# Effects of Dietary Nucleotides on Growth Rate and Disease Resistance of Crustaceans Using Axenic *Artemia* Culture Tests

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## Abstract

*Effects of dietary nucleotides on growth and disease resistance of crustaceans were evaluated using axenic Artemia culture tests. Higher Artemia growth in xenic culture (15.6 ± 2.9 mm) than in axenic culture (9.2 ± 1.9 mm) reaffirmed the need to eliminate microbial populations known to influence growth and disease resistance. The study further demonstrated that dietary nucleotides supplementation at 0.005% significantly improved growth as demonstrated by higher body length (11.3 ± 3.4 mm) compared to the control (9.5 ± 1.3 mm). The nucleotides also led to improved disease resistance as demonstrated by significantly higher survival (38.3 ± 4.4%) compared to the control (16.7 ± 1.7%) after a challenge with Vibrio proteolyticus.*

***Key words:*** *Artemia*, axenic culture, nucleotides, crustacea.

## Introduction

Nucleotides are low molecular weight biological compounds, which are building blocks of DNA and RNA and play vital roles in various physiological and biochemical functions of the body. They are composed of a phosphate group, a five-carbon sugar and a weak basic nitrogenous compound. The body derives nucleotides from three potential sources namely, synthesis de novo; salvage, i.e. recycling of pre–formed bases and diet (Boza, 1998). Nucleotides are often regarded as conditionally essential nutrients particularly during periods of rapid growth and physiological stress (Uauy *et al.*, 1994). Dietary nucleotides are more preferential because de novo synthesis and salvage of nucleotides are metabolically costly processes which account for 5-10% of the energy used in synthesis of tissue protein (Carver, 1994; Grimble, 1994).

Dietary nucleotides in aquaculture have shown a number of beneficial effects. Feed intake enhancement was observed in largemouth bass (Kubitza *et al.*, 1997). Improvement in growth was observed in Tilapia (Ramadan and Atef 1991) and salmonids (Adamek *et al.*, 1996; Burrells *et al*., 2001a). Increased resistance towards pathogens has been observed in salmonids (Burrells *et al,*. 2001a; Leonardi *et al.,* 2003) and hybrid striped bass (Li *et al.,* 2004). Dietary nucleotides have also been reported to increase resistance towards stress in salmonids (Burrells *et al*., 2001a; Leonardi et al., 2003). There is however little information on the effects of supplementation of nucleotides in crustacean diets.

Artemia has been used as a model for studying nutrition and disease aspects of crustaceans (Overton and Bland 1981; Criado-Fornelio *et al.*,1989; Verschuere *et al.*, 1999). Their resting stage in the form of a cyst makes them easily available, convenient and cheaper compared to the use of animals that require long period of culturing. Moreover, *Artemia* have physiological capabilities to withstand extreme culture conditions like low oxygen concentrations (De Wachter *et al*.,1992) and high salinity (Sorgeloos *et al*., 1986). Artemia can also be easily cultured in small containers under axenic conditions in order to eliminate microorganisms to which are known to influence both growth (Verschuere *et al*., 1999) and disease resistance (Verschuere *et al.,* 2000a). This study was

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aimed at using axenic *Artemia* culture tests to evaluate beneficial effects of feeding nucleotides on growth and disease resistance in crustaceans.

## Materials and methods

### Sterile Artemia nauplii for axenic culture

The sterile *Artemia nauplii* for the axenic culture were obtained through a modification of a technique by Sorgeloos *et al,* (1986). Ten grams of Artemia cysts (EG grade, INVE Aquaculture NV, Dendermonde, Belgium) were hydrated for 1 h in 89 ml of well-aerated tap water after which 3.3 ml sodium hydroxide (32%) was added to maintain pH above 10. Decapsulation was initiated by addition of 50 ml of sodium hypochlorite and monitored under a stereomicroscope until 80% done and then 50 ml of sterile sodium thiosulphate was added to stop the process. All reagents used were placed in a refrigerator overnight to cool in order to avoid poor hatching due to excessive heat released during the process. The decapsulated cysts were rinsed with autoclaved artificial seawater of 33g/l (Instant Ocean, Aquarium Systems, Sarrebourg, France) and then put into sterile 50 ml Falcon tubes (Becton Dickinson Labware, Lincoln Park, NJ, USA) containing 30 ml of sterile seawater. The tubes were placed on a rotor and incubated for 24 hrs with sufficient light. All processes were carried out in a laminar flow chamber to ensure sterility.

### Rationale for axenic condition

The rationale for axenic conditions was determined by comparing *Artemia* growth under axenic and xenic culture conditions in triplicates for 6 days at a stocking density of 0.6 *artemia*/ml. The *Artemia* were fed with a commercial diet PROLON® (Inve Technologies NV, Dendermonde, Belgium) rate of 0.25 mg/day/nauplii on day 0 and 1 and 0.28 mg/day/nauplii from day 2 onwards.

For axenic culture, 20 sterile *Artemia nauplii* were stocked in a sterile falcon tube containing 30ml of sterile seawater (33ppt). The culture tubes were kept at 25$^{o}$C on a rotor. The feed was sterilized by an overnight gamma irradiation at 10 kGray from a particle accelerator and suspended daily in autoclaved seawater before being administered. All routine procedures were done in a laminar flow to avoid bacterial contamination. Sterility of the culture was assessed every other day by plating 100µl of the culture solution from each falcon tube on two petri dishes containing marine agar 2216 (MA) (Difco laboratories, Detroit, Mich.).

For xenic 480 non-sterile *Artemia nauplii* were cultured in 800 ml conical cylinders with sufficient aeration as described by Sorgeloos *et al.*, (1986). The cylinders were put in a water bath so as to maintain the temperature at 25oC. The *Artemia nauplii* were fed with a non-sterile feed at rate was similar to that used for the axenic culture.

At the end of the culture period Artemia from both cultures were immobilized with Lugol solution and individual body length was measured using a stereomicroscope fitted with a camera lucida (SM-1). The measured lengths were then digitized using a Graphtec Digitizer (KD 3320, Japan) and converted to actual lengths in centimetres.

### Feeding trial

Feeding trial was conducted using axenic culture to determine effects of nucleotides on growth. The commercial diet PROLON® was supplemented with ASCOGEN® at 0.002% and 0.005% as a source of nucleotides. ASCOGEN® is a natural biogenic performance enhancer comprised of purified RNA, purified nucleotides and their precursors, specific organic acids and inactivated yeast. The commercial diet without nucleotide supplement served as control. After 6 days *Artemia* were immobilized and individual body length was measured.

### Challenge test

A virulent Artemia pathogen, Vibrio proteolyticus was used for the challenge test. *Artemia* were axenically cultured for 4 days during which they were fed the commercial diet supplemented with nucleotides at 0.005%; while unsupplemented diet served as control. A bacterial suspension with density of approximately 1 × 103 cells/ml was added into the cultures on day five. The bacterial density was determined spectrophotometrically at 550 nm according to MacFarland standard (BioMerieux, Marcy l'Etoile, France). Percentage of surviving *Artemia* was determined after 24 and 48 hours.

**Statistical analysis**

One-way ANOVA and t-test was used to test differences in body length and survival respectively of *Artemia* fed diets containing different inclusion levels of ASCOGEN®. The differences were deemed significant at $p < 0.05$. Duncan's multiple range test was used for post-hoc analysis where significant differences existed in one-way ANOVA. Data presented as means ± standard error.

## Results and discussion

**Rationale for Axenic Conditions**

*Artemia* growth in axenic conditions (9.2 ± 1.9 mm) was significantly lower ($p<0.005$) compared to that in xenic conditions (15.6 ± 2.9 mm) (Figure 1). These results are similar to those reported by D'Agostino (1980) in which *Artemia* cultured in xenic conditions had higher biomass production than in axenic conditions. Xenic conditions harbour microbial populations, which may have several positive effects to *Artemia.* It is hypothesized that dissolved nutrients normally unavailable to *Artemia* are converted into bacterial biomass, which consequently becomes available to *Artemia* (Verschuere *et al.*, 1999).

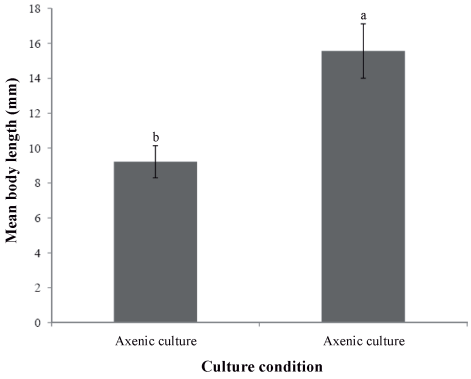


**Figure 1: Mean body length of Artemia cultured in xenic and axenic conditions. Error bars indicate standard deviation. Different letters above each bar denotes significant differences in length ($p<0.05$)**

Furthermore, the bacteria may prevent proliferation of opportunistic pathogens through release of chemical substances that have bactericidal or bacteriostatic effects (Verschuere *et al.,* 2000b). All these demonstrate the positive influence of microbial populations on *Artemia* and hence the rationale for axenic culture when evaluating effects of feed additives on growth and disease resistance.

**Effects of nucleotides on growth**

Effects of different inclusion levels are given in fig.2. The treatment with ASCOGEN® supplementation at 0.005% resulted into significantly higher body length (11.3 ± 2.4 mm) ($p<0.05$), almost 18% better than the control (9.5 ± 1.3 mm). Similar results were also observed by Hernandorena (1990) where *Artemia* nauplii reared in a medium rich in RNA grew faster than those reared in a medium devoid of RNA.

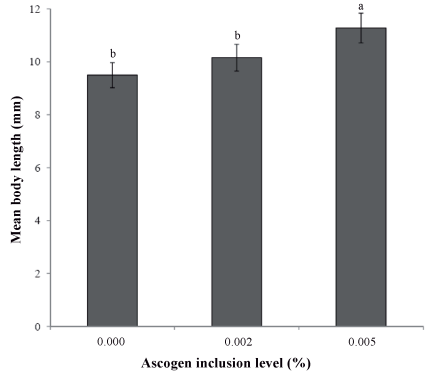


**Figure 2: Mean body length of axenically cultured Artemia after 6 days of culture. Error bars indicate standard deviation (n=20). Different letters above each bar denotes significant differences in length ($p<0.05$).**

Nucleotides appear to influence growth in several ways. They tend to enhance feed intake as many external sensory organs in aquatic organisms contain receptors for nucleotides. Mackie (1973) hypothesized that nucleotide (AMP) and nucleoside (inosine) components were the main chemo – attractants for crustaceans like lobsters. Improved feed intake due to nucleotide inclusion has been observed in puffer fish (Kiyohara *et al*., 1975), jack mackerel (Ikeda *et al*., 1991), rainbow trout (Rumsey *et al.,* 1992) and largemouth bass (Kubitza *et al*., 1997). They also play a role in development of tgastrointestinal tract leading to increased surface area for nutrient absorption as observed in mice (Uauy *et al*., 1990), Atlantic salmon (Burrells *et al*., 2001b) and sea bream (Borda *et al.,* 2003).

**Effects of nucleotides on disease resistance**

*Artemia* in both treatments were observed to have swimming difficulties within 24 hrs after a challenge with *V. Proteolyticus* but without any significant difference in survival. High mortalities were observed after 48 hrs but survival in the treatment with 0.005% ASCOGEN® supplement was significantly higher (38.3 ± 7.6%) than in the control (16. 7 ± 2.9%) (Fig. 3).

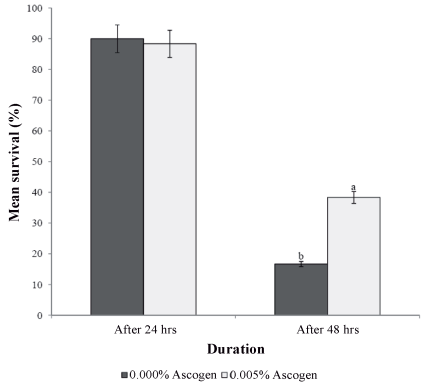


**Figure 3: Survival of axenically cultured Artemia after 24 hrs and 48 hrs challenge with Vibrio proteolyticus (n=20). Error bars indicate standard deviation. Different letters above each bar denotes significant differences in length (p<0.05).**

Improved disease resistance due to dietary nucleotides has been observed in salmonids (Burrells *et al.,* 2001b; Leonardi *et al.*, 2003) common carp (Sakai *et al.*, 2001) and hybrid striped bass (Li *et al.*, 2004). The nucleotide supplement may have contributed in several ways towards the observed higher survival of *Artemia*. Immune response requires cell growth and multiplication and creating demand for nucleotides. At the same time, juveniles have a high rate of growth, which again demands an ample supply of nucleotides for DNA replication. The presence of dietary nucleotides enhances immune response by availing adequate amounts of nucleotides required. Dietary nucleotides are reported to support optimal functioning of metabolically active cells during stressful conditions like infection or gut injury, which requires rapid repair (Rudolph *et al.,* 1990). This has been observed in humans supplemented with Uracyl, which resulted in optimal functioning of rapidly proliferating lymphocytes (Carver and Walker, 1995). Dietary nucleotides are also reported to promote intestinal microflora, which impedes proliferation and/or growth of pathogenic bacteria (Carver and Walker 1995; Burrels *et al.,* 2001b). Dietary nucleotides are believed to be vital for immunity in shrimps hence lessening harmful effects of *V. proteolyticus*, which is known to penetrate through the gut epithelium causing damage to the underlying tissue and eventually death (Devresse, 2000; Verschuere *et al.,* 2000a).

## Conclusion

This study has shown that exogenous nucleotides can significantly improve growth and disease resistance in *Artemia*. It is recommended that further studies be conducted to understand the exact mode of action, the dose – response relationship and the duration of the effects after intake of nucleotides.

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