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Morphological characteristics of the digestive tract of gnotobiotic *Artemia franciscana* nauplii

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ABSTRACT

Cysts of *Artemia franciscana* were hatched and nauplii were reared under gnotobiotic conditions (gnotobiotic *Artemia* rearing system). Stereomicroscopy, computer assisted three-dimensional reconstruction, light microscopy, and transmission electron microscopy were used to study the structural and cellular morphology of their digestive tracts. The alimentary tract of gnotobiotic *Artemia* nauplii, fed with dead *Aeromonas hydrophila* and wild type strain of *Saccharomyces cerevisiae*, is a hooked, tubular structure which is composed of three clearly distinguishable parts, i.e. the foregut, midgut and hindgut that are freely suspended in haemolymph. The epithelium lining of the entire gut consists of a single cell layer. Enterocytes of the foregut and hindgut are cuboidal and lined by a thin cuticle, whereas midgut enterocytes are cuboidal to columnar and possess an apical brush border. The fore- and hindgut mainly display characteristics suggestive for mechanical functions, whereas the midgut shows characteristics of absorption, storage and secretion.
The gnotobiotic *Artemia* rearing system is most useful to investigate the effects of micro-organisms on the development of nauplii. The knowledge acquired in this study potentially facilitates the evaluation of gut morphology when specific micro-organisms are introduced into the culture system, as compared to the gnotobiotic counterparts.

1. Introduction

Successful aquaculture is still hampered by diseases of the larval phases, leading to massive mortalities and considerable economic losses (Marques et al., 2005). Antibiotic therapy and disinfectants have only had limited success in the prevention or cure of aquatic diseases (Defoirdt et al., 2004). Moreover, the frequent use of these chemicals results in rapid development of resistance (Dias et al., 1995; Molina-Aja et al., 2002; Vattanaviboon et al., 2003; Vivekanandhan et al., 2002). Therefore, it is of major importance to have alternative disease control techniques in aquaculture, focusing especially on prevention, which is likely to be more cost-effective than curative treatments (Subasinghe, 1997). Managing microbiota in larvae aquaculture is an effective preventive strategy. Hence, the study and control of harmful micro-organisms and the promotion of beneficial micro-organisms are of utmost importance.

A powerful experimental approach to study the function of the microbiota uses a gnotobiotic rearing system in which animals are reared in germ-free conditions and monitored after introducing defined microbes (Falk et al., 1998). Although many studies are performed with gnotobiotic terrestrial animals, studies with gnotobiotic aquatic organisms are still scarce. Some constraints hampering the wide use of these organisms in research are the need for disinfection methods to produce germ-free organisms and difficulties in assuring the complete germ-free condition of a culture system (Marques et al., 2006). A successful step forward in the development of such an aquatic gnotobiotic model was made more than twenty years ago when Sorgeloos et al. (1986) and later Marques et al. (2004a) described a gnotobiotic *Artemia* rearing system to obtain sterile *Artemia* cysts and nauplii. Future studies of host–microbe interactions using gnotobiotic aquatic animals should consider possible parameters, such as survival, growth, immunological response and histological development (Marques et al., 2006). The present study is aimed especially at the morphology of the gnotobiotic *Artemia* nauplii.

Although the gut morphology of conventional *Artemia* nauplii was studied in detail using transmission electron microscopy a few decades ago (Hootman and Conte, 1974), to our knowledge, this is the first descriptive study illustrating the digestive tract morphology of gnotobiotic *Artemia franciscana* nauplii. The present study aims to describe the histology, the three-dimensional architecture, and the cellular morphology of the gut of gnotobiotic *Artemia* nauplii during

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the early stages of the life cycle. The knowledge gained from this study will facilitate future research which aims at comparison of the digestive tract morphology between gnotobiotic *Artemia* nauplii and their counterparts at same age after introduction of either one or more definite strains of micro-organisms to the culture system.

2. Materials and methods

2.1. Culturing and harvesting of yeast

Wild type (WT) strain of baker's yeast (*Saccharomyces cerevisiae*) (BY4741 [genotype, *Mata his3Δ 1 leu2Δ0 met15Δ0 ura3Δ0*]), kindly provided by the European *Saccharomyces cerevisiae* Archive for Functional Analysis (University of Frankfurt, Frankfurt, Germany), was used as live feed for *Artemia*. It was cultured in minimal yeast nitrogen based (YNB), with amino acids (histidine HCl: 10 mg/l; methionine: 20 mg/l; tryptophan: 20 mg/l; inositol: 2 mg/l-Sigma, 0.67% w/v), supplemented with uracil (Sigma, 0.002% w/v), leucine (Sigma, 0.002% w/v) and D-glucose (Sigma, 0.5% w/v), agar. Harvested yeast cells were counted following the methodology described by Marques et al. (2006) and Gunasekara et al. (2010b). Yeast suspensions were stored at 4 °C and used to feed *Artemia* until the end of the experiment.

2.2. Culturing, harvesting, and killing of bacteria

The *Aeromonas hydrophila* strain (LVS3) selected for the experiment is a rod shaped, motile, gram negative, Aeromonadaceae bacterial strain. LVS3 was cultured and harvested, and the density was determined according to the methodology described by Marques et al. (2005, 2006) and Gunasekara et al. (2010b). As LVS3 cells were used for feeding, they were killed by autoclaving at 120 °C for 20 min. Bacterial suspensions were stored at 4 °C and used to feed *Artemia* until the end of the experiment.

2.3. Culturing and feeding of gnotobiotic Artemia

The experiment was carried out using *A. franciscana* originating from the Great Salt Lake, Utah. *Artemia* cysts were hydrated and decapsulated following the procedures described by Defoirdt et al. (2005) in order to obtain sterile cysts and subsequently sterile nauplii. Sterilization of necessary equipment, decapsulation of cysts and setting of culture tubes for hatching were done according to the procedures described by Gunasekara et al. (2010b). After about 20–24 h, 30 nauplii were transferred to fresh 50 ml sterile tubes containing 30 ml filtered autoclaved sea water made with Instant Ocean (Aquarium systems, Sarrebourg, France). All the culture tubes received living germ-free WT yeast and dead germ-free LVS3 as daily feed and were placed on a rotor turning at 4 rpm. Daily feeding was done ad libitum. As such 140.1 μg WT strain (ash free dry weight content) and 1401 μg dead bacteria were administered per culture tube during the six days of the study period as described by Marques et al. (2004a).

Sampling was done at days two, four and six for stereomicroscopy, light microscopy, and transmission electron microscopy and tested with four replicates for each.

2.4. Verification of axenity

The axenity of the decapsulated cysts, the *Artemia* culture water and the dead LVS3 suspension (after autoclaving) was verified using plating. Absence or presence of bacterial growth was monitored after five days of incubation at 28 °C of 100 μl of decapsulated cysts, culture medium or feed plated on Marine agar (n = 2).

2.5. Stereomicroscopy

Immediately after fixation, the specimens were viewed and photographed using a stereomicroscope (Olympus SZX 7, Olympus, Belgium) to study the gut segments in relation to external structures.

2.6. Light microscopy

Nauplii were fixed, pre-stained and further processed for histological sections following the procedures described by Gunasekara et al.

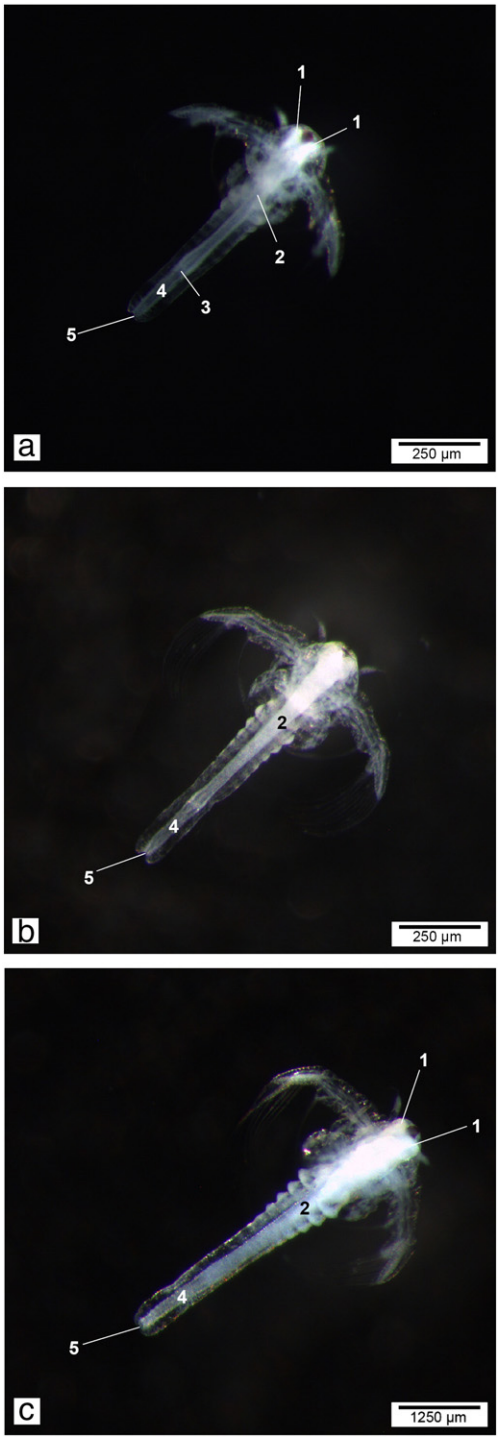

Fig. 1. Stereomicroscopic images of two day-old (a), four day-old (b), and six day-old (c) gnotobiotic *Artemia nauplii*. 1, gastric caeca; 2, midgut; 3, midgut–hindgut transition; 4, hindgut; 5, anal opening.