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Cell proliferation and growth in *Zoothamnium niveum* (Oligohymenophora, Peritrichida) – thiotrophic bacteria symbiosis

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Abstract
Chemolithoautotrophic, sulphide-oxidizing (thiotrophic) symbioses represent spectacular adaptations to fluctuating environmental gradients and survival is often accomplished when growth is fuelled by sufficient nourishment through the symbionts leading to fast cell proliferation. Here we show 5'-bromo-2'deoxyuridine (BrdU) pulse labelling of vegetative growing *Zoothamnium niveum*, a colonial ciliate obligately associated with thiotrophic ectosymbionts, and demonstrate age related growth profiles in three heteromorphic host cell types. At the colony's apex, a large top terminal zooid performed high proliferation activity, which decreased significantly with increasing colony age but was still present in old colonies indicating that this cell possesses lifelong cell division potential. In contrast, terminal branch zooids proliferated independent of colony age but appeared to be limited by their cell division capacity predetermined by branch size, thus leading to the strict, feather-shaped colony form. Appearance of labelled terminal branch zooids allowed us to distinguish a highly proliferating apical colony region from an almost inactive, senescent basal region. In macrozooids attached to the colony, extensive BrdU labelling suggests that DNA synthesis occurs in preparation for a new generation. As motile swarmers, the macrozooids seem to be arrested in the cell cycle and mitosis and cell division occur when the swarmer settles and transforms into a top terminal zooid building up a new colony.

Keywords: Cell proliferation, growth, bromodeoxyuridine, ciliate, *Zoothamnium*, symbiosis, chemoautotrophy, thiotrophy

1. Introduction

Beneficial associations between bacteria and eukaryotes exist in large numbers and varieties in marine habitats. Prominent hosts of chemolithoautotrophic, sulphide-oxidizing (thiotrophic) bacteria are invertebrates and protists occurring in sulphide rich habitats from the intertidal zone to the deep sea (Cavanaugh et al., 2006; Ott et al., 2004). Mutual benefits driving these interactions are mainly nutritional, as the host is provided with a food source and the symbiont is able to exploit sulphide and oxygen gradients (Cavanaugh et al., 2006; Fenchel and Finlay, 1989; Fisher and Childress, 1986). In order to maintain a well functioning mutualistic association, cell division and reproduction of the host must be synchronized with the life cycle of the symbiont. However, our understanding of growth dynamics in thiotrophic symbioses is in its infancy.

The host of a remarkable shallow-water thiotrophic ectosymbiosis is the colonial, peritrich ciliate *Zoothamnium niveum* (Ciliophora, Oligohymenophora) first described by Hemprich and Ehrenberg (1831) and Ehrenberg (1838) from the Red Sea. *Z. niveum* grows to more than one centimetre in length and is therefore, the largest known representative of a diverse genus that comprises over 70 described species (Ji et al., 2006). The sessile ciliate is obligately associated with the sulphide-oxidizing Gammaproteobacterium *Candidatus Thiobios zoothamnicoli* (Rinke et al., 2006; 2007). This bacterium covers almost the entire colony's surface in a strict monolayer (Bauer-Nebelsick et al., 1996a) and gives the ciliate its conspicuous white colour.

Zoothamnium niveum colonies are feather-shaped, consisting of a central stalk from which branches appear in

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an alternating arrangement, bearing three heteromorphic cell types, termed zooids (Bauer-Nebelsick et al., 1996b): (1) terminal zooids, (2) microzooids and (3) macrozooids. The colony results from a strict ramification pattern that is achieved through a specific series of longitudinal fissions: First, on the tip of each colony a top terminal zooid divides and generates a new terminal zooid which initiates the formation of a new branch. Thus, the number of branches is equivalent to the divisions of the top terminal zooid (Rinke et al., 2007) which is responsible for the length growth of the colony. Second, the new terminal zooid continues to divide and generates up to 20 structurally similar microzooids on a new segregated branch. These microzooids are left behind as feeding zooids and are equipped with a complete cytopharynx. Third, macrozooids asexually originate mitotic divisions and develop at a well determined place, generally at the bases of the branches. These voluminous zooids have the potential to transform into motile swarmers that can leave the colony and initiate a new colony after settlement (Bauer-Nebelsick et al., 1996a).

Zoothamnium niveum typically inhabits mangrove peat walls along tidal channels in the Caribbean Sea. Patches of up to hundred colonies settle at fast appearing and disappearing mini-vents, where the surface of the sulphide laden peat wall is disturbed occasionally and only allows for settlement at the oxic-anoxic interface. Patch life span is limited to approximately 20 days as these spatial habitats are overgrown rapidly by a microbial community, thus closing the vents after a short time (Ott et al., 2004). Hence, this ectosymbiosis faces the challenge of habitat instability, and survival should be characterized by common life history traits, such as fast growth and early and high reproduction capabilities.

In general, detailed information of life history traits in symbiotic partners is scarce due to the limited success in cultivating thiotrophic symbioses. However, the development of an artificial gradient system allowed the cultivation of the *Zoothamnium niveum* symbiosis for several generations (Vopel et al., 2001). By simulating natural conditions in a flow through aquarium, experiments with these artificial sulphide-producing systems revealed a 6–8 days life span of the Caribbean host ciliate. Starting with a motile swarmer that had settled within a few hours of migration, *Z. niveum* developed from a single cell to a full grown colony within 4 days (Ott et al., 2004). Furthermore, the cultivation of the symbiosis from the Mediterranean Sea in an automated flow-through respirometer during its entire lifespan allowed documentation of asexual reproduction and showed an average life span of 11 days when optimal sulphide conditions were provided (Rinke et al., 2007).

A distinctive characteristic of colonial species within the genus *Zoothamnium* is the strict ramification pattern. Taking this into consideration, *Z. niveum* provides a particularly useful system for tracing proliferating cells. Recent studies have shown that labelling with the thymidine analogue 5'-bromo-2'deoxyuridine (BrdU) is a suitable tool for studying proliferation kinetics in aquatic invertebrates (Alexandrova et al., 2003; Gschwentner et al., 2001; Nakayama et al., 2005). Originally developed for cells in culture, this labelling technique has also been applied to whole-mount preparations (Plickert and Kroiher, 1988). BrdU is only incorporated into the DNA of cells that undergo DNA replication during the S-phase of the cell cycle and can be detected immunocytochemically by a specific antibody (Gratzner, 1982). Since replicative DNA synthesis is central to any increase in cell number (Moore et al., 1994), the percentage of BrdU labelled cells (proliferation index, PI) can hence serve as a dynamic measure of proliferation activity (Morris, 1993). Regarding ciliates, BrdU labelling studies have so far focused on the nuclear architecture and function of macro- and micronuclei in solitary species (Postberg et al., 2005; Tanaka and Watanabe, 2003).

Investigating the host's growth profiles is one approach that can enhance our understanding of dynamic symbiotic entities such as the *Zoothamnium niveum* symbiosis, particularly at the cellular level. For this reason, the present study aims to identify proliferating zooids in *Zoothamnium niveum* by labelling whole mount colonies with BrdU. We report spatial distribution patterns of BrdU labelled zooids within the colony, and use this data to reveal their proliferation activity during the colony's life span to elucidate growth profiles in this uniquely large symbiotic protist.

2. Material and Methods

Sampling

Specimens of *Zoothamnium niveum* were collected in the main channel at the north end (Batfish Point) of the mangrove island Twin Cays (16°48'N, 88°05'W; Belize Barrier Reef, Caribbean Sea) in February 2006. Colonies were cut from vertical mangrove peat walls in 0.5 to 3 m depth and transported 3.5 km seawards to the Carrie Bow Cay Marine Field Station (CCRE program of the National Museum of Natural History, Washington, DC), where labelling experiments were conducted.

Proliferation labelling

For pulse labelling experiments, living specimens were treated with 5'-bromo-2'deoxyuridine (BrdU, SIGMA). Twelve specimens each were incubated for 15 min, 30 min, and 1 h in 1 mM BrdU in 0.2 µm filtered seawater. At the end of each pulse period, whole specimens were rinsed with sterile seawater (3 x 5 min), fixed with 3.2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2) overnight, dehydrated in 30, 50, and 70%

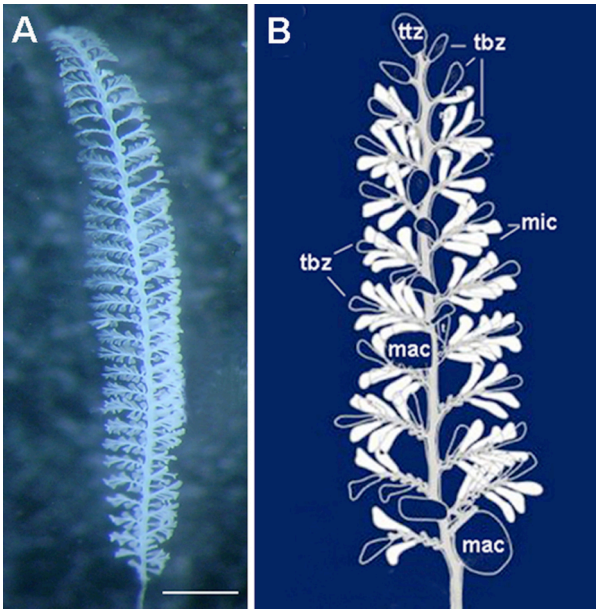


Figure 1. *Zoothamnium niveum* colony. A. General view of an old colony with more than 50 branches, showing the characteristic ramification pattern; scale bar 0.5 mm. B. Schematic drawing demonstrating the heteromorphic cell types: top terminal zooid (ttz), terminal branch zooids (tbz), microzooids (mic), and macrozooids (mac).

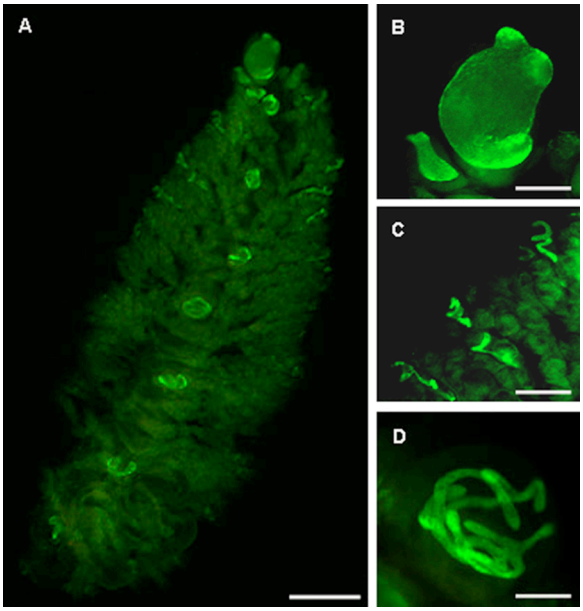


Figure 3. Proliferating zooids. A. General overview of colony demonstrating the characteristic, spatial distribution pattern of BrdU labelled zooids. Note that label decreases from the apical to the basal colony region; scale bar 100 µm. B. Enlargement of the top terminal zooid showing a large, labelled macronucleus; the smaller, labelled terminal branch zooid on the left side originated from the division of the top terminal zooid; scale bar 25µm. C. Enlargement of labelled terminal branch zooids in the apical colony region; BrdU-positive nuclei are elongated and often show corkscrew structure; scale bar 25µm. D. Enlargement of a labelled macrozooid. Note intense staining of the large, irregularly branched macronucleus extending through the whole cell; scale bar 25 µm.

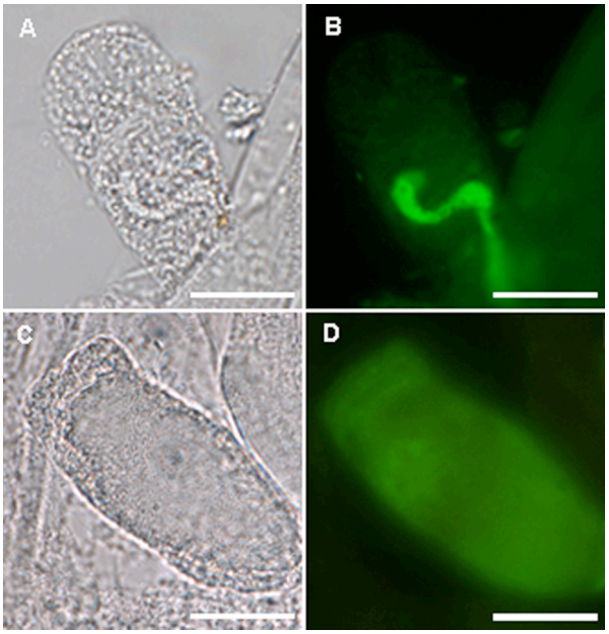


Figure 2. Light (A,C) and corresponding epifluorescence (B,D) micrographs showing the specificity of anti-BrdU antibody in macronuclei. A, B. BrdU-positive S-phase macronucleus (bright green label); scale bar 20 µm. C, D. Non-labelled macronucleus; scale bar 20 µm.

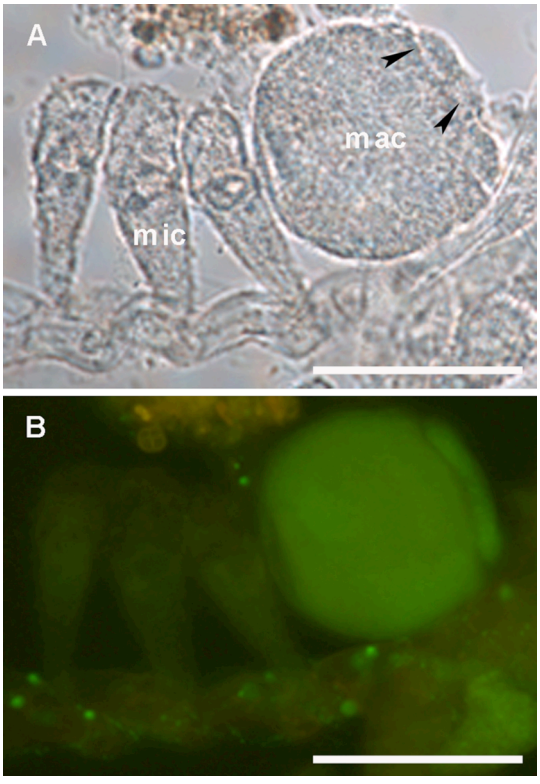


Figure 7. Macrozooid morphology. Note the morphology of the mature macrozooid (mac; arrows indicate furrow where ciliary wreath develops) compared to the slender microzooids (mic); macrozooids in this transformation stage with developed somatic ciliature never showed BrdU label. A. Light micrograph; scale bar 50 µm. B. Corresponding epifluorescence micrograph; scale bar 50 µm.

ethanol (each 5 min) and stored in 70% ethanol until further treatment.

Immunocytochemical detection

BrdU treated specimens were rehydrated in 50 and 30% ethanol (each 10 min), transferred to PBS, permeabilized in 0.1% Triton X-100, PBS for 4 h at room temperature and then treated with 30 µl/ml proteinase K in PBS for 20 min at 37°C. For DNA denaturation, specimens were exposed to 0.1 M HCl (10 min, on ice) and 2 N HCl (1 h, 37°C). After washing with PBS (3 x 5 min), unspecific staining was blocked by incubation in 3% bovine serum albumin, 0.1% Triton X-100, PBS (blocking solution) for 30 min at 37°C. BrdU incorporation was detected using monoclonal mouse anti-BrdU, Alexa Fluor 488 conjugate antibody (MOLECULAR PROBES) at 1:20 dilution in blocking solution overnight at 4°C. After 2 wash steps (each 10 min) in Tris high salt buffer (29.2 g/l NaCl, 4.4 g Tris/HCl, pH 7.5 and 0.5% Tween 20), whole mount specimens were mounted in PBS and sealed with nail varnish. All necessary positive and negative controls were carried out.

Analyses of proliferating zooids

Specimens were examined with a Nikon Eclipse E 800 epifluorescence microscope, equipped with a digital camera. Images were processed with the software program analySIS (3.2) and Adobe Photoshop (7.0). To determine a relationship between colony age and presence of proliferating zooids, we first counted the number of branches per colony in BrdU treated specimens (number of colonies n = 34). Branch number ranged from 6 to 128. Since length growth in *Zoothamnium niveum* colonies is accomplished by increasing the number of branches over time, we used the branch number to determine the approximate age of a colony and arbitrarily classified colonies with less than 50 branches as young specimens and colonies with more than 50 branches as old specimens (Fig. 1a). As only the top terminal zooid at the tip of the colony is responsible for length growth in the colony (Fig. 1b), we distinguished it in the present study from the terminal zooids of the branches (terminal branch zooids). Subsequently, we discriminated an apical, a middle and a basal colony region and recorded the spatial distribution of BrdU labelled zooids within each colony (n = 34). The percentage of BrdU labelled zooids of total zooids counted is given as the proliferation index (PI) for each zooid type, respectively. In order to increase the power of statistical comparison, similar treatments were combined into a short pulse (15 and 30 min pulse, n = 24) and a long pulse (1 h pulse, n = 10) period. Since data sets did not meet the assumption of normal distribution, differences in PI were statistically analyzed using nonparametric chi-square and Mann-Whitney's U test. Multiple data sets, such as the PI among colony regions, were compared by using the Kruskal-Wallis, followed by Tamhane`s post hoc multiple comparison test for unequal variances with a 95% confidence interval (SPSS 11.5). A probability of $p<0.01$ is considered to represent a significant difference except for Tamhane post hoc multiple comparisons where $p<0.05$ is recommended. Results are shown as means ± SE (standard error of mean).

3. Results

Incorporation of BrdU

Incubations of living *Zoothamnium niveum* colonies in 1 mM BrdU solution resulted in labelled S-phase nuclei, even after a pulse period as short as 15 min. Zooids that incorporated the Alexa 488 conjugated antibody to BrdU displayed bright green staining in the macronuclei, while none of the conducted negative controls showed any positive staining (Fig. 2). In addition, we used 30 min and 1 h BrdU pulses to increase the number of labelled S-phase zooids and to follow proliferation patterns. We followed BrdU staining in macronuclei only, because micronuclei are quite small and could not be detected reliably. Fig. 3 provides a typical distribution pattern of BrdU labelled zooids in a *Z. niveum* colony and shows that the macronuclei of the top terminal zooid, the terminal branch zooids in the apical region, and the macrozooids are heavily stained.

Proliferating zooids

Typically, the BrdU labelled top terminal zooid had a large macronucleus (about 50 µm) appearing as a compact mass that filled almost the whole zooid body (Fig. 3b). To investigate age-related S-phase activity in the top terminal zooid, we compared the proliferation index (PI) of the top terminal zooid between young specimens (n = 22) with less than 50 branches and old specimens (n = 12) with more than 50 branches (see Fig. 4). The chi-square test revealed that differences in PI of short 15 and 30 min pulse-labelled and long 1 h pulse-labelled specimens are not statistically significant. Therefore, we combined data from both, short and long incubation treatments and found that in young specimens the PI of the top terminal zooid (81%) was significantly higher ($x^2 = 6.5$, df = 1, $p = 0.01$) than in old specimens (45.5%).

BrdU incorporation was also detected in the terminal branch zooids located at the tip of the branches. S-phase labelled macronuclei of terminal branch zooids were elongated and often appeared as a corkscrew structure (Fig. 3c). Labelled terminal branch zooids were most abundant in the apical region and decreased towards the basal region of a colony (Fig. 3a). This spatial distribution pattern of

labelled zooids was evaluated by comparing the PIs of terminal branch zooids in the apical, middle, and basal colony region of young and old specimens (Table 1). After a short pulse, the PIs of terminal branch zooids in the three colony regions were significantly different from each other (Kruskal-Wallis, $p<0.01$). As depicted in Fig. 5, the PI of terminal branch zooids was highest in the apical region (9.1 ± 2.4%), followed by the middle region (1.7 ± 0.9%) and the basal region (1.2 ± 0.5) (Tamhane post hoc, $p<0.05$). These findings were independent of colony age (Mann-Whitney U, $p>0.01$) and also apparent as a trend, after a long pulse labelling period (Fig. 5).

In our whole mount preparations, the number of macrozooids varied within a *Zoothamnium niveum* colony (Fig. 6). Overall however, old specimens had significantly (Mann Whitney U, $p<0.01$) more macrozooids (15.5 ± 2.8) than young specimens (5.7 ± 1.3). These large, irregularly branched macronuclei displayed also intense staining when labelled with BrdU (Fig. 3c). We observed no characteristic distribution pattern of labelled macrozooids within a colony and there was no significant difference in the PI of macrozooids between young and old specimens (Mann Whitney U, $p>0.01$). After short pulse labelling, macrozooids showed a PI of 45.5 ± 8.0% which increased to 85.2 ± 11.3% after 1 h pulse labelling. Based on the morphology and incorporation of BrdU in macronuclei, we additionally observed different transformation stages of macrozooids within old and young *Z. niveum* specimens. Unlike all other stages (Fig. 3a), roundish and mature macrozooids with a partly developed ciliary wreath never showed BrdU label (Fig. 7).

4. Discussion

Here, we present incorporation of BrdU in proliferating zooids of vegetative growing *Zoothamnium niveum* colonies that have been found exclusively in obligatory association with ectosymbiotic bacteria. By investigating proliferation activity, for the first time on the scale of single cell analysis, we discovered different growth profiles in the heteromorphic zooids of this uniquely large ciliate. During a colony's life span (1) the top terminal zooid decreases its high proliferation activity with increasing age but appears to posses unlimited cell division potential until the death of the colony. (2) The terminal branch zooids show an age independent proliferation limited by the cell division capacity on growing branches in the apical colony region. (3) Highly proliferating macrozooids are built throughout the entire life span. In preparation for a new generation, extensive growth and DNA synthesis occur while macrozooids are attached to the colony and nourished, while during the dispersal stage, the cell cycle is arrested and resumes activity upon settlement, including mitosis and cell division.

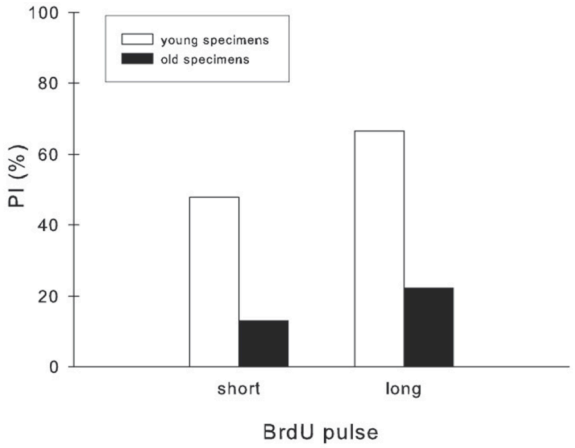


Figure 4. Proliferating top terminal zooid. Proliferation index (PI) of the top terminal zooid in young (n = 22) and old (n = 12) *Z. niveum* specimens, after short and long BrdU pulse labelling.

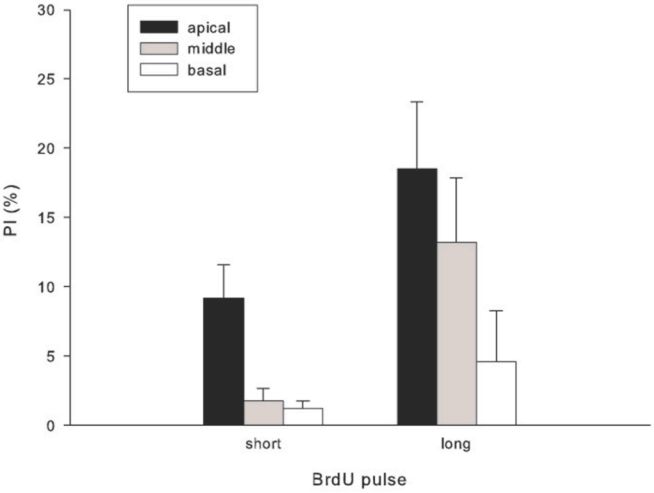


Figure 5. Proliferating terminal branch zooids. Proliferation index (PI) of terminal branch zooids (mean + SE) in the apical, middle and basal region of *Z. niveum* colonies (n = 34), after short and long BrdU pulse labelling. Independent of colony age, PI was highest in the apical region and decreased towards the basis of the colony.

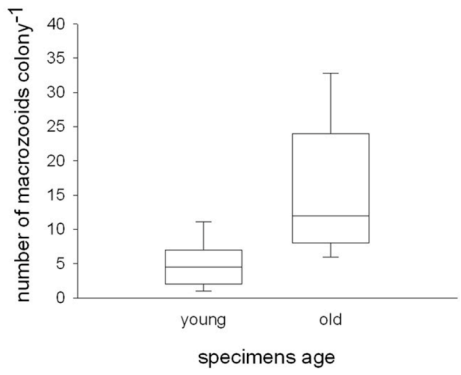


Figure 6. Box-whisker plot showing that the number of macrozooids varied among differently aged colonies; plotted are the median, the first and the third quartile.

Table 1. Age-related proliferation dynamics within colonies. Proliferation index (PI) of terminal branch zooids (mean ± SE) in the apical, middle and basal region of young (less than 50 branches, n = 22) and old (more than 50 branches, n = 12) specimens, after short (15 and 30 min) and long (1 h) BrdU pulse labelling. Comparisons between pulse periods and colony age were tested with the Mann-Whitney U test; no significant differences were found. Comparisons between apical (a), middle (m) and basal (b) region within each colony were tested with the post hoc Tamhane test (confidence level 95%) which revealed significant differences (a>m>b).

Pulse period	Colony age	Colony region apical (%)	Colony region middle (%)	Colony region basal (%)
Short (15–30 minutes)	Young	11.4 ± 3.4	2.3 ± 1.3	1.8 ± 0.8
	Old	4.6 ± 2.1	0.6 ± 0.6	0.0 ± 0.0
Long (60 minutes)	Young	16.1 ± 7.2	14.4 ± 6.6	5.6 ± 5.6
	Old	23.3 ± 0.7	10.7 ± 6.4	2.6 ± 2.6

High proliferation activity in the top terminal zooid is consistent with outstanding growth rates in symbiotic *Zoothamnium niveum* colonies. Ott et al. (2004) reported a period of exponential growth period until the third day and suggested a division rate of about 20 times per day in laboratory reared *Z. niveum* colonies. Colonies were shown to grow slower and live longer in the more temperate waters of the Mediterranean Sea. Experiments in a flow-through respirometer resulted in an 8 days rapid growth phase with division rates of up to every 4.1 hours, followed by a 3 day degeneration phase during their 11 days live span (Rinke et al., 2007). Assuming such a high division rate in growing *Z. niveum* colonies, we hypothesized the presence of fast cycling zooids and found that almost 50% of the top terminal zooids in young specimens were labelled even after a short 15 min BrdU pulse. Such a high percentage of top terminal zooids in S-phase clearly demonstrate that a fast proliferating top terminal zooid is responsible for the remarkable length growth in young *Z. niveum* colonies. Although the PI was significantly lower than in young colonies, we still found S-phase labelled top terminal zooids in old colonies, characterizing a top terminal zooid with almost unlimited cell division potential throughout a colony's life span.

Little is known about proliferation and growth rates of other peritrichs (Kusuoka and Watanabe, 1987; Sudo and Aiba, 1971). *Zoothamnium alternans* is a close non-symbiotic relative of *Z. niveum* (Clamp and Williams, 2006; Fauré-Fremiet, 1930) that is regularly found in the same habitat but distinguished from the latter by its smaller size (Vopel et al., 2001). Summers (1938) reported a division rate of 8 h in developing *Z. alternans* colonies. Interestingly, aposymbiotic *Z. niveum* colonies reared from swarmers, which had lost their bacteria in the laboratory, did not display rapid growth and appeared similar in size to *Z. alternans* (Vopel et al., 2001). In addition, ultrastructural studies (Bauer-Nebelsick et al., 1996b) indicate that *Z. niveum* gains at least part of its nutrition from its chemoautotrophic bacteria. Therefore, we suggest that the high proliferation activity of symbiotic *Z. niveum* colonies reported in our study is only possible in the presence of ectosymbionts. The ectosymbionts in return have to maintain the bacterial monolayer by efficiently covering free host surface in a highly specific pattern. Rinke et al. (2007) demonstrated that the highest frequency of dividing cells for the ectosymbiont was indeed observed on the top terminal zooid of cultured *Z. niveum* specimens.

The terminal branch zooids are limited by their cell division capacity on the branches independent of the colony's age. After a long BrdU pulse, labelled zooids were concentrated in the apical region, appeared less frequently in the middle and seldom in the basal region of a colony. These labelled zooids corresponded to terminal branch zooids proliferating at the tip of the branches. Our spatial labelling pattern indicates that proliferation activity in terminal branch zooids ceases, when the branch reaches its maximal length according to its spatial position within the colony (up to 20 microzooids, Bauer-Nebelsick et al., 1996a). Therefore, we detected high PI's only in the apical region where branches were still growing, but hardly any proliferating terminal zooids on short branches in the basal colony region. Our proliferation data give striking support that these basal colony parts are the primary regions where aging within the colony takes place, since they are the first to become regenerative inactive. Senescence is also seen in the appearance of a dense, non-specific microbial epigrowth and subsequent loss of zooids (Bauer-Nebelsick et al., 1996a; Ott et al., 1994; Rinke et al., 2006). Such phenomena are known also in sessile, colonial animals such as marine bryozoans, for which a decrease in divisions and onset of heavy microbial fouling in older zooids is reported (Palumbi and Jackson, 1983).

BrdU pulse labelling revealed that macrozooids of *Z. niveum* are fast proliferating zooids throughout the entire colony's life span that display an extremely high PI independent of their spatial position within the colony. Macrozooids are considered as fundamental dispersal units that are released to build up a new colony. In order to leave the colony they have to undergo several morphological changes such as the excessive growth in size and the production of a ciliary wreath at the aboral pole region (Bauer-Nebelsick et al., 1996a). Our findings show that

macrozooids perform DNA synthesis in preparation for building a colony after migration in the water column and settlement. Arrested in the cell cycle throughout the motile swarmer phase, mitosis and cell division occur when the swarmer settles and transforms de facto into the top terminal zooid building a new stalk. Our findings reinforce prior reports that DNA synthesis never has been found in large macrozooids with developed ciliary wreath but only in cells without somatic ciliature. The possibility of an arrested cell cycle in swarmers is supported by a molecular study of another ciliate, which shows that in the dimorphic *Eugolliculina uhligi* the expression of genes involved in the progression of the cell cycle is low or absent in swarmers, but high in trophonts (Markmann-Mulisch et al., 1999).

5. Conclusion

Thiotrophic symbioses represent spectacular adaptations to environmental gradients. They are often confronted with unstable oxic-anoxic interfaces lasting only for short times and survival can only be accomplished when growth is fuelled by sufficient nourishment through the symbionts, leading to fast cell proliferation. Microbes and hosts share overlapping life histories in the context of a dynamic standoff, each looking after its own genetic interests. In summary, the BrdU whole mount approach allowed us to gain insights into *Zoothamnium niveum* host growth profiles. In agreement with former *in situ* and cultivation studies, our cell proliferation data contributes to a better understanding of adaptations of thiotrophic associations within a broader evolutionary and ecological framework.

Acknowledgements

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