

# Developing and understanding the use of pre-biotics in Homarid lobster culture

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## Abstract

Limited use of antibiotics in culture environments has led to the search for alternative substances which can not only protect against disease but also enhance performance. Immunostimulants have shown in recent years to act as excellent alternative substances for antibiotics especially in agriculture, this has opened a market for their potential use in aquaculture situations where high mortalities during larval phases cause high levels of loss in stock production.

## Introduction

The application of antibiotics in both aquaculture and agriculture has become limited in recent years due to usage restrictions brought about by concerns of antibiotic resistance and food quality. With the use of antibiotics having diversified from not only disease control but to growth promotion (Waldroup et al., 2003) there is huge pressure on the industries to find suitable alternatives. These alternatives must therefore offer both protection from disease and performance enhancement.

Natural immunostimulants (both pre and pro-biotic substances) have been shown, as dietary supplements in agriculture, to reduce the risk of disease via activation of an organism's innate immune response as well as improving digestibility of various dietary substances. Similar results have also been found in a small number of aquacultural species (Daniels et al., 2006), however research is limited. Examples of such alternative substances include oligosaccharides such as mannan-oligosaccharide and fructo-oligosaccharide (Lji et al., 2001). These have shown the potential to reduce the common diseases such as those cause by *Vibrio* spp. which cause huge problems in all cultured species. There is therefore a clear calling for alternative products such as this in aquaculture.

Pre and pro-biotics have been shown to possess immunostimulant properties. Pre-biotics are indigestible carbohydrates which stimulate the growth and activity of beneficial bacteria of the intestine and can activate the innate immune responses of cultured organisms when used as a dietary supplementation. Pre-biotics have also

increased the efficiency of the digestive tract in many organisms. This is done by increasing the regularity, height and integrity of the gut villi (Hooge, 2004) and acting as an alternative binding site for pathogenic growth inhibiting microbes (bacteria) inhabiting the gut (Lji et al., 2001). Examples of pre-biotic immunostimulants include Mannan Oligosaccharide, which is derived from the cell wall of the yeast, *Saccharomyces cerevisiae* (Miguel et al., 2002; Fritts and Waldroup, 2003), and various forms of Fructo-oligosaccharides. Pro-biotic immunostimulants are cultures of living beneficial bacteria which, with oral application, have improved the host's health by inhibiting the colonisation and growth of some pathenogenic micro organisms, and compete with other pathenogenic micro organisms for resources such as nutrients and space within the digestive tract (Vine et al., 2006).

Unlike agricultural studies, which used pre-biotics as a direct dietary supplementation in dry feed, the inclusion of immunostimulants in larval diets for aquaculture must occur via indirect bio-encapsulation. Live *Artemia* shrimp are non-selective obligate filter feeders so it is possible to manipulate them during rearing, through enrichment or bio-encapsulation, to replicate the specific nutritional requirements of the species being cultured (Dhont and Stappen, 2003). This trait allows for the indirect inclusion of immunostimulants, into larval diets.

Initial dietary supplement studies, using Mannan Oligosaccharide pre-biotics at a recommended dosage for dry feed, showed increased growth and survival during the larval stages of European lobster (*Homarus gammarus*) culture (Taylor, 2005). This demonstrated the potential of pre-biotics to reduce the effects of bacterial diseases which cause low larval survivability within crustacean culture and also to improve larval growth by increased food breakdown and so nutrient uptake.

Research was conducted on *H. gammarus* at the National Lobster Hatchery (NLH) in 2005 to determine the effect of various dietary concentrations of Mannan Oligosaccharide (Bio-Mos<sup>®</sup> aquagrade – Supplied by Alltech, Lexington, Kentucky, US) on their growth and survival. The results showed short term effects of increased larval survival to stage IV, with concentrations of 2ppt and 20ppt, in comparison to larvae fed the control diet excluding Mannan Oligosaccharide (Daniels et al., 2006). However the results also identified high concentrations of Mannan Oligosaccharide to have negative effects on the survival of lobster larvae. This indicated that only certain dietary concentrations of immunostimulants positively effect the survival of *H. gammarus* throughout their larval stages of development.

Trials were therefore conducted at the NLH in 2006 to further investigate the use, and improve the understanding of dietary immunostimulat supplements throughout crustacean culture.

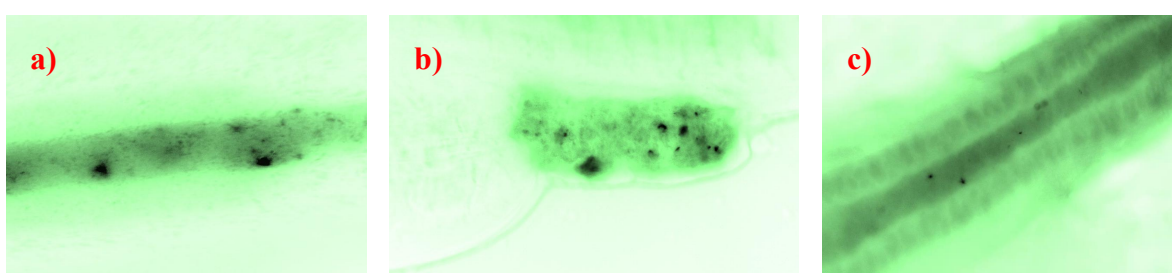
### **Fluorescent labelling trials**

Research was conducted to determine how, and in what concentration immunostimulants were taken up by *Artemia*. The indirect inclusion of enrichments through *Artemia* makes it difficult to determine at what concentration the given substance is reaching the cultured organism. Fluorescent dye was chosen to label Bio-

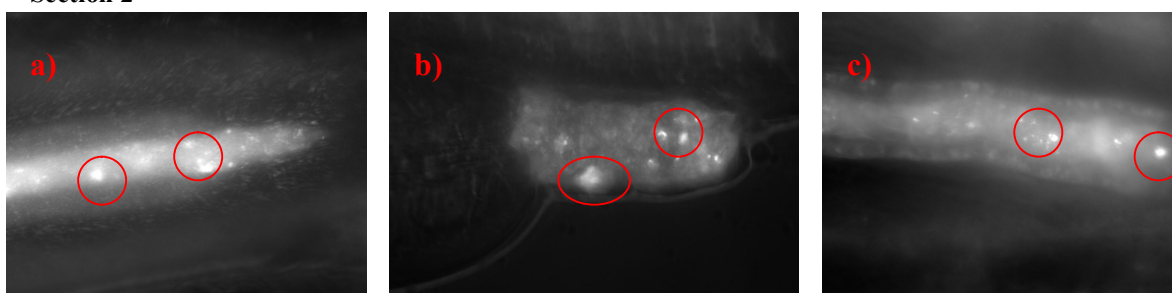
Mos<sup>®</sup> in order to trace its route and presence through *Artemia* and subsequently lobster larvae.

*Artemia* were enriched with fluorescently labelled Bio-Mos<sup>®</sup> over a 24 hour period and the process was analysed under fluorescence microscope at 0, 1, 5, 16, 20 and 24 hours. Fluorescently labelled Bio-Mos<sup>®</sup> particles appeared present in the *Artemia* guts from as early as one hour, with evidence of excretion of Bio-Mos<sup>®</sup> seen at around 16 hour. As enrichment time progresses the particle size of Bio-Mos<sup>®</sup> appears reduced (see Figure 1), this is supported by particle size analysis, where with elapsing time a reduction in particle size occurs with the presence of *Artemia* as shown in figure 2. The control solution, lacking *Artemia*, does not show this pattern, so removing suspicion that reduction in particle size may be due to Bio-Mos<sup>®</sup> dissolving.

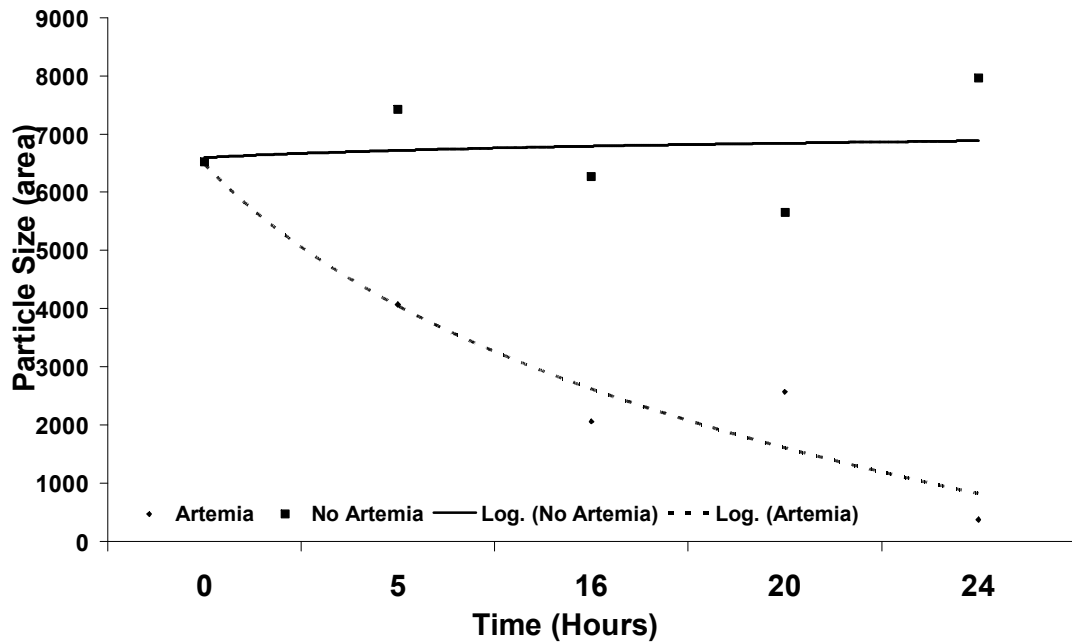
### Section 1



### Section 2



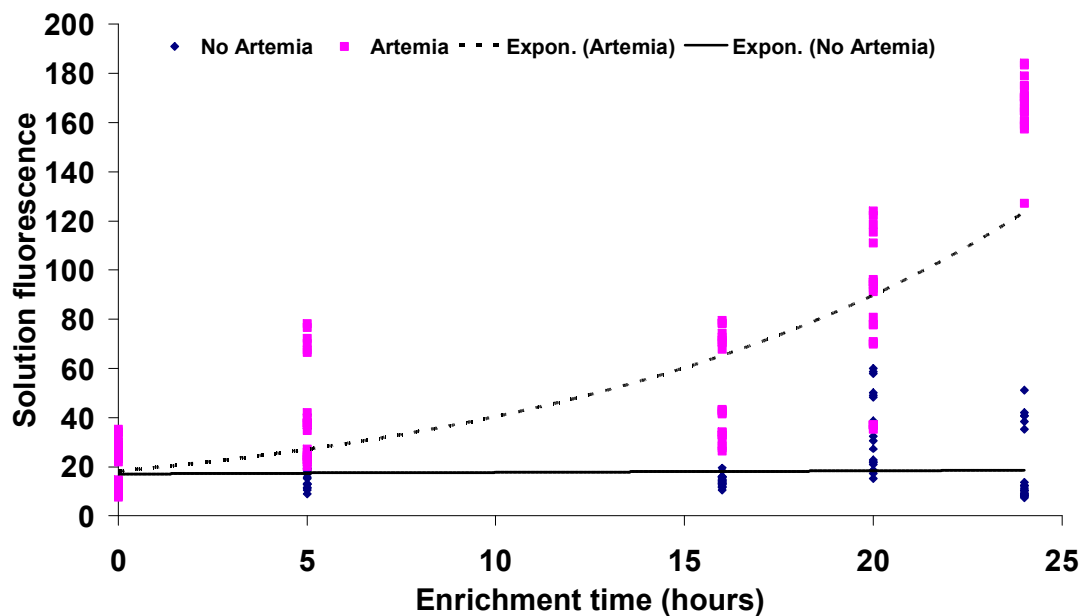
**Figure 1.** **Section 1** Negative Fluorescence microscope photos in the green spectrum depicting the internal gut sections and external views of *Artemia* at various stages through a 24 hour enrichment period. **Section 2** Fluorescence microscope photos depicting the internal gut sections and external views of *Artemia* at various stages through a 24 h enrichment period. Red circles depict the fluorescently labelled particles of Bio-Mos<sup>®</sup>. **a)** Photo taken 1h into the enrichment period **b)** Photo taken 16h into the enrichment period depicting an external view of *Artemia* faeces at the hind end of the organism **c)** Photo taken at the end of the enrichment period (24h).



**Figure 2.** Effects of the presence of *Artemia* on the particle size of fluorescently labelled Bio-Mos<sup>®</sup> in a solution over 24 hours.

### Solution fluorescence

*Artemia* proved to increase the background fluorescence of a given solution containing fluorescently labelled Bio-Mos<sup>®</sup> (see Figure 3) indicating potential breakdown of Bio-Mos<sup>®</sup> by *Artemia*. Fluorescently labelled Bio-Mos<sup>®</sup> was found to be present through the gut of the *Artemia* and in the excretion, as shown in Figure 1. The indication of increased solution fluorescence caused by Bio-Mos<sup>®</sup> breakdown by *Artemia* is also supported by the clear reduction in particle size.



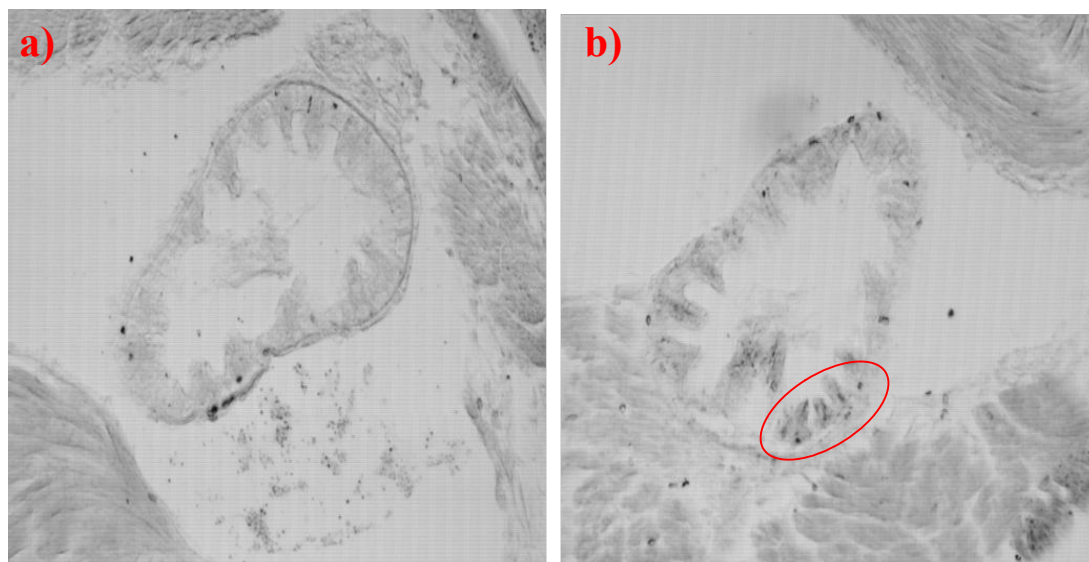
**Figure 3.** Effects of the presence of *Artemia* on the background fluorescence of a solution containing fluorescently labelled Bio-Mos<sup>®</sup> over a 24 hour period.

Bio-Mos<sup>®</sup> is undoubtedly orally ingested by *Artemia* and slowly broken down over a 24h enrichment period. With Bio-Mos<sup>®</sup> appearing to pass through *Artemia* within 16 Hours of enrichment it may therefore be suggested that an optimum enrichment time may be lower than previously used in the culture situation. However, further work would be needed to determine optimum enrichment time of other substances in the enrichment media, such as Selco<sup>™</sup>.

### **Gut morphology**

Lobster larvae were then fed with *Artemia* containing fluorescently labelled Bio-Mos<sup>®</sup> for 10 days. Throughout this period samples were taken for analysis to evaluate the presence of Bio-Mos<sup>®</sup> in the gut. Larval tails were set in wax and sections of 10µm were cut at the University of Plymouth, UK. Sections were mounted onto microscope slides and the wax removed. These unstained sections were analysed for the presence of fluorescently labelled Bio-Mos<sup>®</sup> and also for physical properties of gut wall lining, using laser scanning confocal microscopy (carried out at the Marine Biological association, Plymouth, UK).

From initial viewing of gut section photos there appears to be darker patches in the epithelial folds, this indicates fluorescence. These darkened patches are not visible in the control fed samples. It may be that the Bio-Mos<sup>®</sup> particle size is too small for the specified magnification and so appears as patches of dark where many particles are present. To validate such conclusions further research is needed to analyse gut villi sections in the epithelial folds.



**Figure 4.** Laser scanning confocal microscopy gut section photos negative colours from larval lobsters tail sections a) fed on a control diet b) fed a fluorescently labelled Bio-Mos<sup>®</sup> diet. Red circles denote areas of Bio-Mos<sup>®</sup>.

### **Synergistic use of immunostimulants**

Bio-Mos<sup>®</sup> is one of numerous immunostimulants which have shown the ability to activate innate immune responses when used as a dietary supplementation. It was

therefore logical to further study the additional effects of  $\beta$ -glucans and other pre-biotics. These could potentially, as a supplement to Bio-Mos<sup>®</sup>, have greater positive effects on the growth and survival of European lobsters during high stress larval periods where resistance to pathogens appears innately low.



**Larval European lobster**

Four larval lobster diets were trialled over a two week period. Mixed origin larvae were reared in a 12 Kreisel cone recirculation system with a maximum of 2500 larvae per cone. At the moult to stage IV (approximately two weeks of growth) the miniature lobsters were separated into individual rearing pens to prevent cannibalism.

Larvae were fed daily from hatching through to stage IV of growth on 5 *Artemia* per ml of the specified enrichment. *Artemia* were harvest and enriched over a 48h protocol (de-capsulated cysts re-hydrated and hatched for 24h, then hatched *Artemia nauplii* enriched for a further 24h). Four different enrichments were used, as described in Table 1.

Data collected from this trial showed no significant effect on larval survival and growth with the simultaneous dietary use of pre-biotics and  $\beta$ -Glucans. However, the potential simultaneous use of pre and pro-biotics is a top priority for investigation in future studies. Pre-biotics decrease the number of harmful pathenogenic bacteria in the digestive tract whereas pro-biotics increase the number of beneficial bacteria in the digestive tract. Therefore, simultaneous use could potentially have greater positive effects on the growth and survival of cultured crustaceans during high stress larval periods where resistance to pathogens appears innately low.

**Table 1.** Daily enrichment solution for *Artemia* of four experimental diets.

Diet	Enrichment quantities					
	Selco (g) <sup>1</sup>	Distilled water (g)	Bio-Mos <sup>2</sup> (g) 20ppt	Beta MAK C85 <sup>3</sup> 5mg/g (g)	Aquacite <sup>4</sup> 0.25mg/g (g)	Macroguard <sup>5</sup> 0.5mg/g (5% solution) (g)
Control	6	3	0.072	0	0	0
1	6	3	0.072	0.03	0	0
2	6	3	0.072	0	0.015	0
3	6	3	0.072	0	0	10.26

<sup>1</sup> Bio-Mos<sup>®</sup> Aquagrade, Alltech, Lexington, Kentucky US

<sup>2</sup> Beta MAK C85<sup>™</sup>, James. A. Mackie (Agricultural), Clackmannanshire, UK.

<sup>3</sup> Aquacite<sup>™</sup>, James. A. Mackie (Agricultural), Clackmannanshire, UK.

<sup>4</sup> Macroguard, Biotec Pharmacon ASA, Tromsø, Norway.

## **Immunostimulant inclusion into juvenile diets**

In order to understand the use of immunostimulants in aquaculture it is important to consider and examine their exploitation at all stages throughout a culture period. The consideration of artificial diets for juvenile stages of growth which require larger food sources to satisfy nutritional and physical dietary requirements was essential especially when considering the inclusion of immunostimulants. Frozen natural foods have proved very successful as juvenile diets, however, are not practical when the inclusion of dietary additive are taken into account. A pellet was therefore formulated (Table 2) based on previous formulations trialled in tropical spiny lobster (*Panulirus ornatus*) culture (Smith et al., 2003, Barclay et al., 2006).

Four lobster diets were initially trialled, a dry and a wet formulation of each of the two experimental pellets (Control and Bio-Mos<sup>®</sup>). These preliminary trials showed non suitability of the wet pellet due to lack of physical stability during the feeding process. A dry pellet feeding experiment was therefore set up and run for a duration of 8 weeks.



**Juvenile European lobster**

730 juvenile lobsters were placed into individual Orkney pots, to prevent cannibalism, and sorted by age into three groups; stage V, VIII and X. These were then separated evenly between two raceways, one for each experimental pellet type. The raceways were provided with flow through filtered seawater at 19°C with salinity at 35 g L<sup>-1</sup>. Prior to the experiment the juveniles were fed on a diet of frozen adult brine shrimp for between 8 and 24 weeks dependent on age. During the experimental period juveniles were then fed once every two days with one-two pellets, dependent on size. The response of the juveniles in terms of survival and growth were monitored between the two experimental pellets over a 30 day period. Microscopy photographs were taken at day one, ten and thirty in order to determine growth rates over the 30 days.

### **Diet formulation**

Two 1mm extruded pellet diets were made up with the inclusion of Bio-Mos<sup>®</sup> manipulated at the expense of starch (cornstarch). Diets were prepared by mixing the dry ingredients, after which the warm distilled water was added to form soft dough. The dough was thoroughly mixed and pressed through a 1 mm die attached to electrohydraulic sausage filler. The spaghetti like strands were subsequently reduced to 5 mm in length while wet. This is noted to be an appropriate size for juvenile lobsters due to observed ingestion with minimal wastage and fragmentation occurring (Smith et al., 2003), preliminary trials on juvenile *H. gammarus* also showed this to be the case. The pellets were separated while wet then dried overnight in a slow cook oven and stored at room temperature until used.

**Table 2.** Growth and survival responses of Juvenile *H. gammarus*

Lobster Diet and Age	Development response				
	Initial Length (mm)	End Length (mm)	Total Growth (mm)	Growth Rate (GR) (% day)	Survival (%)
<b>Control-S5</b>	6.12	6.87	0.75	0.41	94.32
<b>Bio-Mos-S5</b>	5.99	7.20	1.22	0.68	93.18
<b>Control-S8</b>	8.04	8.53	0.49*	0.20*	100
<b>Bio-Mos-S8</b>	7.76	8.96	1.20*	0.51*	100
<b>Control-S10</b>	10.04	10.17	0.13*	0.04*	98.75
<b>Bio-Mos-S10</b>	9.69	11.19	1.51*	0.52*	98.13

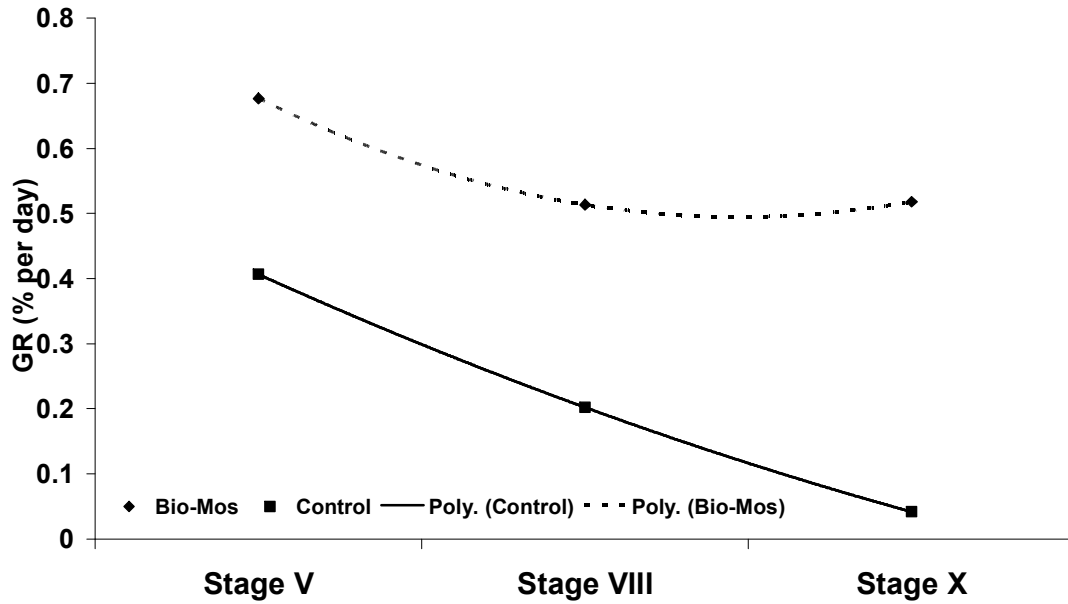
S = Stage of growth

\* denotes significance differences (p<0.05)

In this experiment survival rate was high but unaffected by the diet fed. Despite this, the potential for effects on survival must not be discounted. The scope for a longer trial, which was not feasible in the time scale of this study, would provide the ability to monitor survival over a more realistic period probably up to a year.

Lobster growth did exhibit a clear response to dietary inclusion of Bio-Mos<sup>®</sup> during juvenile stages of development studied, with the benefit of increased size being apparent. Maximal growth responses occurred with the inclusion of Bio-Mos<sup>®</sup> at later stages of juvenile lobster development (VIII-X), with juveniles growing, on average, nearly twice (1.5\*) the rate of the control feed at stage V and a huge 12.5 times the rate at stage X of development (Figure 5).

The growth rates (GR) obtained in this study are much higher (0.68 %/day) compared to that reported by other studies (Jones et al., 2001., Smith et al., 2003) trialling extruded feeds on lobsters (0.15-0.2 %/day). However, there is a dearth of research pellet feeds and European lobsters and these studies consider different species, so may not be directly comparable. Even so, there are still definite increases in growth with the inclusion of Bio-Mos<sup>®</sup> into juvenile lobster diets in this study which are directly comparable.



**Figure 5.** Effects of dietary presence of Bio-Mos<sup>®</sup> on the GR response of clawed European lobsters fed diets with and without the addition of Bio-Mos<sup>®</sup> for 40 days.

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