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Factors Controlling the Length of Autogamy-Immaturity in *Paramecium tetraurelia*

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ABSTRACT—Autogamy-immaturity is the period during which autogamy can not be induced by natural starvation; in *Paramecium tetraurelia*, autogamy first becomes induceable at about 7 fissions after the previous autogamy, and thereafter the percent of cells undergoing autogamy increases gradually to almost 100% at the clonal age of about 20 fissions and remains at 100% thereafter. The length of autogamy-immaturity (LAI), determined by plotting the percent of cells in autogamy versus the number of fissions, was found to be similar in two cultures grown at different fission rates at 25°C and 30°C. This indicates that paramecia count LAI by the number of fissions, not by the calendar time. LAI estimated from the peaks of percent autogamy through successive autogamous generations was also similar in two continuous cultures grown with different cycles of growth and starvation at 25°C and 30°C, indicating stability of LAI under ordinary laboratory conditions. However, LAI was affected by the cultural age of paramecia from which the new autogamous generation was derived: advanced cultural age shortened the LAI in the following generation.

INTRODUCTION

Autogamy is a form of sexual reproduction in a single cell, consisting of meiosis and self-fertilization, and is usually induced by starvation. In *P. tetraurelia* cells, autogamy can not be induced by natural starvation for a certain period after autogamy, the period called "autogamy-immaturity". Berger (1990) showed that autogamy can be induced even in very young cells by intensifying the starvation level, indicative of the absence of autogamy-immaturity in a strict sense. He showed that the threshold level of starvation decreases with clonal age, so that autogamy becomes induceable by natural starvation after a certain number of cell divisions.

Under ordinary laboratory conditions where autogamy is induced by natural starvation, the period during which autogamy can not be induced persists for about 7 fissions, and is followed by a transitory period lasting for 10-13 fissions, and then followed by autogamy maturity, which has been referred to as "senescence" (Sonneborn, 1957, 1974), at the age of about 20 fissions and thereafter. The length of autogamy-immaturity (LAI) can therefore be defined in several ways: in the above case, for example, the shortest LAI is 7 fissions, indicating the period during which no cells respond to natural starvation by autogamy; the longest LAI is 20 fissions, indicating the period when the cells come to respond to natural starvation by autogamy at constant and high levels; and the average LAI is an intermediate number of fissions, when 50% of the cells are in autogamy. The profile plotting the percent of cells in autogamy versus the number of fissions, the LAI profile, can be used to determine any of the above defined LAIs and is useful to compare the LAIs of cells in different culture conditions.

The autogamy-immaturity is distinguished from sexual immaturity which is the period during which conjugation does not occur. They are of interest because they may both be related to the length of the clonal life span. If this were the case, time-consuming and laborious studies to determine the clonal life span might be very much reduced. The following examples show that LAI or the length of sexual immaturity and the clonal life span in *Paramecium* are somehow related to each other, although not by a causal relationship.

In the *jumyo* mutant of *P. tetraurelia*, which has a short clonal life span, the LAI profile is shifted to younger clonal ages (Takagi *et al*., 1987b; Maruyama and Takagi, unpublished data). A rough correlation between the length of sexual immaturity and life span has been suggested in ciliates (Smith-Sonneborn, 1981). Parental clonal age has an effect on the length of progeny life span (Smith-Sonneborn *et al*., 1974). It also has an effect on the length of sexual immaturity (Siegel, 1961) and of the autogamy-immaturity (Iizima *et al*., 1997) in the next generation.

Also of interest is the relationship between LAI and the cultural aging, i.e., the process of physiological changes in division-arrested cells under starvation. Aging in *Paramecium* has two aspects: clonal aging and cultural aging (Smith-Sonneborn, 1985). The former is the process in dividing cells that is related to the limit of the number of cell divisions, and the latter is the process in division-arrested cells that is related to the limit of time of cell survival. The relationship be-

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tween these two processes has so far not been studied, although cultural aging itself has been studied separately (Fok and Allen, 1981; Fok *et al.*, 1981; Maruyama and Takagi, 1997; Yamamoto *et al.*, 1997).

In this study, we first examined the effect of starvation level on LAI under laboratory conditions and the relationship between LAI and the number of fissions. We then studied the effect of parental cultural age on the LAI of the next generation.

MATERIALS AND METHODS

Cells

Paramecium tetraurelia, stock 51 (mating type VII), was used.

Culture medium

Cells were cultured in 0.5% phosphate-buffered Wheat Grass Powder (Pines Int., USA) medium that had been inoculated with *Klebsiella pneumoniae* 2 days before use. Culture vessels for mass cultures were flasks (HARIO) capped with a Silicon Sponge Closure (Sigma) and those for isolation cultures were 3-well depression slides housed in moist chambers. The volume of the culture was 40% or less of the flask volume. Cultures were handled on a clean bench and incubation was at 25 ± 1°C unless otherwise stated. Single cells were manipulated with a micropipette under a dissecting microscope.

Culture

Single cells were isolated into 800 μl of culture medium and allowed to proliferate until food bacteria were exhausted. When the cells had become starved, more than 100 cells were sampled from each of the cultures and stained with Dippell's stain (Dippell, 1955) to search for cultures in which all of the sampled cells had macronuclear fragments, which is indicative of autogamy. In the 100% autogamous culture, the cells are reset to time zero of the clonal age, and they are genetically identical because inbreeding *P. tetraurelia* cells are homozygous for all of the genetic loci, and thus the loci remain unchanged after autogamy except for some heterozygous loci produced by mutation (Sonneborn, 1957, 1974).

In Exp. 1, 25 μl of a 100% autogamous culture was added to 775 μl of culture medium, and the 800 μl culture was incubated for 2 days. Thus the diluted cells could undergo 5 fissions on average, before reaching a maximum concentration of about 4,000 cells in 800 μl of culture medium (5 cells/μl). On the second day, 25 μl of the culture was again transferred to 775 μl of culture medium; this procedure was repeated every 2 days to maintain continuous cultures for 18 days (9 transfers). Twenty continuous cultures were run in parallel, with 10 cultures at 25°C and 10 at 30°C. Average fission rates of *P. tetraurelia* cells measured in daily reisolation culture (see Exp. 3) in a separate experiment were 4.0 and 5.1 fissions per day at 25°C and at 30°C, respectively. After every transfer, the remaining cells were monitored to see if they were in autogamy. In this growth-starvation cycle, in which the number of fissions is limited to 5, the cultures grown at 30°C for 2 days would have a shorter growth period and a longer starvation period than those grown at 25°C for 2 days.

In Exp. 2, the genetically identical cells of a 100% autogamous culture were allowed to advance their clonal age (i.e., to undergo fissions) at 25°C and at 30°C. Cultures of a definite clonal age were monitored for percent autogamy after 1 and 2 days of natural starvation. The higher value of percent autogamy was adopted. Cultures of a definite clonal age were obtained by adjusting the initial concentration of cells in the 100% autogamous culture so that the cells would undergo the desired number of fissions. For example, the 100% autogamous culture was diluted 1:8 into culture medium in order to allow 3 fissions, or, a single cell was placed in 200 μl of culture medium to allow 10 fissions (2^{10} = 1024), or, 8 cells were placed in 100 μl of culture medium to allow 6 fissions (8×2^6 = 512), and so on. Daily reisolation culture was combined with the above methods to achieve more than 10 fissions. Different methods were applied to produce cultures of a given clonal age and their percent autogamy was examined. Similar experiments were conducted in parallel at 25°C and 30°C.

In Exp. 3, a 100% autogamous culture was allowed to advance its cultural age and cells of different cultural ages were used to start a new clonal life cycle in daily reisolation cultures: 9 cells from each of the cultural age groups were placed separately in 100 μl of culture medium, one of the fission products (N) was transferred to fresh culture medium daily, and the remaining culture was allowed to grow to the maximal cell density (about 500 cells/100 μl: approximately 9 fissions' product of a single cell) to monitor percent autogamy. The clonal age was calculated from the daily number of fissions given by: $\log_2 N$ plus 9 fissions.

RESULTS

Exp. 1: LAI in continuous cultures with different cycles of growth and starvation at different temperatures

The LAI profiles in the continuous cultures at 25°C and 30°C are shown in Fig. 1. The average profile is shown at the bottom of each column. Starting with a 100% autogamous culture, no or almost no autogamy was observed in the 25°C and 30°C cultures on the first and second transfers, corresponding to 5 and 10 fissions, respectively. The first peak was on the fourth transfer at 25°C and on the third or fourth transfer at 30°C, indicating a slightly shorter LAI in the 30°C culture than in the 25°C culture. However, in both the 25°C and 30°C cultures, the second peak was on the seventh transfer, although the height of the peak of the 30°C culture was low, reflecting the first blunt peak. In the second autogamous generation, therefore, the difference of LAI in the two groups practically disappeared. This shows that a difference of starvation of this extent has little effect on the LAI.

Two sharp peaks in the 25°C culture may indicate that the LAI in the second generation becomes shorter than in the first generation. However, the mode of inheritance of LAI through autogamous generations remains to be studied.

Exp. 2: LAI in cultures grown with different fission rates at different temperatures

Starting with a 100% autogamous culture, the LAI profiles were compared in two cultures incubated at 25°C and 30°C. Temperature (25°C versus 30°C) had little effect on LAI measured in the number of fissions, although a given number of fissions was attained in fewer days at higher temperature, autogamy being induced first at 6 ~ 7 fissions and maximally at 19 ~ 20 fissions (Fig. 2). This result indicates that *Paramecium* measures LAI by some mechanism associated with the number of fissions rather than the calendar time.

Exp. 3: LAI in progeny derived from parental cells of different cultural ages

The cells in a 100% autogamous culture, if kept unfed, undergo cytological changes resulting in death with the advance of calendar time. The change in division-arrested cells under starvation is called cultural aging. When a culture shifted

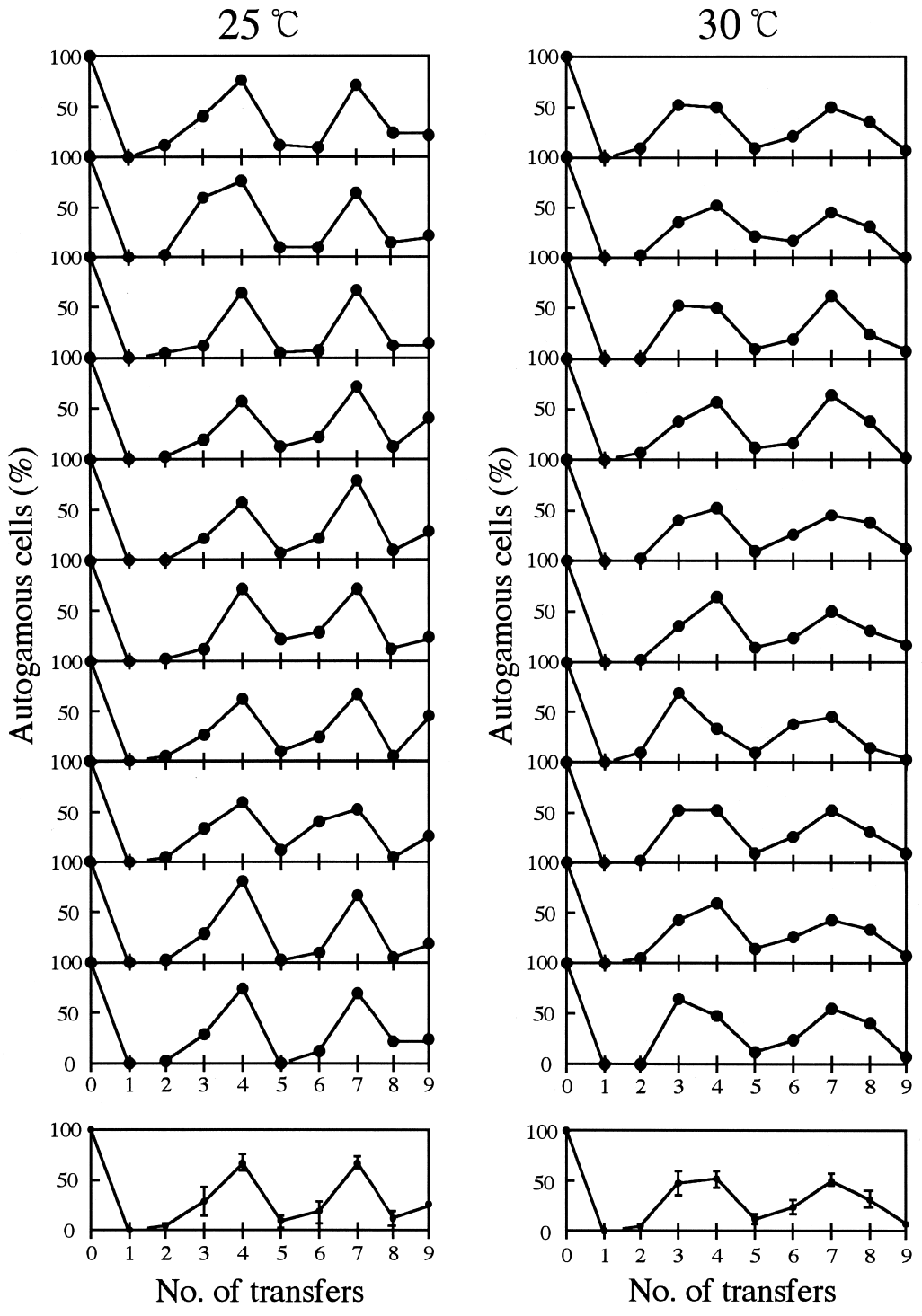

Fig. 1. LAI profiles in continuous cultures with different cycles of growth and starvation at different temperatures. Twenty-five μl of a 100% autogamous culture was added to 775 μl of culture medium, and the 800 μl culture was incubated for 2 days, during which the cells were expected to divide 5 times on average, and then to starve. On the second day, 25 μl of the culture was again transferred to 775 μl of culture medium; this procedure was repeated every 2 days to maintain continuous cultures for 18 days (9 transfers). After every transfer, the remaining cells were monitored for autogamy. Ten continuous cultures were maintained at 25°C and 10 at 30°C. The average profile (± SD) is shown at the bottom of each column. Note that in this growth-starvation cycle in which the number of fissions is limited to 5, the starvation period is longer at 30°C than at 25°C.

from the log to stationary phase, indicated by the time when the culture became transparent as a result of exhaustion of food bacteria, the cultural age was taken as 0. To rule out the possibility that cell divisions might occur even under starvation condition, it was confirmed that all of the sampled cells contained two macronuclear anlagen throughout cultural ag-

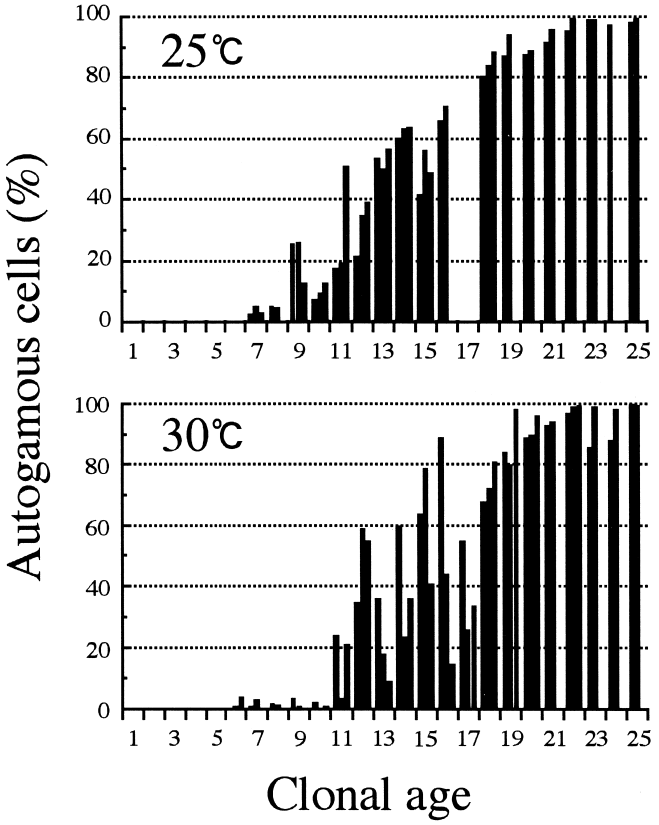

Fig. 2. LAI profiles in cultures grown with different fission rates at different temperatures. Cells from a 100% autogamous culture were allowed to undergo a definite number of fissions and percent autogamy was examined after 1 and 2 days of natural starvation. The higher value was adopted. Similar experiments were done in parallel at 25°C and 30°C. See Materials and Methods for the methods to obtain the cultures of a given clonal age. No cultures of age 17 at 25°C were available.

ing. One hundred percent autogamous cells of different cultural ages were transferred to culture medium to start the progeny generation, and the LAI profiles were compared. The experiments were done three times using different 100% autogamous cultures; the cultural ages compared were 1, 6 and 12 days in the first experiment, 1, 7 and 11 days in the second experiment, and 1, 7 and 10 days in the third experiment.

The results are shown in Fig. 3, in which the values of percent autogamy for 3 successive clonal ages are averaged. In every experiment, LAI tended to become shorter as the cultural age advanced. To make this clear, the data from the three experiments were averaged, and the LAI profiles were compared between two groups, namely the group of cultural age 1 day and that of cultural age 10 ~ 12 days (Fig. 4). The difference of percent autogamy during the clonal ages from 8 to 13 fissions was highly significant between the two groups ($P < 0.01$, Dunn's multiple comparison rank sum test; Dunn, 1964). This is the first direct demonstration of an association between the cultural age and LAI.

DISCUSSION

Studies on sexual immaturity in *P. multimicronucleatum*, *P. caudatum* and *P. bursaria*, in which autogamy does not occur and thus there is no autogamy-immaturity, have shown that 1) its length is measured by some mechanism associated with the number of fissions (Kroll and Barnett, 1968; Miwa and Hiwatashi, 1970; Takagi, 1970), 2) its length is shortened by mytomicin (Miwa and Hiwatashi, 1970) or UV (Takagi, 1974) or codominant gene mutation (Myohara and Hiwatashi, 1978), 3) its length correlates with the length of the clonal life span (Smith-Sonneborn, 1981), 4) the transition from immaturity to maturity is stepwise and genetically controlled (Siegel, 1967; Takagi, 1988), and 5) immaturin, a protein of molecular weight 10,000 daltons, is responsible for maintaining the immature state (Haga and Hiwatashi, 1981; Miwa, 1984).

Studies on the clonal life span in *Paramecium* have shown that 1) its length is measured by some mechanism associated with the number of fissions (Smith-Sonneborn and Reed, 1976; Takagi and Yoshida, 1980; Takagi *et al.*, 1987a), 2) its length is shortened by UV but elongated rather than restored by photoreactivation following UV irradiation (Smith-Sonneborn, 1979), and 3) its length is genetically controlled (Takagi *et al.*, 1987b, 1989).

The present study on the autogamy-immaturity in *P. tetraurelia* has shown that 1) its length is measured by some mechanism associated with the number of fissions, 2) its length is shortened by intensifying the starvation level to some extent, and yet to a negligible extent under ordinary laboratory conditions, and 3) its length is considerably shortened by starting the new generation from a parent of more advanced cultural age. These results are closely related to the earlier studies on the autogamy-immaturity in *P. tetraurelia* showing that 1) its length is measured by some mechanism associated with the number of macronuclear DNA replications (Mikami and Koizumi, 1983), 2) its length is shortened by intensifying the starvation level (Berger, 1990), and 3) its length is shortened by starting the new generation from a parent of more advanced clonal age (Iizima *et al.*, 1997). The first two results from our study show that the LAI profile can be used as a reliable marker for comparative studies such as the isolation of mutants with long or short LAIs under ordinary laboratory conditions. The third result from our study is the first direct demonstration of an association between the cultural age and LAI.

The mechanism by which the cultural age affects the LAI remains unknown, but a possible interpretation may be as follows. During starvation period in *P. tetraurelia* exconjugants, macronuclear fragments are individually and selectively autolysed, and surviving fragments continue to synthesize some DNA (Berger, 1974). If this is also true for *P. tetraurelia* exautogamous cells, the number of DNA replications may be counted as a manifestation of the cultural age, carrying it over to the following generation to result in shortened LAI.

We recently reported that UV sensitivity increased with the advance of cultural age as well as of clonal age (Yamamoto *et al.*, 1997). The present study suggests that there may be

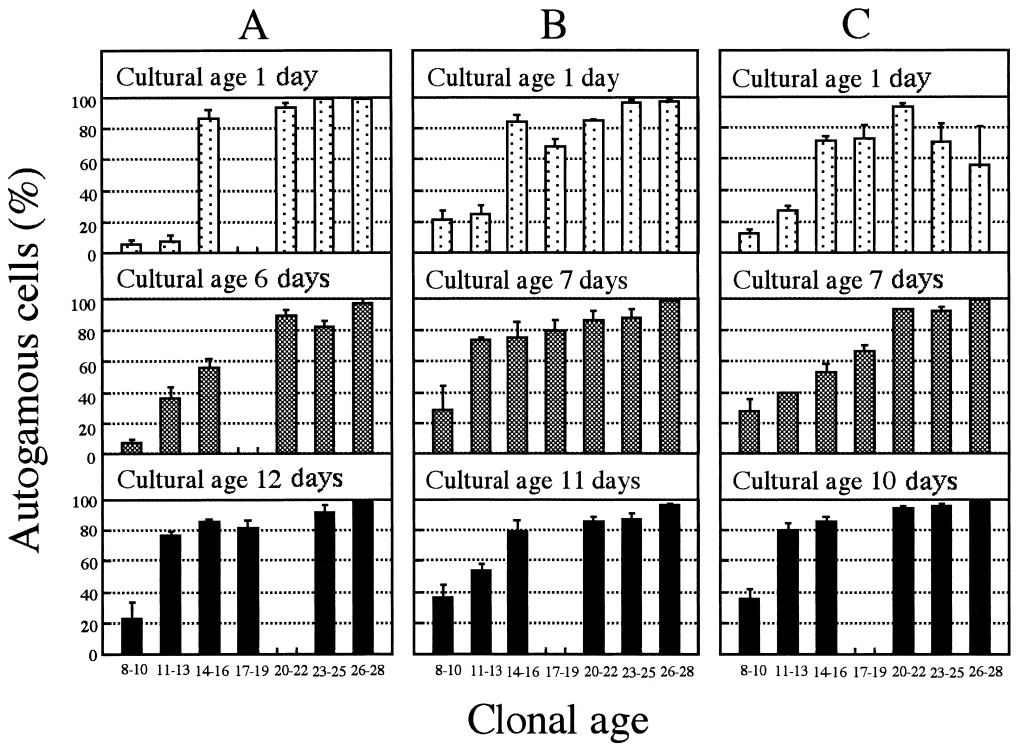

Fig. 3. LAI profiles in progeny derived from parental cells of different cultural ages. A 100% autogamous culture was allowed to advance in cultural age, and resultant cells of different cultural ages were used to start new clonal life cycles. Percent autogamy at a given clonal age was examined 1 and 2 days after starvation, and the higher value was adopted. Three 100% autogamous cultures were used: new clonal life cycles were started from cells of the cultural ages of 1, 6 and 12 days in **A**, those of 1, 7 and 11 days in **B**, and those of 1, 7 and 10 days in **C**. Nine cells were used for each age group, and data were averaged for 3 successive clonal ages. Bar indicates standard error. Blank indicates no data.

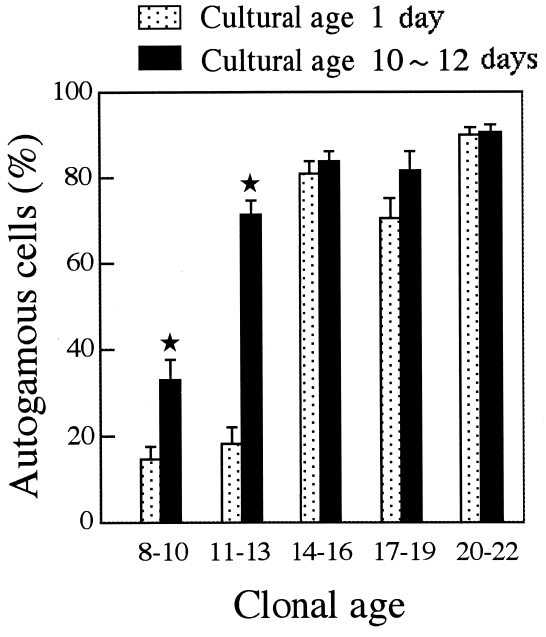

Fig. 4. Comparison of the LAI profiles in progeny derived from parental cells of cultural age 1 day and those of cultural ages 10 ~ 12 days. The data of the three cultures shown in Fig. 3 were averaged, and the two most extreme age groups were depicted. Bar indicates standard error. Star indicates that the difference between the two age groups is significant by Dunn's multiple comparison rank sum test ($P < 0.01$).

some relationships among the length of autogamy-immaturity (LAI), clonal life span and cultural life span.

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