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Some Implications of Ageing in a Recently Isolated "Wild" Ciliate: an Example Using a Marine Planktonic Ciliate

D.J.S. Montagnes

Port Erin Marine Laboratory, University of Liverpool

Abstract

Ciliated protozoa are an ecologically important component of the plankton, and clonal "blooms" of ciliates may transfer a substantial amount of matter through aquatic food webs. To model clonal population dynamics we require estimates of growth and death at various food concentrations (i.e., a numerical response), and this response is typically obtained using laboratory experiments. Ageing of ciliate clonal cultures can occur. Although such ageing is unlikely to have serious consequences under natural conditions, it is an observable event in laboratory cultures, and must be accounted for. Furthermore, recognising ageing and the way in which it is prevented, in some cases, tells us much about the ecology of natural populations. In this work, I have developed a numerical response for the marine planktonic ciliate *Strombidinopsis cheshiri*. During the course of experiments, ageing occurred in some of the clonal, laboratory cultures, and recognising the cause and impact of this phenomenon was essential to developing an accurate data set. This work reviews the processes which cause ageing in laboratory cultures and applies these concepts to help explain variation in the growth response of *S. cheshiri*. I then use some of the observations of ageing and sex of *S. cheshiri* to explain the possible impact on the population dynamics of this ciliate under feast and famine conditions.

Introduction

Ciliated protozoa play a key role in many aquatic food webs (Pierce & Turner 1992). In the plankton, ciliates can form monoclonal "blooms" which at times are so dense they discolour the waters (e.g., Montagnes and Humphrey, in press). Not surprisingly, there is a need to understand the growth and demise of these blooms if we wish to assess the role of ciliates in planktonic food webs. As the direct observation of plankton is difficult at best and impossible at worst, computer simulations are often used to understand population dynamics. But to model clonal population dynamics we require estimates of growth and death at various food concentrations (i.e., a numerical response), and this response is typically obtained using laboratory experiments. Inevitably, laboratory experiments produce some results that differ from those expected to occur in nature; one such result is the ageing of clonal cultures. This paper is an ecologists attempt to place in context the inevitable ageing process that ciliate clones undergo. It is a combination of laboratory research which determined a numerical response (published in detail elsewhere Montagnes 1993, Montagnes et al. 1996) and a review of the key concepts of protozoan ageing (reviewed in detail by Bell 1988, Nanney 1980).
There are few examples of determinations of planktonic ciliate numerical responses, and even these often fail to measure mortality, due to starvation, at low food concentrations (Montagnes 1996). I must emphasise at this point that mortality due to starvation is distinct from mortality due to ageing. Protozoa are often considered to be immortal, but this is a misconception. Death of protozoa may occur, as mentioned above, due to starvation; this is similar to a human starving. Death of a protozoan population, maintained in a closed culture vessel, may also occur due to the accumulation of waste toxins, and this may be similar to ageing caused by the accumulation of toxins within the human body. Finally, ageing caused by the degeneration of genetic and physiological control also affects clonal populations of protozoa, as it affects individual humans. Although, for reasons which will be explained later, such ageing is unlikely to have serious consequences to most protozoan populations under natural conditions, it is an observable event in laboratory cultures, and must be accounted for. Furthermore, recognising ageing and the way in which it is prevented, tells us much about the ecology of natural populations.

To ensure that we are dealing with common terms and concepts, at this point, I will make a brief digression into the ciliate life and cell cycles (Fig. 1). The cell cycle for most ciliates consists of binary fission, accompanied by nuclear division. However, ciliates are unique in possessing two types of nuclei: a macronucleus containing multiple copies of functionally haploid genetic fragments (used for protein synthesis during asexual reproduction) and a micronucleus, composed of a more typical nuclear structure (used for sexual reproduction and maintaining the cell genome). Thus, the ciliate genome is analogous to the human genome, in that the germinal genome is distinct from the somatic genome, and the rate of ageing of the latter may extrinsically but not necessarily intrinsically affect the former.

During ciliate cell division, the cell cycle, the macronucleus divides amitotically, while the relatively small micronucleus divides mitotically. This is the typical form of reproduction, or increase in numbers, and this division of nuclear labour, between the somatic, macronucleus and the germinal micronucleus, is one factor that allows ciliates to grow rapidly and thus form blooms in the plankton.
Conjugation (sex) can be induced by either internal or environmental stimuli; this is the initiation of the ciliate life cycle. Two cells, generally of complementary mating types, fuse together, sometimes after elaborate recognition behaviour. After the cells join, their micronuclei undergo meiosis to form haploid pronuclei. Although there are variations on the theme, the general process that follows is that one of the two pronuclei remains in each ciliate. The other pronucleus becomes migratory and travels to the other conjugant. The two pronuclei then fuse, and the two cells separate. Then, the newly formed zygotic (diploid) nucleus in each cell generates a new macronucleus, and the old macronucleus degenerates; thus a new life cycle begins. The last point, that a new macronucleus forms, is crucial to the phenotypic expression of clonal rejuvenation.

During the cell, cycle ageing processes (e.g., mutations and chromosomal aberrations) occur which affect the macronucleus (see Discussion). Such ageing can be manifest as reduced growth and feeding rates, decrease in cell size, cell distortion, and ultimately clonal death. But ageing is overcome when a new, post-conjugation, macronucleus replaces the aged macronucleus. If, however, for some reason, such as lack of an appropriate mate, conjugation is prevented, ageing continues, and the clone may die. In some rare cases, ciliates are able to overcome this problem by conjugating with their own mating type, selfing conjugation, or even by undergoing a meiotic division and re-fusion of the micronucleus within a single cell, autogamy. In these cases, rejuvenation of the macronucleus occurs, but inbreeding is an obvious consequence which may also lead to the ultimate demise of the population.

In this work, I have developed a numerical response for the marine planktonic ciliate Strombidinopsis cheshiri. However, during the course of experiments, ageing, occurred in the clonal lab cultures, and recognising the cause and impact of this phenomenon was essential to developing an accurate data set. This work thus provides a practical example of the problems associated with ciliate ageing.

Clonal ageing has been well documented for ciliates like Paramecium that are easily maintained in the laboratory (Nanney 1980, Smith-Sonneborn 1981, Bell 1988) but has been poorly documented for planktonic ciliates. I indicate below some of the problems caused by and possible reasons for clonal ageing. Using such ciliates as a model of ageing may help us to understand ageing at a unicellular rather than a system-metazoan level.

Materials and Methods

Strombidinopsis cheshiri (Fig. 2) was isolated in the summer from coastal subsurface British Columbian waters and a clonal culture was established by triplicate single cell isolations. Experiments were conducted to determine the numerical response (growth rate versus food concentration) of S. cheshiri grazing on Thalassiosira pseudonana (a centric diatom on which the ciliate grows well on). The diatom was maintained in continuous culture, under constant conditions, thus removing biases potentially caused by varying prey quality. Ciliates were maintained in a semi-continuous culture by transferring ciliates to new prey and medium on a daily basis; this ensured that prey concentration remained relatively constant (for details of these procedure see Montagnes 1993).

Fig. 2. A scanning electron micrograph of Strombidinopsis cheshiri. The cell is ~60 µm long.
The numerical response data were fit to Eq. 1, which is homologous to Michaelis-Menten enzyme kinetics and is a good predictor of the numerical response and is based on sound theoretical mechanisms (Fenchel 1986). Curves were fit to the data using the Marquardt-Levenberg algorithm (Sigmaplot, Jandel Scientific, Calf.). For biological data sets, this method may be more accurate and precise than methods of curve fitting that transform the model to linear forms (Berges et al. 1994).

\[
\mu = \frac{\mu_{\text{max}} \times ([P] - x')}{k + ([P] - x')} 
\]

where, \( \mu \) = growth rate (d\(^{-1}\)), \( \mu_{\text{max}} \) = the maximum growth rate (d\(^{-1}\)), \( [P] \) = prey concentration (No. ml\(^{-1}\)), \( x' \) = the x intercept or threshold concentration (the prey concentration where \( m = 0 \)) (No. ml\(^{-1}\)), \( k \) = a constant (No. ml\(^{-1}\)).

The method, described above, was followed for three separate experiments. As experiments were performed, modifications were made in the procedure to determine the cause of unanticipated variation in the growth response. The methods of these three sets of experiments are outlined below.

**Experiment 1.** Growth rate was determined at 25 prey concentrations ranging from 2x10\(^3\) to 10\(^5\) prey ml\(^{-1}\). It was impossible to run 25 treatments simultaneously, so these rates were determined in three independent sub-experiments. The sub-experiments were run days to weeks apart, on different batches of prey and on different batches of ciliates (ciliates from the same clone but different sub-clones); it was assumed that combining these experiments would not cause any bias.

**Experiment 2.** In Experiment 1, there was variation in the growth responses between treatments which may have been caused by combining the three separate sub-experiments (see below). To examine this, a single experiment was run with 20 treatments using one "batch" of prey and one "batch" of ciliates. As the growth response was asymptotic by >104 prey ml\(^{-1}\) (see below), this experiment was conducted from 0 to 4x10\(^4\) ml\(^{-1}\).

**Experiment 3.** The modifications made in Experiment 2 did not reduce the variation of the growth rates (see below). Experiment 3 was conducted to test if the variation was due to differences in ciliate cell lines. Newly isolated sub-clones were established by placing a single ciliate from the original clone in each of 48 wells containing 10 ml of medium with saturating prey (10\(^4\) ml\(^{-1}\)). At ~1 division d\(^{-1}\), this provided sufficient numbers of progeny, from a single cell, to perform experiments within 8-10 days.

Three such sub-clones, were grown in each of six treatments, ranging between 3.7x10\(^{-2}\)-1.9x10\(^4\) prey ml\(^{-1}\). Some ciliates in one of these sub-clones were found to be conjugating on day 10 of the isolation period. Only non-conjugating individuals from this sub-clone were used, but these may have been exconjugants. Another of the isolated cells produced >360 cells within eight days; this produced sufficient numbers for six replicates at the six concentrations.

**Results**

**Experiment 1.** Growth rate followed a rectangular hyperbolic response (Fig. 3a)

At concentrations above 7x10\(^{-4}\) prey ml\(^{-1}\) the response was less predictable: some replicates lay near the asymptote predicted by the hyperbolic function, but others lay well below it. The variance of the treatment response increased as prey concentration increased. At prey concentrations below 2.7x10\(^3\) ml\(^{-1}\) ciliate net mortality (henceforth referred to as mortality) occurred due to starvation. Equation 1 was fit to the data (Fig. 3a, Table 1).

**Thalassiosira pseudonana** for prey concentrations (k, x'), and d\(^{-1}\) for growth rate (m\(_{\text{max}}\)). The number of data points (n) used to fit the line is reported for each fit. The data were not evenly scattered around the predicted curve. Variation between treatments existed and was related to the different sub-experiments (i.e., over a range of
similar prey concentrations, sub-experiments yielded distinctly different values from each other. It was speculated that this was due to uncontrolled, changing, conditions between the three sub-experiments.

That the variation seen in Experiment 2 was due to a physiological heterogeneous population within the harvested "batch" culture. This prediction was supported by what appeared to be three discernible numerical response curves formed by the data (Fig. 3b).

**Table 1.** Growth and grazing data for *Strombidinopsis cheshiri*; parameters and associated standard errors (SE) of the numerical response equation (Eq. 1) presented in Fig. 3 and Fig. 4. Values are in units of numbers ml$^{-1}$ of the diatom.

<table>
<thead>
<tr>
<th>Fit from figure</th>
<th>$m_{\text{max}}$ (SE)</th>
<th>$k$ (SE)</th>
<th>$x'$ (SE)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 3a</td>
<td>0.734</td>
<td>2420</td>
<td>2720</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>(0.083)</td>
<td>(1550)</td>
<td>(523)</td>
<td></td>
</tr>
<tr>
<td>Fig. 3b</td>
<td>0.731</td>
<td>1670</td>
<td>774</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>(0.071)</td>
<td>(506)</td>
<td>(201)</td>
<td></td>
</tr>
<tr>
<td>Fig. 3</td>
<td>1.180</td>
<td>4060</td>
<td>1750</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>(0.167)</td>
<td>(1210)</td>
<td>(265)</td>
<td></td>
</tr>
<tr>
<td>Fig. 3c,</td>
<td>0.199</td>
<td>1.35 x $10^4$</td>
<td>1.07 x $10^4$</td>
<td>36</td>
</tr>
<tr>
<td>triangles</td>
<td>(0.164)</td>
<td>(4520)</td>
<td>(4270)</td>
<td></td>
</tr>
<tr>
<td>Fig. 4a,</td>
<td>0.729</td>
<td>4840</td>
<td>2530</td>
<td>186</td>
</tr>
<tr>
<td>solid &amp; open</td>
<td>(0.077)</td>
<td>(1260)</td>
<td>(4520)</td>
<td></td>
</tr>
<tr>
<td>circles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. 4a,</td>
<td>0.985</td>
<td>4090</td>
<td>1570</td>
<td>148</td>
</tr>
<tr>
<td>solid circles</td>
<td>(0.046)</td>
<td>(615)</td>
<td>(157)</td>
<td></td>
</tr>
</tbody>
</table>

Rather than reducing the overall variance in growth response, seen in Experiment 1 (by conducting Experiment 2 under a single set of conditions), this experiment produced data with greater variance (*cf.* Figs. 3a and 3b). For instance, at comparable food concentrations (~2x10$^5$-2x10$^6$ cells ml$^{-1}$) the average of the variances of growth rate, measured at each food concentration, in Experiment 1 was ~0.025 while that in Experiment 2 was 0.123. This suggested that the variation seen in the previous experiment was not necessarily linked to variable culturing technique. It was speculated
growth at all treatment levels. In the cell line that exhibited maximum growth (open circles, Fig. 3c) ~20% of cells were found to be conjugating when the cells were harvested. This alone is remarkable as it indicated that S. cheshiri was capable of selfing conjugation (for a review of sex in ciliates see Dini & Nyberg 1993).

Equation 1 was fit to all the data in the two sets that exhibited positive growth (open circles and triangles, Fig. 3c, Table 1). For both curves, the growth rate followed a rectangular hyperbolic response. For the fast-growing clone (open circles, Fig. 3c), the variance within replicates was considerably lower than that observed in Experiments 1 or 2 (in Fig. 3c there are triplicate measurements at each food concentration); between ~2x10^3-2x10^4 prey cells ml^-1 the average variance of the growth rate for the fast growing clone was ~0.006. Mortality occurred below 1.8x10^3 prey ml^-1. Note: this clone, which was conjugating prior to the experiment, produced cells that grew as fast as the maximum rates determined from Experiments 1 and 2. For the slower growing (non-conjugating) clone (triangles, Fig. 3c), the variance of growth rates was greater than that of the fast-growing clone; between ~2x10^3 - 2x10^4 cells ml^-1 the average variance was ~0.051. For this clone, mortality occurred below 1x10^4 prey ml^-1.

Synthesis of Experiments 1-3. Observation of conjugating cells showed them to have the highest growth rates. When the data from the two sub-clones that grew in Experiment 3 (open circles and triangles, Fig. 3c) were compared to those in Experiment 2, over the range of ~2x10^3-2x10^4 prey cells ml^-1, there were significant differences in the average variance of the growth response (µ, d^-1; Kruskal-Wallis ANOVA, p = 0.060). The most rapidly growing clone, which was conjugating prior to the experiment, had a significantly lower variance in growth response than the ciliates examined in Experiment 2 and those not conjugating in Experiment 3 (triangles Fig. 3c) (Dunn's multiple comparison, µ = 0.05). Assuming that the rapidly growing cells isolated in Experiment 3 (open circles, Fig. 3c) were exconjugants, these data suggest that conjugation influenced the growth rate.

Discussion

To determine a numerical response for S. cheshiri, it was necessary to assess the effects of ageing and selfing conjugation on the clonal culture. The following sections assess the results above, examine the life cycle of S. cheshiri, establish a numerical response of a "young" and healthy population of S. cheshiri, and discuss some of the implications of this response in terms of the growth, death and ageing of planktonic ciliate populations.

Life cycle of S. cheshiri. During clonal growth, mechanisms exist that reduce the quality of the macronucleus and thus reduce clonal vitality (Bell 1988). There are four main reasons for the decrease in vitality in small populations: i. during division, random assortment in the macronucleus causes a dilution and/or change of frequency of functionally haploid genetic fragments (i.e., heterozygosity verges towards homozygosity or a loss of a gene); ii. repeated autogamy or selfing conjugation fractions a heterozygotic population into homozygotic lines; iii. recessive mutations in the micro- and macronuclei become expressed through random assortment in the macronucleus and/or repeated autogamy and selfing (i. and ii. above); and iv. Muller's ratchet (the accumulation of deleterious mutations) works on the mico- and macronuclei. In small cultures, maintained in the laboratory, the probability of extinction and the rate at which the above problems occur, due to stochastic processes, is also increased substantially (i.e., this probability increases exponentially with decreasing population size).

These processes increase the likelihood of: i. expression of lethal or sublethal recessive genes; ii. homozygosity, which may reduce hybrid vigour; iii. the accumulation of mutations; and iv. dilution out of healthy stock from cultures. All of these would increase the likelihood of a culture dying out and help to explain why so many ciliates do not survive well in culture (see...
Ciliate conjugation generates a new macronucleus from the micronucleus (Fig 1). Since the macronucleus mediates growth rate, it follows that conjugation should increase the growth rate and decrease the variability in growth rates in a culture that has undergone ageing; this is precisely what was observed in this study (Fig. 3) and what has been seen for other non marine ciliates (Bell 1988).

Typically, isolated ciliate clones maintain a constant growth rate for >100 generations, and by ~200 generations after conjugation, most clones are extinct (Bell 1988). This is particularly interesting as there are ~50 cell divisions between egg and adult in humans (Nanney 1980). However, the older the parent ciliate at the time of conjugation, the shorter the life span of the progeny line (Smith-Sonneborn 1981). Thus, a series of delays in the onset of conjugation reduces the number of cell divisions, post conjugation, and clonal lines maintain a constant growth rate for <<200 generations. Possibly, analogous situations may be observed in humans.

Strombidinopsis cheshiri was maintained in culture for >250 days. If the ciliate grew near its maximum rate in stock cultures (~1.4 division d-1), then it exceeded the 200 generations typical of many viable laboratory cultures. Since conjugation occurred in some cultures, the life of the culture may have been prolonged, but conjugation may not have entirely rejuvenated the culture. When Experiment 3 was conducted, S. cheshiri was near the end of its life; several months later the culture was dead. This may explain why only 10% of the cells isolated in that experiment survived.

The above data suggest that over short incubation periods (5-10 d) experimental manipulation was not the cause of high variation of growth rates at or near a single food concentration. Instead, intrinsic features, possibly mutation load, caused the variation. If reduced growth rates were due to mutational load (caused by ageing, accelerated by maintaining small populations in culture), then the higher growth rates observed in all three experiments may have been representative of natural populations, assuming that natural populations regularly conjugate and thus do not age. The assumption that reduced growth rates were due to mutational load provides a criterion for removing outliers from the data prior to constructing a numerical response.

A numerical response. One goal of this study was to establish a numerical response for S. cheshiri, but it was hindered by the variance in the growth response, and this variance appeared to be due to ageing within the culture. Assuming that natural populations regularly conjugate and thus do not age (see below), some of the lower growth rate data may be ignored; accordingly, outliers were removed. Equation 1 was fit to the data presented in Experiments 1-3, (Fig. 4, solid line). The residuals from the fit were not normally distributed around zero (data not shown). To adjust for this, lower growth rates were omitted until the residuals were normally distributed around zero. The second curve, after outliers were removed, (Fig. 4, broken line) provides a higher numerical response with a more rapid response to food concentration than the fit to all the data. This may be considered to be the growth response of a non-aged population of S. cheshiri.

The experiments revealed four pieces of information: i. the concentration of T. pseudonana where growth rate is zero was ~10^3 ml^-1; ii. the major increase in growth rate occurred between 10^3-10^4 prey ml^-1; iii. the maximum growth rate was μ = 0.985 (~1.4 divisions d^-1); and iv. S. cheshiri was capable of selfing conjugation.

During the summer in the waters where S. cheshiri was collected, plankton of similar size to T. pseudonana (5-10 µm) ranged from 102-104 cells ml-1. If these plankton are the dominant food for S. cheshiri, then food levels were generally sufficient to support the ciliate, but at times it would starve, and for short periods it would grow at its maximum rate.
Thus, it appears that this ciliate could form blooms under natural conditions.

Fig. 4. The numerical response of *Strombidinopsis cheshiri* from a combination of Experiments 1-3. All data points represent growth rates (m, d⁻¹) at respective prey concentration, presented in terms of prey cell numbers (No. ml⁻¹). The solid line represents the fit of Eq. 1 using data represented by both open and solid circles. The broken line represents the fit using only data represented by the solid circles. Table 1 presents the parameters associated with the curves.

The implications of ageing and selfing conjugation. The notion that sub-clonal variation occurs in experiments which use recently established clonal cultures has far-reaching implications. Ciliate clones are often used to study ecological processes (e.g., growth rates, grazing rates). The above data suggest that variation observed in such studies may be partially attributed to clonal variation caused by ageing, rather than experimental error. Thus, care should be taken to ensure that ageing does not occur in laboratory cultures. Continually isolating new clones from the field might be one method of ensuring young, healthy ciliate populations. Furthermore, to recognise if ageing occurs, monitoring of some physiological parameter (e.g., growth rate) should be regularly conducted while clones are maintained and experiments are being performed.

We must also consider the extent of and the reason for ageing in natural populations. First, why should ageing occur? Ageing, as indicated above, is at least in part due to the accumulation of deleterious mutations. However, not all mutations are deleterious. Expression of some mutations either by trends toward homozygosity (e.g., by selfing conjugation) or by recombination (e.g., breeding with other genotypes) can provide a selective advantage to clones, especially if environmental conditions change; this is a fundamental tenet of evolution. Thus, the production and expression of mutations can be beneficial under changing conditions, and ageing (due to the accumulation of deleterious mutations) must be viewed as a necessary by-product of evolutionary adaptation.

What is the extent of such ageing? This would depend primarily on the regularity of sex in natural populations. Conjugation, or sex, is likely to be a regular occurrence, assuming that it is stimulated by starvation (Nanney 1980), that ciliates become starved after blooming and exhausting their prey (Montagnes 1993), and that ciliate blooms are common in the plankton. We can also ask, how predisposed is *Strombidinopsis* to selfing conjugation?

Gifford (1985) maintained *Strombidinopsis* (cf. *acuminatum*) for almost a year. Although her culture was not indisputably monoclonal, it was the only one of 21 isolated planktonic ciliate species that conjugated. Possibly these cultures were selfing or were at least predisposed to conjugate in culture. I have also observed selfing conjugation in a monoclonal culture of *Strombidinopsis multiauris* (unpublished data). Similar observations have not been reported for the other major taxa of planktonic ciliates. Thus, *Strombidinopsis* may be a useful organism to culture for future research on planktonic ciliate ageing and mating, for which there are virtually no data.

Not all ciliates are able to have sex with themselves. Why might *Strombidinopsis* be predisposed to selfing conjugation? We know that selfing conjugation is a trait acquired to allow conjugation when ciliates are unable to find other clones, and starvation stimulates conjugation (Nanney 1980). In the plankton, a ciliate bloom could develop from a single cell finding itself in optimum-food conditions and
the progeny of this ciliate could then deplete the food, and starvation would ensue. This in turn would stimulate conjugation. Moreover, the bloom would be monoclonal and selfing would be necessary.

The above scenario, under natural conditions, parallels that observed in laboratory cultures. It is possible that repeated blooms of a single clone could cause ageing, and its deleterious effects, if conjugation did not occur. Thus, the observations of ageing and selfing conjugation in *Strombidinopsis* circuitously suggest that this organism may be especially suited to form blooms. The next steps in this work will be to i. find natural blooms of *Strombidinopsis* and determine if conjugation does occur near their demise and ii. establish other *Strombidinopsis* clones and examine ageing and mating patterns in these.

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**References**


