

# Glycogen storage in the *Riftia pachyptila* trophosome: contribution of host and symbionts

Angelika Sorgo<sup>1</sup>, Françoise Gaill<sup>2</sup>, Jean-Pierre Lechaire<sup>2</sup>,  
Cordelia Arndt<sup>2,3</sup>, Monika Bright<sup>1,\*</sup>

<sup>1</sup>Department of Marine Biology, Institute of Ecology and Conservation Biology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

<sup>2</sup>Biologie Cellulaire et Moléculaire du Développement, Biologie Marine UMR 7622 CNRS, Université P. et M. Curie, 7 Quai Saint Bernard, 75252 Paris, France

<sup>3</sup>Marine Biology Research Division-0202, Scripps Institution of Oceanography, La Jolla, California 92093, USA

**ABSTRACT:** Glycogen storage in host tissue and symbiotic bacteria in the anterior trophosome of the vestimentiferan tubeworm *Riftia pachyptila* Jones, 1981, was investigated using transmission electron microscope and stereological methods. The relative glycogen content (RGC) of each partner was calculated from the percentage of host and bacterial cytoplasm area taken up by glycogen and from the percentage area that host and symbionts occupy within a trophosome lobule section. Our results show that host and symbionts contribute equally to the total glycogen content of the trophosome; this ratio remains similar for up to 40 h of hypoxia. Furthermore, there is a glycogen gradient in the lobule. In both symbiotic partners, the glycogen content increased from the lobule center toward the periphery, implying different metabolic activities of host cells and bacteria depending on their location within a trophosome lobule.

**KEY WORDS:** Glycogen · *Riftia pachyptila* · Symbiosis · Trophosome · Chemoautotrophic bacteria · Hydrothermal vents

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## INTRODUCTION

The giant tubeworm *Riftia pachyptila* Jones, 1981 (Vestimentifera) is one of the most conspicuous members of the hydrothermal vent fauna at the East Pacific Rise (see Desbruyères & Segonzac 1997). Its enormous tube length (up to 1.5 m; Jones 1981) and high growth rate (Lutz et al. 1994) depend on a successful nutritional symbiosis with endosymbiotic, chemolithoautotrophic, sulfide-oxidizing bacteria (Fisher & Childress 1986, Felbeck & Jarchow 1998, Bright et al. 2000). The symbionts are harbored in the trophosome, a specialized organ in the trunk of *R. pachyptila* that is differentiated into lobules. Each lobule consists of 4 distinct areas: the axial blood vessel, the symbiont-containing host-cell area (termed bacteriocyte area), the sheath

cells, and the peripheral blood vessels (see Bright & Sorgo in press). Most of the symbionts occur individually in specialized vacuoles termed symbiosomes; they are embedded in the peribacterial space and surrounded by the symbiosome membrane of the host (Cavanaugh et al. 1981). They are  $\gamma$ -Proteobacteria of a single phylotype (Nelson et al. 1984, Stahl et al. 1984, Distel et al. 1988), but display a great variation in size and shape, depending on their location within the bacteriocyte area.

The pleomorphy of the symbionts is morphologically expressed in 4 concomitant but distinguishable bacterial types (rods, and small, large, and degrading cocci) occurring in distinct zones (central, median, peripheral, degrading; Bright & Sorgo in press), and has led to the 'gradient' and the 'cell cycle' hypotheses: (1) Biochemical gradients within each lobule result from the worm's circulatory system (with the blood flow directed from the periphery toward the center of each lobule; van der

\*Corresponding author. E-mail: monika.bright@univie.ac.at

Land & Nørrevang 1975, Felbeck & Turner 1995) that supplies the symbiotic bacteria with nutrients to differing degrees (Hand 1987). (2) A coordinated cell cycle of host bacteriocytes and symbionts would lead to a permanent process of renewal through division in the center and degradation through lysis on the periphery (Bosch & Grassé 1984a,b, Bright & Sorgo in press).

In *Riftia pachytila*, glycogen reaches concentrations of up to 100  $\mu\text{mol}$  glycosyl units  $\text{g}^{-1}$  fresh wt (Arndt et al. 1998). TEM studies have shown that the trophosome tissue of *R. pachytila* contains numerous glycogen particles at different densities (Bosch & Grassé 1984a). These are electron-dense isodiametrical  $\alpha$  particles (30 nm) usually clustered into rosettes of up to 120 nm, and are easily distinguishable from other inclusions or organelles such as ribosomes (20 nm, roundish) or polysomes (chains or rings of ribosomes) (Bozzola & Russel 1992). Glycogen serves as an important substrate for glycolysis in pro- and eukaryotes and, therefore, as an important energy source during periods of nutrient limitation. Additionally, glycogen is the only long-term energy fuel available for anaerobic metabolism (Bryant 1991).

This study determined the distribution of glycogen in different areas of the trophosome of *Riftia pachytila* kept under various conditions in high-pressure flow-through aquaria (see Arndt et al. 1998). We analyzed the glycogen density in host and bacteria separately, using quantitative morphological methods to determine the contributions of host and symbionts to the glycogen pool. The *R. pachytila* symbiosis exhibits great tolerance toward hypoxia (Childress et al. 1984, Arndt et al. 1998). Short-term experiments under similar hypoxic conditions (already known not to affect the glycogen pool as a whole; Arndt et al. 1998), were carried out to determine whether the glycogen pools of the symbiotic partners, considered separately, were affected. An increase of glycogen in the symbionts and a decrease in the host, for example, would maintain the level of the glycogen pool for the symbiosis as a whole.

In addition, we examined differences in the glycogen storage between symbiont morphotypes and host bacteriocyte zones to determine whether the gradient hypothesis or the cell cycle hypothesis is consistent with the recorded glycogen distribution. We also recorded data on the metabolism of the host cells and bacterial morphotypes, and on metabolic interactions between the symbiotic partners.

## MATERIALS AND METHODS

**Collection and incubations.** Twenty-five adult *Riftia pachytila* were collected during the 'Hot Times 1997' expedition to the hydrothermal vent site at 9° N at the

East Pacific Rise. After recovery from depths of approximately 2500 m by the submersible 'Alvin' on Dives 3183, 3184, 3190 and 3191 in an insulated collection container at ambient pressure, the tubeworms were transferred to 210 atm in a flow-through aquarium system simulating natural conditions. To maintain them in a healthy condition, the flow-through aquaria were supplied with cold sea water (15°C) containing 2 mM  $\Sigma\text{CO}_2$  (total concentration of all ionic species of inorganic carbon), 300  $\mu\text{M}$   $\Sigma\text{H}_2\text{S}$  (total concentration of all ionic species of sulfide), 120  $\mu\text{M}$   $\text{O}_2$  and 400  $\mu\text{M}$   $\text{N}_2$ . Before starting an experiment, all worms were kept at these 'maintenance conditions' for 24 h. A total of 19 specimens were incubated for different periods of time in hypoxic, sulfidic conditions, according to Arndt et al. (1998) (2 mM  $\Sigma\text{CO}_2$ , 300  $\mu\text{M}$   $\Sigma\text{H}_2\text{S}$ , 10 to 16  $\mu\text{M}$   $\text{O}_2$  and 400  $\mu\text{M}$   $\text{N}_2$ ): (1) 'short-term' hypoxia (11, 15, 24 h; n = 9), (2) 'long-term' hypoxia (40 h; n = 5), and (3) 40 h hypoxia with subsequent recovery under maintenance conditions for 17 h (n = 5). Six specimens were held in maintenance conditions for 24 h as controls. At the end of each experiment, the worms were sacrificed and samples were taken from their anterior trophosome tissue.

**Specimen preparation.** Pieces of anterior trophosome were fixed on board ship in 1.5% acrolein, 3% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) with 10% w/v sucrose added, rinsed in the same buffer, postfixed in 2% osmium tetroxide in the same buffer, dehydrated in a series of ethanol, and embedded in Spurr's epoxy resin for transmission electron microscopy. Ultrathin sections were cut on a Reichert ultracut, placed on Formvar-coated slot-grids and stained in a Reichert stainer with uranyl acetate and lead citrate.

**Stereological analysis.** Trophosome lobules were examined for local distribution of glycogen, which was found in the axial peri-sinus epithelium, in the host, in the bacteria, and in the peribacterial spaces of the bacteriocyte zones and the sheath cells. Further investigations did not include (1) the axial peri-sinus epithelium (which contributes only 1.6% glycogen to the trophosome [Bright & Sorgo in press] and in which only a few glycogen particles were visible), (2) the degrading bacteriocyte zone (in which the glycogen is already degraded and therefore not unambiguously attributable to host or bacteria) and (3) the peribacterial spaces (in which only a few glycogen particles were visible and only in those worms incubated under hypoxic conditions).

**Glycogen density (GD):** In this study, the term 'density' expresses the percentage of the cytoplasm area (i.e. volume fraction) taken up by glycogen in an ultrathin section. However, in comparing host and symbiont contributions in each area, similarities or differences

in the GD reflect trends in whole cells. Five photographs each of bacterial and host tissue in each of the bacteriocyte zones and 5 images of the sheath cells of 25 worms were taken with a cooled slow-scan camera under the electron microscope LEO 912 and printed at a final magnification of 36 000 $\times$ . As the glycogen rosettes are not uniform in color (light to dark gray) and size, they could not be identified and counted by a computer analysis program, so the stereological Chayé's dot-counting technique (Weibel & Elias 1967) was used to calculate the GD in bacteria and host in the glycogen-containing zones: a grid of 30 dots spaced at 0.65 cm intervals and covering an area of 8.45 cm<sup>2</sup> was twice laid randomly on the images.

**Relative glycogen content (RGC):** To calculate the glycogen content of each symbiotic partner, the GD was determined as a proportion of the percentage lobule area taken up by host and symbiont in the various ultrathin sections; i.e. the RGC for the host and symbionts in each area (3 bacteriocyte zones and the sheath cells) was determined, based on the GD for that area, as a percentage of the lobule area taken up by the host (A/h) or symbionts (A/s) (A/h and A/s calculated by Bright & Sorgo in press): The RGC (Table 1) was calculated with following formula:  $RGC (\%) = GD (\%) \times A (h/s)(\%) \times 10^{-3}$ .

**Statistical analysis.** The Tamhane post hoc multiple comparison test for unequal variances (SPSS<sup>®</sup>8.00) was used (CI = 95%) to compare the GD of both of the multiple data sets: (1) symbiont GD (central, median, peripheral bacteriocyte zones, separately) after maintenance, short-term and long-term hypoxia, and recovery; (2) host GD (central, median, peripheral bacteriocyte zones, and sheath cells separately) after maintenance, short-term and long-term hypoxia, and recovery. Since no significant differences were found, all 25 worms were com-

bined and the GD data sets for the central, median and peripheral zones (+ sheath cells for host tissue) were each compared between symbiont and host. The Kendall rank-correlation test checked for correlation of the host and bacterial GD within the 3 bacteriocyte zones. All values are means  $\pm$  SD.

**Size of bacteriocytes and bacteria in trophosome ultrathin sections.** Photographs of the central, median and peripheral bacteriocyte zones were made under the transmission electron microscope LEO 902, and the negatives were scanned. The mean sizes of the bacteria in a cross-section of the central (n = 500), median (n = 250) and peripheral (n = 250) zones were determined using analysis PRO<sup>®</sup>3.00. Since the cell membranes of the bacteriocytes were not continuously visible, the mean size of the bacteriocytes in each zone was based on the proportional area of bacterial and host cytoplasm in a zone (calculated by Bright & Sorgo in press) and on the average number of bacteria in a cell.

## RESULTS

**Experiments.** No significant differences in the GD between the 4 experiments (aerobic sulfidic maintenance, short- and long-term sulfidic hypoxia, and recovery) were detected in the host and bacterial tissues of the trophosome lobules. Therefore, all further GD and RGC calculations were made using the mean percentage of all 25 worms.

**Glycogen density (GD).** The different GDs in both partners of the trophosome are shown for the central and peripheral bacteriocyte zones in the transmission electron micrographs in Fig. 1. GD was not correlated between host and symbionts, and showed an inversely related trend between the symbiotic partners (Fig. 2). Bacterial GD increased from the center to the periphery, with a significant increase between the rods and the large cocci and the small and large cocci ( $p < 0.05$ ). Host tissue GD decreased from the center toward the periphery, with a significant decrease between host tissue in the central and peripheral bacteriocyte zones, and in the the median and peripheral zones ( $p < 0.05$ ). The GD in the sheath cells was significantly lower ( $p < 0.01$ ) than in any bacteriocyte host tissue.

**Relative glycogen content (RGC).** The sums of the RGCs in host and symbionts show a 1:1 ratio (1.57 and 1.46%, respectively). Since only 2% of the symbionts are rods and the remaining 98% are cocci (Bright & Sorgo in press), the vast majority of bacterial glycogen is of coccoid origin.

**Host tissue area, bacteria and peribacterial spaces and glycogen content of a single bacteriocyte in ultrathin sections.** A central rod takes up an area of  $0.81 \pm 0.54 \mu\text{m}^2$ , a small coccus in the median zone  $4.13 \pm$

Table 1. *Riftia pachyptila*. Trophosome lobule areas and mean percentage  $\pm$  SD of the relative glycogen content (RGC) in host and symbionts in the glycogen-containing zones (n = 25, subsamples = 5)

Trophosome lobule	RGC
Central bacteriocyte zone	
Host	0.08 $\pm$ 0.04
Symbionts	0.01 $\pm$ 0.02
Median bacteriocyte zone	
Host	0.66 $\pm$ 0.36
Symbionts	0.47 $\pm$ 0.46
Peripheral bacteriocyte zone	
Host	0.54 $\pm$ 0.35
Symbionts	0.98 $\pm$ 0.81
Sheath cells (host)	0.29 $\pm$ 0.25
Sum host	1.57
Sum symbionts	1.46

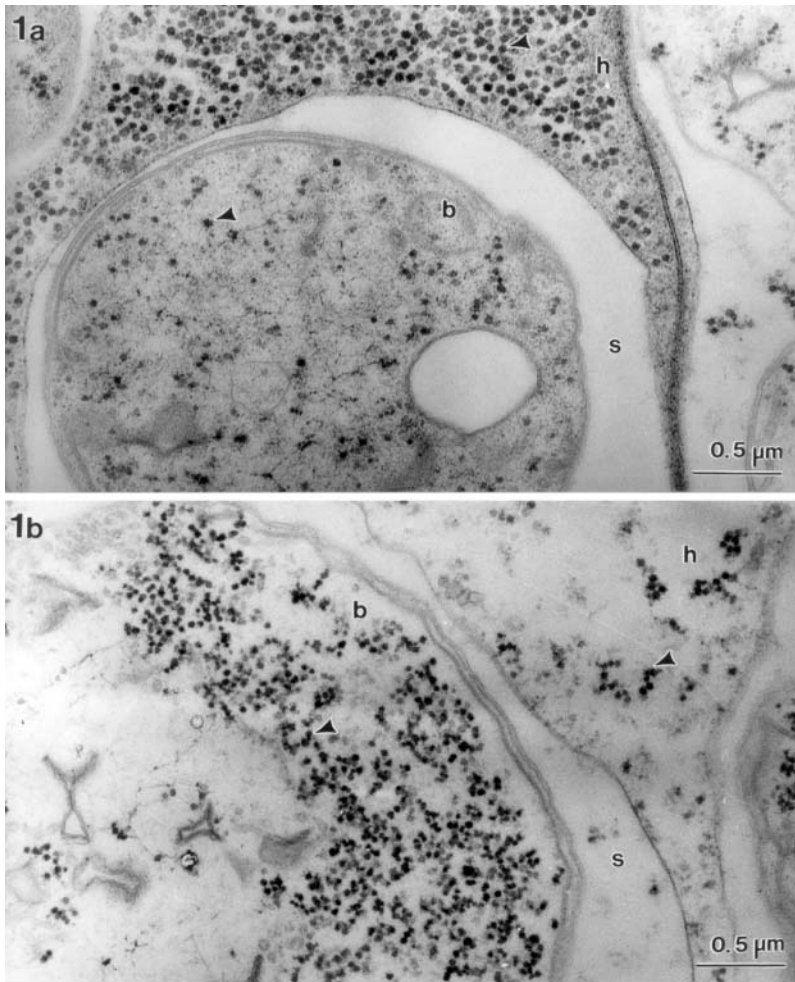


Fig. 1. *Riftia pachyptila*. Transmission electron micrographs of the trophosome, showing glycogen density in host and bacteria in (a) central and (b) peripheral bacteriocyte zones. h: host cell; b: bacterium; s: peribacterial space in symbiosome surrounding the bacterium; arrow heads: glycogen particles

$1.9 \mu\text{m}^2$ , and a large coccus at the periphery  $17.0 \pm 5.6 \mu\text{m}^2$ . Thus, from the center to the periphery of a lobule, a single bacterium increases in size by 21-fold. In a single bacteriocyte, the average number of bacteria is 36.6 in the center, 30.5 in the median zone, and 19.8 at the periphery (Bright & Sorgo in press); the area occupied by all bacteria taken up in a single bacteriocyte is  $30 \mu\text{m}^2$  in the center,  $126 \mu\text{m}^2$  in the median zone and  $341 \mu\text{m}^2$  at the periphery. Thus, the bacterial area in any 1 bacteriocyte increases in size 11 times from the center to the periphery.

The average areas of host tissue in a single bacteriocyte (central,  $38 \mu\text{m}^2$ ; median,  $76 \mu\text{m}^2$ ; peripheral,  $178 \mu\text{m}^2$ ) and the peribacterial spaces (central,  $21 \mu\text{m}^2$ ; median,  $45 \mu\text{m}^2$ ; peripheral,  $62 \mu\text{m}^2$ ) also increase toward the periphery, but only by 5 and 3 times respectively. The bacteria take up increasingly more space within the bacteriocyte from the center toward the

periphery, exceeding the host tissue area by a factor of 2 at the periphery (Fig. 3). Although the GD calculated for any defined area decreased in the host tissue, the glycogen content of the host tissue increased within each cell from the center to the periphery.

## DISCUSSION

In *Riftia pachyptila*, the glycogen content of  $100 \mu\text{mol}$  glycosyl units  $\text{g}^{-1}$  fresh wt determined in the trophosome (Arndt et al. 1998) is divided equally between host and symbionts. Although the symbionts take up only 25% of the trophosome, glycogen content is distributed equally between both partners, and this ratio remains similar for up to 40 h of hypoxia. Thus, host and symbiont each contain about  $50 \mu\text{mol}$  glycosyl units  $\text{g}^{-1}$  fresh wt of trophosome. This amount is comparable to that in other host tissues of *R. pachyptila*, e.g. in the body wall ( $35 \mu\text{mol}$  glycosyl units  $\text{g}^{-1}$  fresh wt) or the vestimentum ( $20 \mu\text{mol}$  glycosyl units  $\text{g}^{-1}$  fresh wt) (Arndt et al. 1998), to that of other chemoautotrophic symbiotic animals (Hentschel et al. 2000, Arndt pers. obs.) and to that of nonsymbiotic animals known to be adapted to long-term anoxic periods (Dales 1958, de Zwaan & Zandee 1972, Gäde 1983, Schöttler et al. 1984, Vopel et al. 1998).

The presence of glycogen in all symbionts of *Riftia pachyptila* indicates a surplus of carbon arising from autotrophy. The distinction of different symbiotic morphotypes of the same phylotype is based not only on size and shape modulations and on different locations within the trophosome lobules, but also on different physiological abilities. This has been proven for inorganic carbon fixation and incorporation rates (Bright et al. 2000), but is also evidenced by the increase in the amount of glycogen storage from the central rods to the large peripheral cocci.

Although the glycogen gradient is in accordance with the direction of blood flow and thus with the direction of nutrient supply, it does not support the gradient hypothesis (Hand 1987). Glycogen distribution is not correlated with incorporation rates of organic carbon, which were similarly high in the central rods and the large peripheral cocci after the 1 to

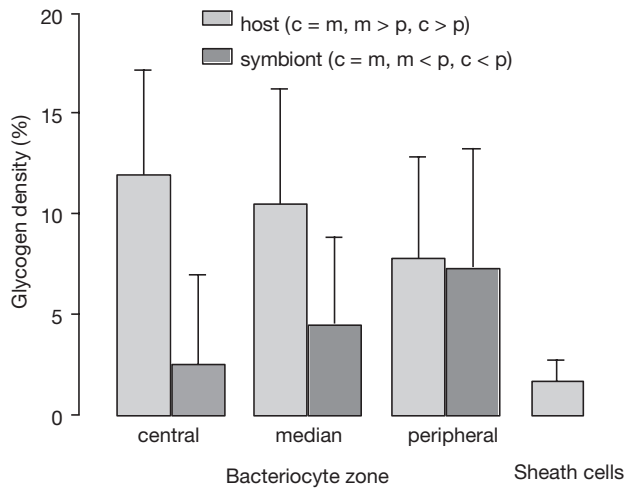


Fig. 2. *Riftia pachyptila*. Glycogen density (% of cytoplasm area taken up by glycogen) in host tissue and symbionts in central (c), median (m) and peripheral (p) bacteriocyte zones and in sheath cells of the trophosome; comparisons between the glycogen density of central, median, and peripheral host and symbiont tissues were each tested with the post hoc Tamhane multiple-comparison test ( $n = 25$ , confidence level 95%). =: not significantly different; >, <: significantly greater or less, respectively

3 h pulse,  $^{14}\text{C}$ -bicarbonate experiments. This organic carbon incorporation shows that there is no gradient in the compounds used in chemosynthesis (Bright et al. 2000). Even if there were some limitation to the formation of a nitrogen or phosphorus gradient (chemicals that, in free-living bacteria, are known to result in glycogen synthesis when organic carbon is available in excess; Preiss 1984, Preiss & Romeo 1989, Lou et al. 1997), any such limitation would result in high glycogen concentrations in the center where the pool would be depleted most and low concentrations at the

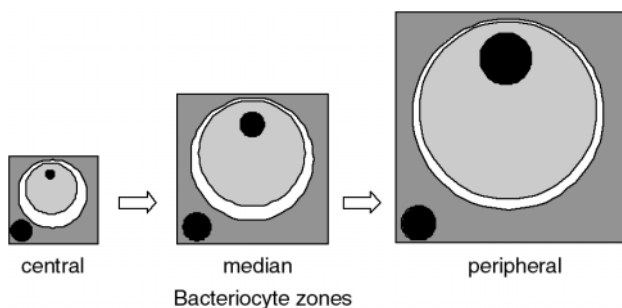


Fig. 3. Single bacteriocyte, showing areas occupied by entire host tissue (dark gray), peribacterial space (white) and symbionts (light gray) in a section, during their migration from central to peripheral bacteriocyte zones. Glycogen content (black) in both symbionts and host increases from center to periphery

periphery. However, the highest values were actually found at the periphery and the lowest in the center.

Therefore, the most reasonable explanation for the rates of incorporation of organic carbon and glycogen in the lobules of the trophosome is the cell-cycle hypothesis (Bosch & Grassé 1984a,b, Bright & Sorgo in press): (1) The incorporation rates of organic carbon reflect the high growth rates of rods and large cocci: as an increase in biomass, growth is highest in the peripheral large cocci; as an increase in cell number, it is highest in the central rods. (2) The glycogen storage may indirectly be correlated with the division rate of the morphotypes: rods (responsible for the production of the whole bacterial population) have a high energy expenditure in division, and consequently can store little glycogen, whereas large cocci, which do not undergo division, can direct surplus organic carbon to glycogen storage.

The glycogen gradient within the host bacteriocytes is the result of positive and negative carbon flows within the trophosome; i.e. the organic carbon the bacteriocytes receive from the bacteria (positive carbon flow) and the carbon used and released by the bacteriocytes (negative carbon flow).

**Positive carbon flow.** (1) Carbon is released from the bacteria into the symbiosome and diffuses further through the symbiosome membrane. This mechanism as well as the amount of translocated carbon is probably controlled by the host itself, as has been shown for many algae-invertebrate symbioses (Muscatine 1967, Taylor 1971, 1973a,b, Muscatine et al. 1972, McDermott & Blanquet 1991). However, it is not known whether the proportion of carbon released corresponds to the amount fixed. (2) After lysis of the bacteria in the degrading zone, the host cell itself degrades and all reduced compounds are released into its blood. Due to the blood flow within the lobules, the degraded compounds are once more available to the bacteriocytes.

**Negative carbon flow.** In this process, carbon is used and released by the bacteriocytes, and only surplus carbon can be stored in the form of glycogen. (1) Carbon is used for metabolic activities, e.g. growth and division. (2) The carbon flow to other host tissues via the circulatory system must be very high, as demonstrated by the high growth rate of the tubeworm (Lutz et al. 1994) and the glycogen stores in the other host tissues (Arndt et al. 1998).

Similar to the symbionts, the host bacteriocytes themselves are produced in the center of each lobule but cease to divide toward the periphery where they terminally differentiate (Bright & Sorgo in press). This indicates different physiological behaviors during the different life stages of these highly specialized host cells, which is also reflected in the differences in glycogen storage.

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