature colonies in the 2×,20°C (daily) treatment remained lower than that of the 2×,20°C (alternate) treatment throughout Phase II (Fig. 4A). When comparing the four treatments at 20°C (Fig. 4B), one sees that colonies in the higher food levels (8× and 16× Liquifry) tended to lag behind colonies at the 2× (alternate) and 4× Liquifry levels with respect to onset of sexual maturity. The time courses for egg production in the 24°C treatments which bore sexually mature colonies (4× and 8× Liquifry) were fairly similar to each other, although initially the 8× food level did lag behind the 4× food level (Fig. 4C).

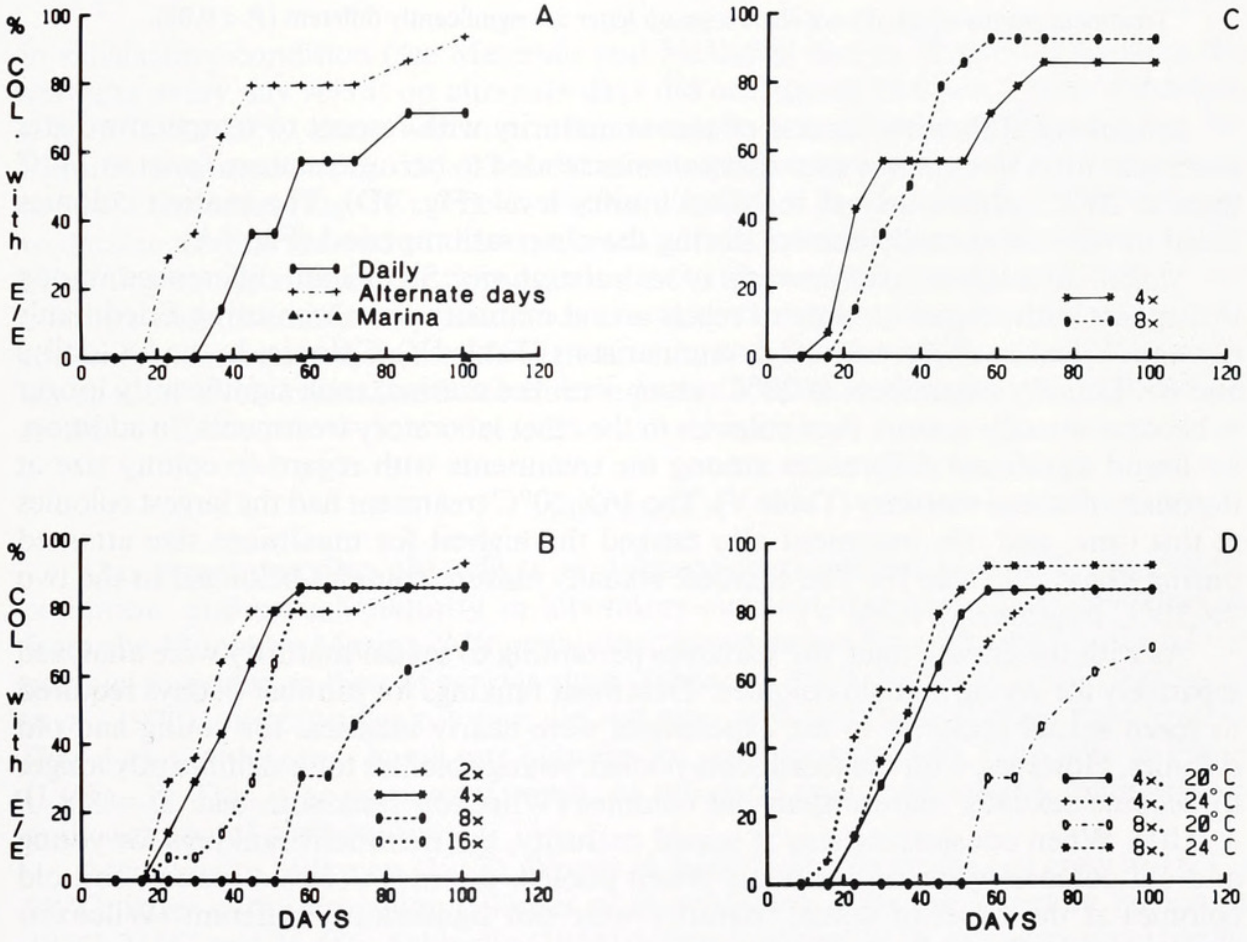

FIGURE 4. Sexual maturity of *Botryllus* colonies in Phase II. The ordinate represents cumulative percentage of colonies with eggs and/or embryos. Day 0 = 12/3/84. (Except as noted in A, laboratory treatments received alternate day seawater change.) A: Comparison of daily seawater change with alternate day seawater change for colonies fed 2× Liquifry at 20°C, and with the marina treatment. B: Colonies fed 2×, 4×, 8×, or 16× Liquifry at 20°C. C: Colonies fed 4× or 8× Liquifry at 24°C. (The 16×, 24°C treatment was terminated on day 58 due to poor colony condition; none of its colonies were sexually mature by this date.) D: Colonies fed 4× or 8× Liquifry at 20°C and 24°C.

TABLE IV

Results of Friedman's test and Wilcoxon-Wilcox multiple comparisons for days to reach sexual maturity in Phase II

Friedman's test: Chi-square = 57.4 d.f. = 7 $P = 0.0000$

Treatment[a]	Mean ± S.E.[b]	Group[c]
8×,24°C	37.5 ± 2.9	A
2×,20°C (alternate)	43.0 ± 6.3	A
4×,24°C	37.0 ± 5.8	A B
4×,20°C	43.4 ± 4.9	A B
16×,20°C	48.8 ± 3.0	A B C
2×,20°C (daily)	54.7 ± 5.9	B C D
8×,20°C	70.7 ± 4.9	C D
Marina	>101	D

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16× for the laboratory treatments. See Materials and Methods for details. The 16×,24°C treatment was not included because it was terminated on day 58 and none of its colonies had become sexually mature by this date.

[b] For the Friedman's test, colonies which survived but did not become sexually mature by the end of the experiment were designated as occupying the highest rank. However, these means do not include such colonies.

[c] Treatment means which do not share a group letter are significantly different ($P < 0.05$).

In looking at the time course of sexual maturity with respect to temperature at a particular food level, it was seen that colonies tended to become mature faster at 24°C than at 20°C, particularly at the 8× Liquifry level (Fig. 4D). The marina colonies failed to become sexually mature during the observation period (Fig. 4A).

Statistical analyses of attainment of sexual maturity. Significant differences among treatments with respect to time to reach sexual maturity were seen using Friedman's test and Wilcoxon-Wilcox multiple comparisons (Table IV). Colonies in the 2× (daily) and 8× Liquifry treatments at 20°C, along with the marina, took significantly longer to become sexually mature than colonies in the other laboratory treatments. In addition, we found significant differences among the treatments with regard to colony size at the onset of sexual maturity (Table V). The 16×,20°C treatment had the largest colonies at this time, and this treatment also ranked the highest for maximum size attained during Phase II (Table II). The smallest sexually mature colonies belonged to the two 2×,20°C treatments (Table V).

As with the growth data, the variables pertaining to sexual maturity were analyzed separately for young and old colonies. Treatment rankings for number of days required to reach sexual maturity in the experiment were nearly identical for young and old colonies. However, with the treatments pooled, young colonies took significantly longer to become sexually mature than old colonies (Wilcoxon rank sum test: $Z = 3.8$, $P < 0.01$). When considering size at sexual maturity, the treatment rankings for young and old colonies were very similar. When pooled, the mean sizes of young and old colonies at the onset of sexual maturity were not significantly different (Wilcoxon rank sum test: $Z = 0.9$, $P > 0.10$).

Phase II: Physical condition of colonies

Along with colony growth and attainment of sexual maturity, it was apparent that the physical condition of the colonies was an important character to consider. Figure 5 depicts the percentage of the initial number of colonies per treatment that were

TABLE V

Results of Kruskal-Wallis test and Wilcoxon rank sum tests for size (# zooids/colony) at sexual maturity in Phase II

Kruskal-Wallis test: H = 25.0 d.f. = 6 $P = 0.0003$

Treatment[a]	Mean ± S.E.	n[b]	Group[c]
16×,20°C	149.5 ± 21.5	11	A
8×,24°C	109.2 ± 25.2	13	A B
4×,24°C	89.8 ± 13.0	12	A B
8×,20°C	118.9 ± 42.2	9	A B
4×,20°C	78.3 ± 15.5	12	B
2×,20°C (daily)	44.7 ± 10.6	10	C
2×,20°C (alternate)	41.8 ± 10.1	13	C

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16× for the laboratory treatments. See Materials and Methods for details. There were no sexually mature colonies in the 16×,24°C or marina treatments, and thus these treatments were excluded.

[b] Decrease in n from an original total of 14 is due to mortality before attaining sexual maturity or failure to reach sexual maturity by the end of the experiment.

[c] Treatment means which do not share a group letter are significantly different ($P < 0.05$).

in satisfactory condition (see Materials and Methods) during Phase II. Changing the seawater every day *versus* on alternate days did not appear to affect colony condition (Fig. 5A). At 20°C, there was intermittent variation in colony condition among the Liquifry treatments (Fig. 5B). There was even greater variation in condition among the 24°C colonies (Fig. 5C). The condition of colonies in the marina (Fig. 5A) began to decline toward the end of the experiment. If the laboratory treatments are ranked according to the number of time points (n = 13) at which greater than 80% of the colonies were in satisfactory condition, one finds that for a given food level the 20°C treatment was better than the 24°C treatment, and that for a given temperature the lower food levels maintained colonies in better condition than did the higher food levels.

DISCUSSION

This paper describes the effects of different temperatures and diets on growth, condition, and sexual maturity in laboratory-cultured *Botryllus schlosseri* colonies from the Monterey Marina. Although other investigators have reported maintaining colonial tunicates on diets of various algae (Sabbadin, 1960; Milkman, 1967; Nakauchi *et al.*, 1979), our *Botryllus* colonies did not grow well with similar algae food sources (Fig. 1A). On the other hand, our Liquifry-fed colonies grew well, especially in Phase II (Fig. 3). This is an important result, as this food source eliminates maintenance and counting of algal cultures.

According to Milkman (1967), Woods Hole *Botryllus schlosseri* can grow at 18°C–28°C. After culturing young colonies of *B. schlosseri* from the Venetian lagoon at 18°C, 21°C, and 26.5°C, Sabbadin (1955) determined that 21°C gave very good results for asexual reproduction. In Japan, Nakauchi *et al.* (1979) found that 25°C allowed for the best growth of inland cultures of the colonial tunicate *Symplegma reptans,* but they had problems with bacterial contamination at that temperature, and thus 20°C became their temperature of choice. Since the normal annual water temperature range in Monterey Marina is about 12°C–17°C, we decided to set up initial comparisons of growth of our colonies at 15°C and 20°C, and found that the Liquifry-fed Monterey

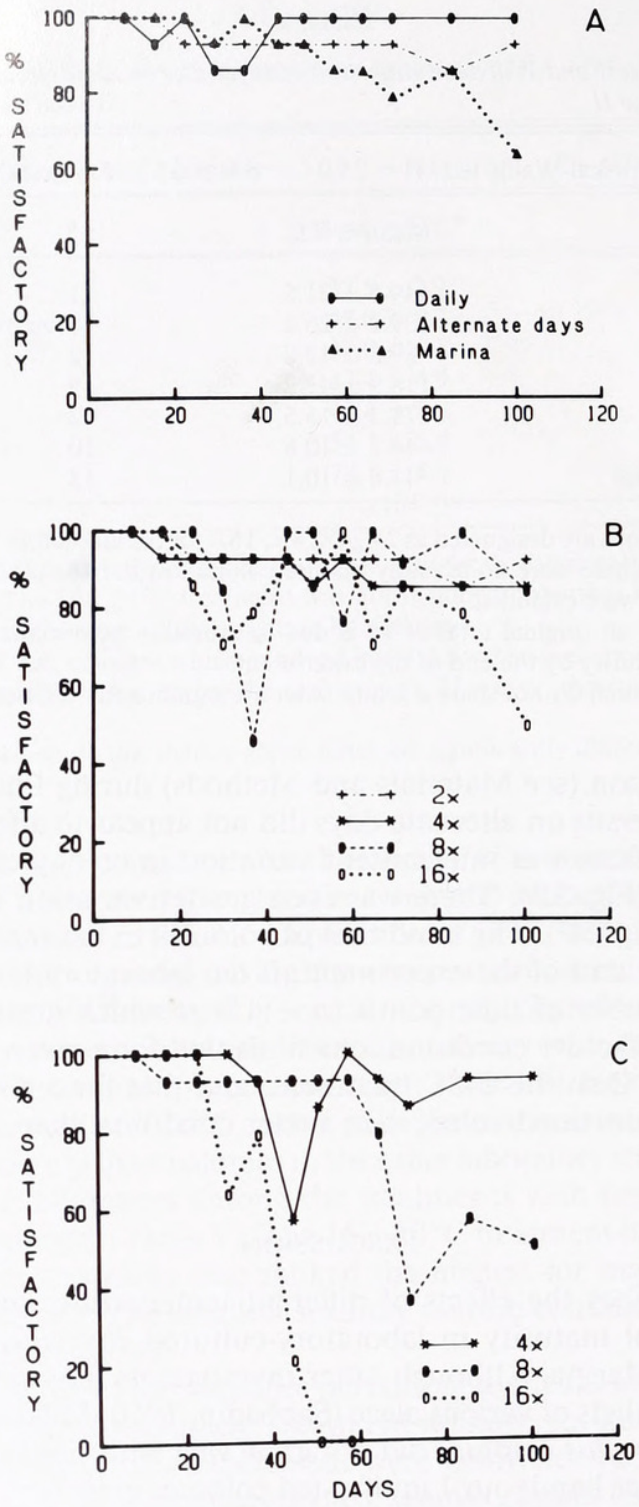

FIGURE 5. Percentage of *Botryllus* colonies in satisfactory condition during Phase II. Day 0 = 12/3/84. (Except as noted in A, laboratory treatments received alternate day seawater change.) A: Comparison of daily seawater change with alternate day seawater change for colonies fed 2× Liquifry at 20°C, and with the marina treatment. B: Colonies fed 2×, 4×, 8×, or 16× Liquifry at 20°C. C: Colonies fed 4×, 8×, or 16× Liquifry at 24°C.

Botryllus grew most rapidly at 20°C (Fig. 1B). Likewise, in Phase II the colonies grown at 24°C grew faster than those at 20°C (Figs. 3D, E; Table III), with the exception of the 16×,24°C treatment. This combination of high food and temperature levels allowed for the growth of many contaminating organisms in this treatment tank, which apparently contributed to the eventual demise of the *Botryllus* colonies.

Temperature is a major factor which influences sexual reproduction in marine invertebrates (Millar, 1971). Thus it was not surprising that the Liquifry-fed colonies at 20°C became sexually mature before those at 15°C in Phase I. The reduction in growth rate of colonies in the 20°C Liquifry treatments beginning around day 57 (Fig. 1B) coincides with the onset of egg production. It is possible that this is due to a shift in the partitioning of resources between asexual and sexual reproductive processes. Reports of this phenomenon occurring in colonies reared in their natural habitat have been made for *Botryllus* (Berrill, 1935; Mukai and Watanabe, 1976; Grosberg, 1982) and for *Tridemnum solidum* (Bak *et al.*, 1981). In Phase II, this decrease in growth rate at the onset of sexual maturity apparently also occurred in all but the 16×,20°C laboratory treatment (Figs. 3B, C; Table IV), which did not exhibit a reduced growth rate until about two weeks after sexual maturation began. In fact, the onset of sexual maturity in colonies in the 16×,20°C treatment occurs at the beginning of the second growth rise in that treatment. Thus it seems possible to provide laboratory-maintained *Botryllus* with sufficient food to develop into sexually mature colonies while continuing to increase in size via asexual reproduction.

Two interesting observations were made when comparing data from the daily *versus* alternate day seawater change treatments (2×,20°C) in Phase II. First, although their maximum sizes attained during Phase II were similar (Table II), colonies in the 2×,20°C (alternate) treatment reached 50% maximum size significantly more rapidly (39.2 ± 2.6 days, Table III) than those in the 2×,20°C (daily) treatment (56.1 ± 1.9 days, Table III) (Wilcoxon signed rank test: $P < 0.01$, see Results section). Second, although their colony sizes at onset of sexual maturity were similar (Table V), the colonies in the 2×,20°C (alternate) treatment became sexually mature earlier (43.0 ± 6.3 days, Table IV) than those in the 2×,20°C (daily) treatment (54.7 ± 5.9 days, Table IV; also see Fig. 4A) These observations have led us to speculate that growth factors and hormones which influence sexual maturation may have accumulated in the tank which received less frequent seawater exchange, thereby enhancing the rates of growth and attainment of sexual maturity of the colonies.

Upon completion of the Phase II culture experiments, the high degree of variability among Monterey *Botryllus* colonies with respect to growth rate and maximum size was evident (see Appendix; Table II). This phenomenon is of practical significance to investigators. The observed variation could be the result of different life history types, as described for *Botryllus schlosseri* in Woods Hole (Grosberg, 1982). In order to investigate the genetic contribution to such differences, one could set up matings between rapidly growing large colonies and slowly growing smaller colonies (three possible mating combinations) and study growth patterns of the offspring.

Our culture research provides ample evidence that laboratory conditions are more favorable than Monterey Marina for maintaining healthy sexually mature *Botryllus* colonies *year-round.* In addition to the results presented here, it was clear that laboratory-raised colonies were much cleaner than those grown in the field, where *Botryllus* must compete with other animals and plants for space. Clean colonies allow for more efficient experimental manipulations with respect to colony transfer and studies of fusion/rejection reactions. Furthermore, the marina environment is unpredictable; for example, the appearance within the last three years of persistent anomalously warm water currents originating off the coast of South America, called El Niño, has had significant biological consequences (Barber and Chavez, 1983), including causing changes in California coastal ecosystems (Dayton and Tegner, 1984). The fact that the marina environment is uncontrolled may have contributed to the sudden, sporadic death of our *Botryllus* colonies. The marina colonies had lower survivorships than the laboratory-grown colonies in both Phases I and II. When Phase II was terminated,

TABLE VI

Laboratory treatment conditions appropriate for some specified uses of Monterey Botryllus *colonies*

Purpose	Treatment conditions[a,b]
(1) Rapidly growing, large colonies for multiple fusion/rejection assays, or to provide material for protein and nucleic acid analysis.	(1) 4×,24°C 8×,24°C 4×,20°C
(2) Colonies with early onset of sexual maturity to develop inbred strains.	(2) 8×,24°C 2×,20°C 4×,24°C
(3) Colonies of sufficient size with early onset of sexual maturity to produce many offspring from defined matings.	(3) 8×,24°C 4×,24°C 16×,20°C
(4) Rapidly growing colonies with early onset of sexual maturity to produce several generations of defined stocks.	(4) 8×,24°C 4×,24°C 2×,20°C
(5) To hold colonies in slow growth until future use.	(5) 2×,15°C

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16×, for the laboratory treatments. See Materials and Methods for details.

[b] The top three treatments are listed in order of preference for each purpose. However, one should remember that colony condition remains good for longer periods of time at the lower food and temperature levels.

the remaining laboratory treatment colonies were transferred to the 4× Liquifry, 20°C condition. Six weeks later, survivorship of these colonies was 85%, whereas only approximately 30% of the marina colonies were still alive. Another advantage of laboratory-reared colonies is that since sexually mature colonies produce new eggs during each asexual cycle, we can obtain two or three times as many sets of progeny from laboratory colonies as compared with matings between marina-maintained colonies in a given time interval, due to the shorter asexual generation times at 20°C and 24°C. This is a significant advantage for the production of genetically defined inbred strains.

We now know how to construct laboratory conditions to obtain *Botryllus* colonies with various life-cycle characteristics to satisfy different experimental needs, as outlined in Table VI. Because it is likely that the high degree of variation among colonies with respect to growth is genetically determined, we will interbreed the healthy rapidly growing colonies as soon as they become sexually mature in order to accomplish our goal of establishing long-term defined genetic stocks in the laboratory. These stocks will be invaluable tools for *Botryllus* research in areas such as immunogenetics, developmental biology, and ecology.

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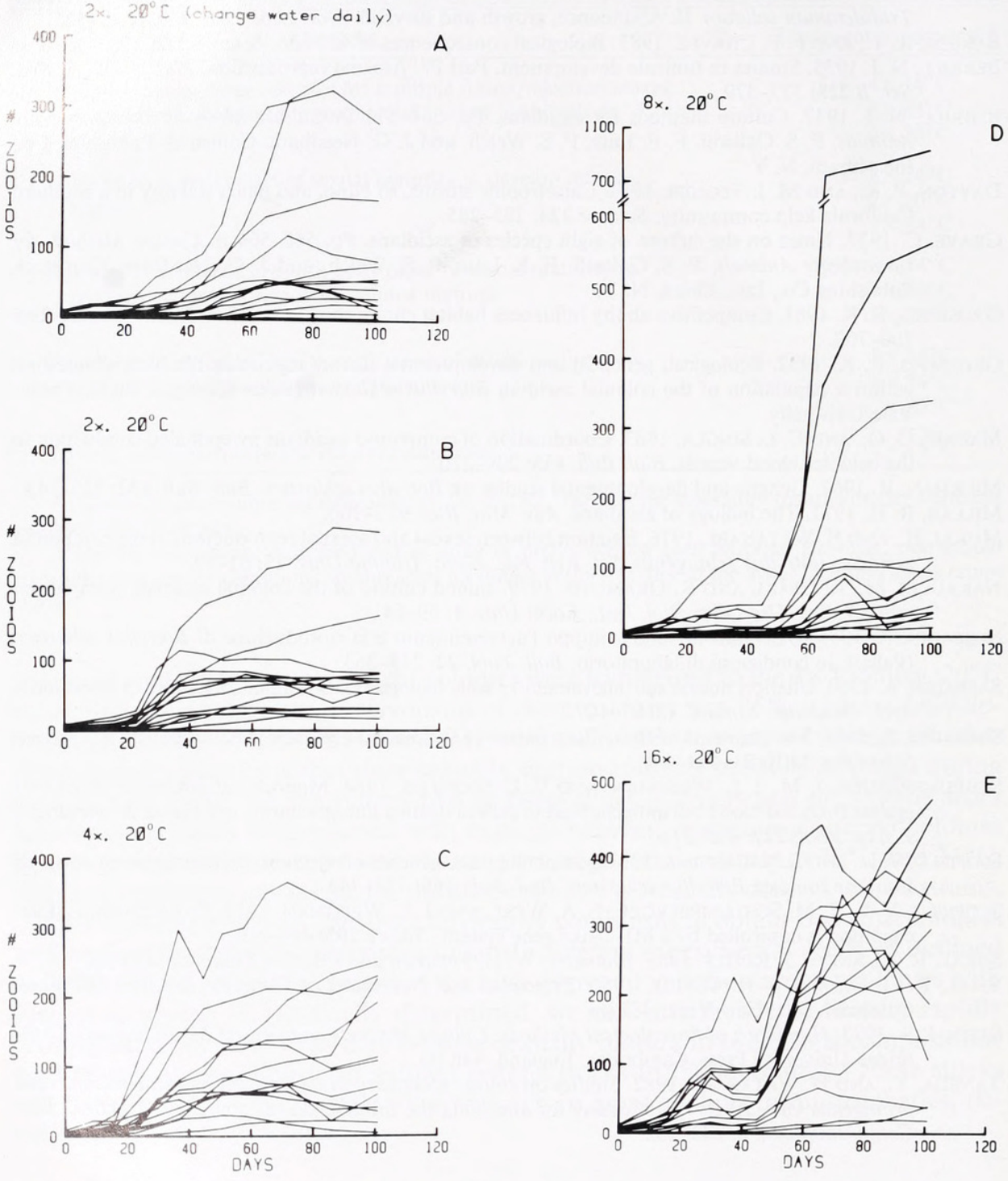
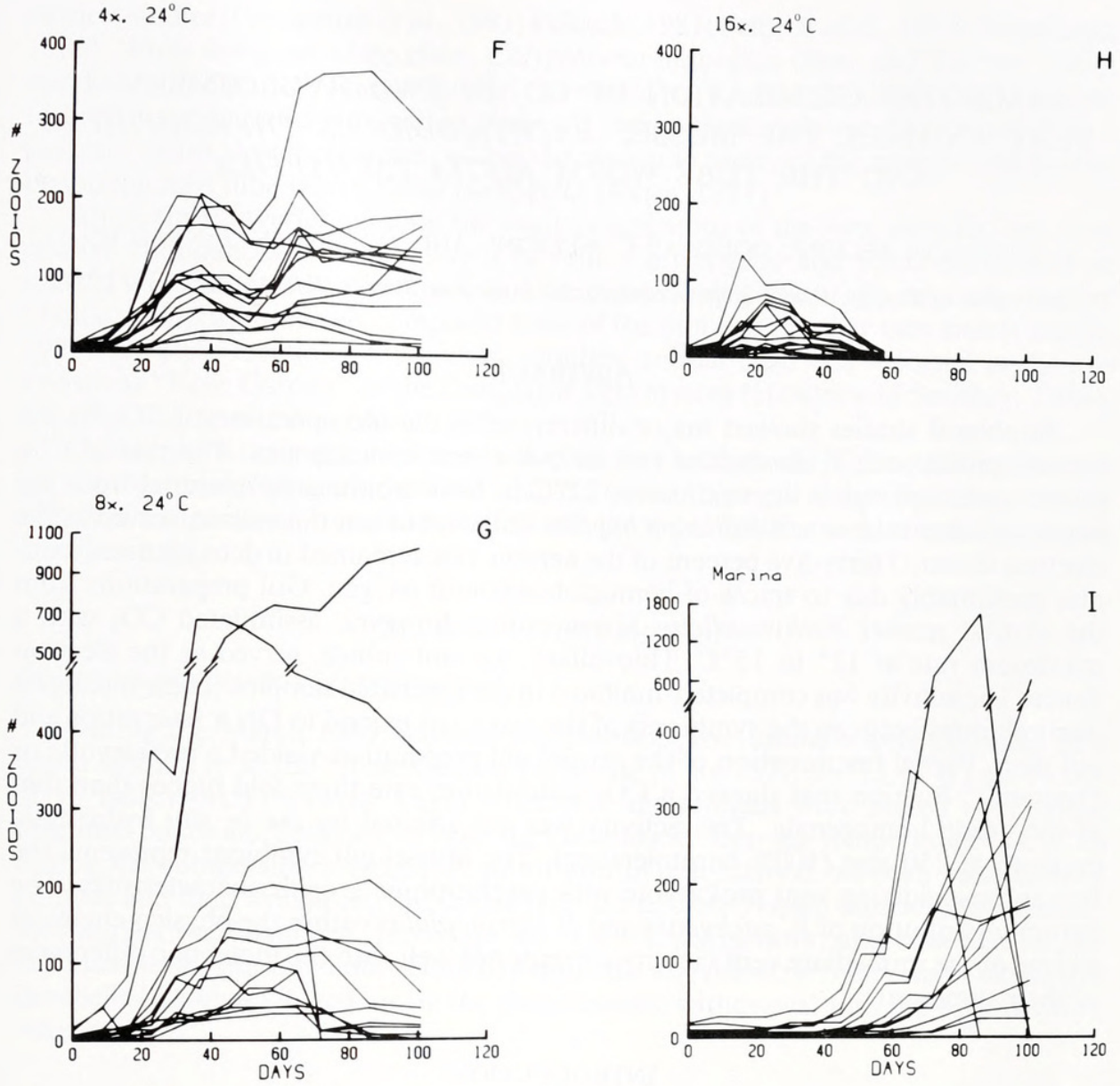
APPENDIX



Growth curves of individual *Botryllus* colonies for the nine treatments in Phase II. Day 0 = 12/3/84. Liquifry concentration is designated as 2×, 4×, 8×, or 16×. (Except as noted in A, all laboratory treatments received alternate day seawater change.)



APPENDIX (*Continued*)