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NOTE

Mitotic indices of zooxanthellae: a comparison of techniques based on nuclear and cell division frequencies

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ABSTRACT: Hitherto, studies of mitotic indices in zooxanthellae have involved identification of doublet cells in the mitotic phase of cytokinesis. This note presents comparative results for zooxanthellae in the temperate sea anemone *Anemonia viridis* Forskal based upon measurements in both cytokinesis and karyokinesis. provides some evidence for the relative duration of these phases of mitosis in *A. viridis* zooxanthellae and highlights the potential benefits of deriving mitotic indices during karyokinesis.

Measurement of mitotic index in cell populations involves an assessment of the proportion of cells which are observed in mitosis (Mitchison 1971). Such measurements have been widely used by those monitoring growth in both animal and plant tissues, though the techniques employed have varied widely depending on the study organism, most methods calculating mitotic index on the basis of the fraction of cells showing paired nuclei or 2 mitotic figures per cell.

Studies of mitosis in symbiotic algae (zooxanthellae) resident in coelenterates have, in contrast, used an index based upon the fraction of cells appearing as doublets with a distinct cell plate (Wilkerson et al. 1983, 1987, Cook & D'Elia 1987, Hoegh Guldberg et al. 1987, Steen & Muscatine 1987). A major aim in these studies has been the calculation of algal population growth rates and doubling times using equations derived from phased cell division in marine phytoplankton (Weiler & Chisholm 1976, McDuff & Chisholm 1982, Wilkerson et al. 1983). In such a context cell plate measurements have provided reasonable estimates of *in situ* algal growth rates that have significantly advanced our understanding of the effects of nutrients on the dynamics of symbiotic algae (Muscatine et al. 1989); temporal patterns of algal division (Wilkerson et al. 1988); and mechanisms of algal regulation (Trench 1987).

More recently the balance between the coelenterate host and its symbiotic algae has been shown to be very sensitive to environmental factors such as temperature increase (Glynn & D'Croz 1990), temperature decrease (Muscatine et al. 1991), salinity and irradiance (Hoegh-Guldberg & Smith 1989). As a result it has been suggested (Brown 1988) that a better understanding of the mitotic cell cycle in symbiotic algae could not only lead to an improved quantitative estimate of 'stress' in coelenterates but also clarification of cause/effect relationships between mitotic responses and specific pollutants.

In the present note the advantages of monitoring mitotic indices for these purposes, using an index which is based on the number of cells showing mitotic figures, are presented using the temperate sea anemone *Anemonia viridis* Forskal.

Materials and methods. Anemones were collected from Millport, Isle of Cumbrae, Scotland, and were maintained under laboratory conditions as described in Suharsono & Brown (1992) in recirculated, artificial seawater at 15 °C. The anemones were fed weekly on fresh *Mytilus edulis* (1 g wet weight mussel flesh per anemone).

Algal division in *Anemonia viridis* has previously been shown to be asynchronous (Suharsono 1990), resembling that in the temperate anemone *Anthopleura elegantissima* which showed considerable variability over the course of a day (Wilkerson et al. 1983). In the present experiment algal division was therefore measured over an 11 h period, between 09:00 and 20:00 h.

In the present experiment 2 methods of measuring mitotic index in *Anemonia viridis* zooxanthellae were compared – one involved identification of 2 mitotic figures per cell (using the Feulgen staining of nuclear DNA) and the other, the counting of doublet cells as used in the majority of coelenterate studies (Wilkerson

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et al. 1983). Feulgen staining of nuclear material is a standard cytochemical technique (Boon & Drijver 1986) and one that has been used to measure nuclear division in both plants (Clowes & Juniper 1968) and animals, including coelenterate material (Muscatine & Neckelmann 1981).

Three tentacles were sacrificed from each of 3 anemones at hourly intervals. The tentacles were fixed in 3:1 alcohol/glacial acetic acid for 30 min and were then transferred to 70% alcohol and stored at 4°C.

Each tentacle was then hydrolysed in 1M HCl at 60°C for 30 min followed by 10 min at 20°C. Serial washing of tentacles in distilled water was carried out to remove any acid; tentacles were then placed in Feulgen stain for 3 h. They were subsequently macerated on a slide in 45% (v/v) acetic acid before being examined as a squash preparation under an Olympus phase contrast microscope at 1000× magnification.

A total of 500 zooxanthellae were counted per slide and the number of algae showing 2 mitotic figures within the cell recorded. This value was then expressed as a percentage of the total number of cells counted to provide a mitotic index based on karyokinesis (nuclear division).

Using the same tentacle samples the numbers of cells showing a cell plate were also scored and a mitotic index value calculated based on cytokinesis (cell division).

Results. In terms of measurement of mitotic index, the identification of mitotic figures proved much easier than that of cell plates. Mitotic figures could be clearly recognised in Feulgen-stained material (Fig. 1) both from the increased contrast and colour which Feulgen staining imparts to the nuclear material in the zooxanthellae. Identification of algae showing cell plates was more difficult both because the cell plate lacked any differential staining and also because any overlapping algae often gave the appearance of an 'apparent' cell plate even after repeated focussing through the depth of field of the sample (Fig. 2).

The values for mitotic indices of the zooxanthellae in karyokinesis were 3 to 5 times higher than those recorded for zooxanthellae in cytokinesis (Fig. 3).

Discussion. The higher numbers of algal cells showing mitotic figures compared with those showing cell plates may be explained in terms of the lengths of the phases of mitosis for *Anemonia viridis* zooxanthellae.

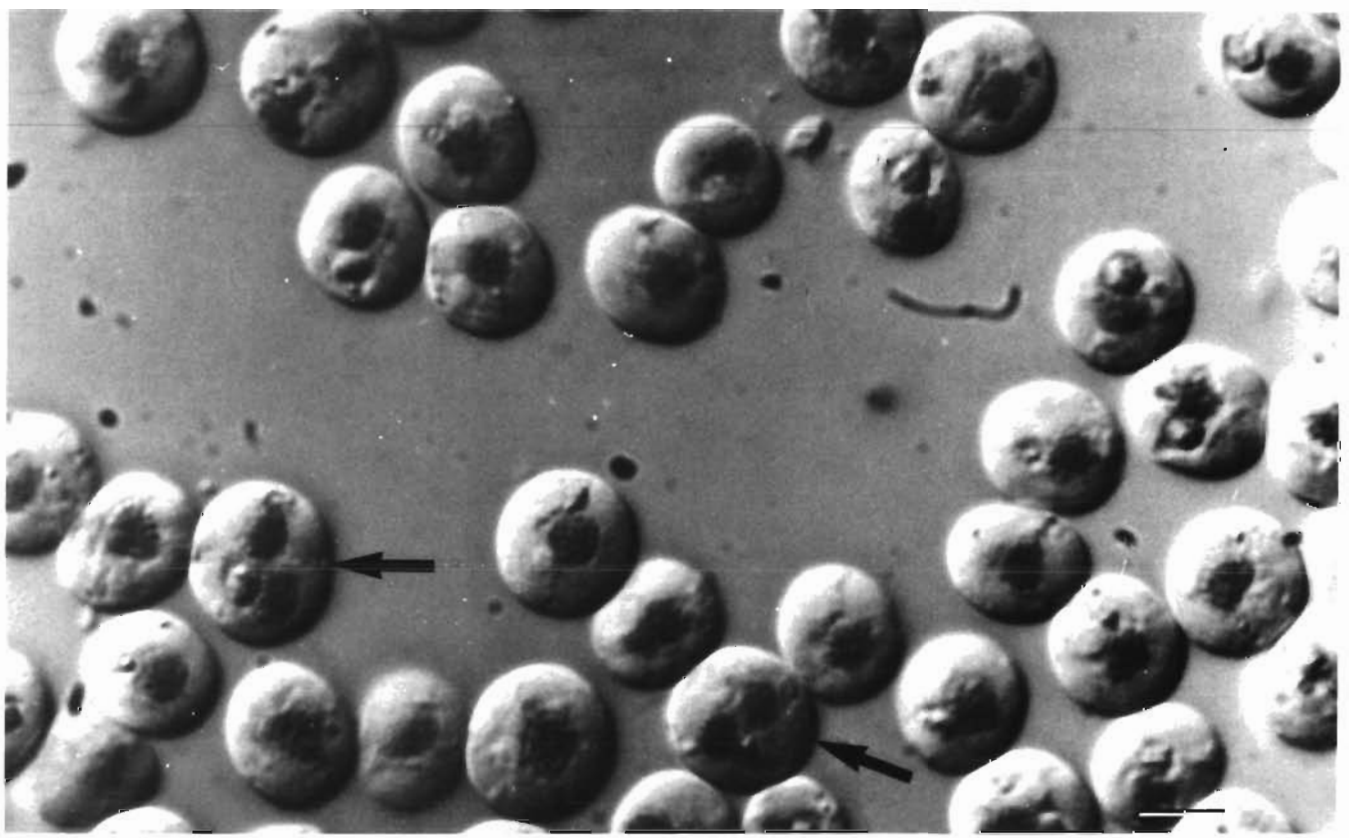

Fig. 1. Zooxanthellae in *Anemonia viridis*. Appearance of a tentacle squash which has been stained with Feulgen reagent to show zooxanthellae with paired mitotic figures (highlighted with an arrow). Photographed using a Nomarski interference contrast device attached to a Leitz orthoplan photomicroscope. Scale bar = 5 μm

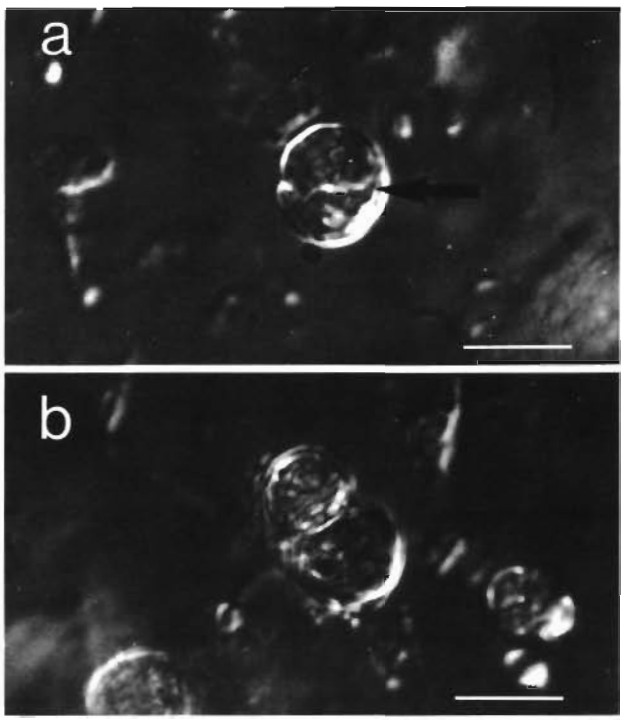

Fig. 2. Zooxanthellae in *Anemonia viridis*. Appearance of dividing zooxanthella in tentacle homogenate, with (a) actual cell plate highlighted with arrow and (b) a profile of 2 overlapping zooxanthellae, where identification of division status of the cell would be difficult. Photographed using a Nomarski interference contrast device attached to a Leitz orthoplan photomicroscope. Scale bars = 8 µm

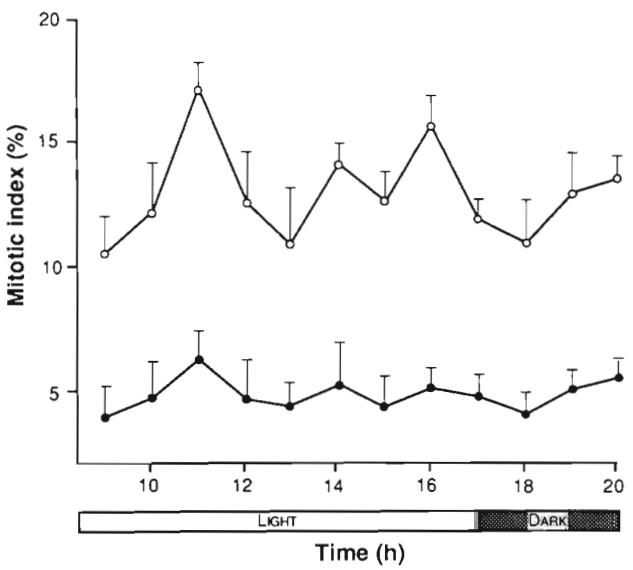

Fig. 3. Average mitotic index, expressed as paired mitotic figures (○) and as cell plates (●), of zooxanthellae in the tentacles of *Anemonia viridis* measured hourly between 09:00 and 20:00 h. Values represent means ± SD (n = 9)

Mitotic figures appear at the end of anaphase/beginning of telophase whereas cell plate formation is a relatively short event at the end of telophase (Dyer 1976a). Since mitotic index values estimated for mitotic figures exceed those obtained for cell plates by a factor of 3 to 5 fold it can be tentatively inferred that the paired nuclei stage in *A. viridis* zooxanthellae is approximately 3 to 5 times longer than the cell plate stage.

In addition to mitotic figures being more easily identifiable than cell plates, the higher mitotic index obtained with mitotic figures would reduce counting errors, particularly where mitotic indices of zooxanthellae are low (< 1% using cell plate methods), as recorded in some tropical corals and anemones (Wilkerson et al. 1983), and/or extremely variable (Wilkerson et al. 1988). The higher visual contrast provided by stained mitotic figures also offers greater scope for automation of counts using image analysis systems. The technique could possibly be further improved by the use of nuclear DNA specific fluorescent stains, such as propidium iodide and ethidium bromide, on living material which could be examined directly.

Recent work on both tropical and temperate symbiotic coelenterates has also indicated that algal mitotic indices (expressed as paired mitotic figures) respond more sensitively to stressors such as heat-shock (Zamani 1991, Miller et al. 1992) and heavy metal ions (Nganro 1992) than mitotic indices assessed on the basis of cell plates. When *Anemonia viridis* were heat-shocked from 15 to 28°C the mitotic index (paired mitotic figures) showed a significant, temporary increase of 2.5-fold compared with a small but non-significant change in mitotic index assessed using cell plates (Zamani 1991).

Such differences in response may be attributed to the varying susceptibility of different stages of the mitotic cycle to perturbation; for example, DNA replication apparently is particularly sensitive to increased temperature and irradiance (Brown 1976). It is also clear, mainly from work on higher plants, that mitotic division of the nucleus is not inevitably linked to cell-plate formation and cell division: either event can take place independently of the other and does so particularly under extreme environmental conditions (Dyer 1976b). Blocking of cell division but not nuclear division occurs in sea-urchin eggs as a result of exposure to heat-shock while direct damage to DNA as a result of UV-B exposure will suppress both nuclear and cell division (Ikeda 1965). Clearly a more detailed fundamental analysis of the duration of different phases of the mitotic cycles of symbiotic zooxanthellae, using a wide range of nuclear labelling techniques, could offer increased scope for highlighting the mechanisms involved in the breakdown of coelenterate symbioses under stress.

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