Author version: *Estuar. Coast. Shelf Sci.*, vol.111; 2012; 147-150

Gut fluorescence analysis of barnacle larvae: an approach to quantify the ingested food

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A B S T R A C T

Gut fluorescence analysis can provide a snapshot of ingested food and has been employed in the feeding studies of various organisms. In this study we standardised the gut fluorescence method utilising laboratory reared barnacle larvae (*Balanus amphitrite*) fed with mono-algal diet *Chaetoceros calcitrans*, a unicellular diatom at a cell concentration of 2×10^5 cells ml^{-1} . The gut fluorescence of IV-VI instar nauplii was found to be $370(\pm 12)$ nanograms chlorophyll *a* larva $^{-1}$ and in faecal pellets it was $224(\pm 63)$ nanograms chlorophyll *a* larva $^{-1}$. A pheopigment concentration in larval gut was found to be $311(\pm 13)$ nanograms larva $^{-1}$ and in faecal pellets it was $172(\pm 61)$ nanograms larva $^{-1}$. The study also included analysis of larval samples collected from the field during different seasons from a tropical environment influenced by monsoons (Dona Paula bay, Goa, west coast of India), with characteristic temporal variations in phytoplankton abundance and diversity. Gut fluorescence of larvae obtained during post-monsoon season was consistently higher when compared to pre-monsoon season, suggesting the predominance of autotrophic forms in the larval gut during post-monsoon season. Whereas, low gut fluorescence obtained during pre-monsoon season indicated the ingestion of food sources other than autotrophs. Such differences observed in the feeding behaviour of larvae could be due to differential availability of food for the larvae during different seasons and indicated the capability of larvae to feed on wide range of food sources. This study presents the utility of fluorescence method in the feeding studies of planktotrophic organisms and in the evaluation of ecosystem dynamics.

Keywords: Barnacle larvae, Gut fluorescence, Phytoplankton, Chlorophyll *a*, Pheopigments, Tropical environment, Monsoons.

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1. Introduction

Barnacles are dominant inhabitants of the intertidal region. Development in these organisms includes planktonic naupliar stages followed by a pre-settling, non feeding cyprid stage. Food available to the nauplii has a deterministic influence on barnacle population in an area (Anil et al., 1995; Anil and Kurian, 1996; Pechenik et al., 1998; Anil et al., 2001; Desai and Anil, 2004). Laboratory studies have shown that diatoms are one of the most preferred food by the larvae (Qiu and Qian, 1997; Anil et al., 2001; Desai and Anil, 2004; Desai et al., 2006). Some of the recent studies have also shown that they can feed on wide range and size of food organisms (Turner et al., 2001; Vargas et al., 2006; Gaonkar and Anil, 2010). However, the food preference and requirements of larvae in the wild is still not well understood.

A measure of gut fluorescence is one approach to quantify the food ingested by the larvae, even though there are certain limitations in the method such as it can detect only the ingested phytoplankton and there is also a possibility of pigment destruction in the guts to non-fluorescent compounds (Conover et al., 1986; Head, 1992; Head and Harris, 1996; McLeroy-Etheridge and McManus, 1999; Pasternak, 1994 and the references therein). Analysis of pigments in the guts of grazers has become a popular tool for the last few decades for measuring the feeding activity of planktonic herbivores (Nemoto, 1968; Mackas and Bohrer, 1976; Baars and Oosterhuis, 1984; Kleppel et al., 1988; Durbin and Campbell, 2007; Lopez et al., 2007 and the references therein). However, this method has not been employed so far in the feeding studies of barnacle larvae.

The larval samples were collected from the Dona Paula bay, Goa (located along the west coast of India). This locality is influenced by monsoons and has characteristic temporal variations in phytoplankton abundance and diversity. In this study, we initially standardised the gut fluorescence technique to assess the larval feeding using laboratory reared larvae. This technique was then employed to evaluate the gut fluorescence of barnacle larvae collected from the field. Through this efforts are made to understand the seasonal variability in availability of food for the larvae.

2. Materials and methods

Gut fluorescence method which was described by Mackas and Bohrer (1976) to measure the *in situ* feeding rate of planktonic herbivores and then by Lopez et al. (2007) to estimate the copepod nauplii ingestion rates on phytoplankton was adapted in this study to analyse the feeding of barnacle nauplii. Prior to analysing the gut fluorescence of field collected larvae, experiments were conducted

with laboratory reared larvae to examine the influence of various factors on experimental procedure and to standardise the gut fluorescence protocol for the analysis of barnacle larval feeding.

2.1. Larval rearing in the laboratory

Larval development in barnacles consists of six naupliar instars and a pre-settling cyprid instar. The I instar nauplius is non-feeding and moults into II instar within a short interval of time. II to VI instars are phytoplanktivorous. Nauplii obtained from the adult broods of the barnacle *Balanus amphitrite* were mass reared in 2 L glass beakers on a daily diet of *Chaetoceros calcitrans*, a unicellular diatom at a cell concentration of 2×10^5 cells ml^{-1} . The cultures were maintained at 20°C ($\pm 1^\circ\text{C}$) temperature in the incubators at 12h:12h, Light:Dark cycle. Larval density was maintained by monitoring the mortality of larvae every day, prior to changing the food.

2.2. Gut fluorescence analysis of laboratory reared larvae

The larvae belonging to IV-VI instars were utilised for the analysis of gut fluorescence. Before analysing the fluorescence, larvae were washed 5-6 times with 0.22μ filtered sea water to remove the adhering phytoplankton cells in which the larvae were reared. In preliminary experiments, to establish the minimum number of larvae needed to obtain valid gut fluorescence measurements, samples with different number of individuals (10, 20, 30, 40 and 50) were analysed and 30 individuals were found to be appropriate for the analysis of gut fluorescence. Background fluorescence of the larvae was also determined by starving the larvae for 24 hours and it was found to be negligible. For each treatment, measurements were taken from 10 different replicates. To avoid gut evacuation and photodegradation of the gut contents, washing of the larvae, sorting and picking processes were carried out in a short interval of time by working under a dissecting microscope with minimum exposure to light.

Pigments were extracted in 2ml of 90% acetone, kept overnight at 4°C in dark condition without homogenization (extraction was also tried with homogenised larvae and the differences in readings were found to be negligible). After extraction overnight, the solution was centrifuged and the upper clear solution was measured using a Turner Design (Model-Triology) fluorometer in the laboratory illuminated with dim light. Fluorescence was measured before and after acidification with HCl (Parsons et al., 1984) and the pigment concentration is expressed as nanogram per individual larva.

2.3. Fluorescence analysis of faecal pellets defaecated by laboratory reared larvae

For the analysis of faecal pellets, larvae were reared till IV-VI instars and sorted (30 larvae) from the mass rearing beaker (2 L glass beaker) into a 50ml beaker containing 0.22 μ filtered seawater and were incubated for 24 hours for the evacuation of gut and to obtain pellets. After incubation, the larvae were separated out and the incubation water containing faecal pellets was filtered on to 0.22 μ filter paper by carefully rinsing the beaker. The pellets were then picked from the filter paper by working under dissecting microscope with minimum exposure to light and were used for pigment extraction following the procedure mentioned above for larvae. Five replicate samples were analyzed for this purpose.

Following the laboratory experiments, larval samples were collected from the field to measure the gut fluorescence of field collected larvae and to study the temporal variations in gut fluorescence of field collected larvae.

2.4. Field collection of larvae

Larval samples were collected from the Dona Paula bay, Goa (west coast of India) with the help of a Haron-Trantor net of mesh size 100 μ m. Horizontal plankton tows were undertaken for the purpose using a mechanised boat. Immediately after collection they were brought back to the laboratory and kept in 5 litre containers with additional seawater (also collected from the same area from where the larvae were collected). The containers were supplied with moderate aeration and maintained at normal room temperature and natural light. Barnacle larvae of stage IV-VI instars were immediately sorted under the dissecting microscope into 0.22 μ m filtered seawater and were washed 4-5 times with 0.22 μ m filtered seawater to clean and remove the adhering phytoplankton cells and dirt from the surface of larvae and were then utilised for the analysis of gut fluorescence following the above mentioned protocol for laboratory reared larvae.

The study area is broadly categorized in to 3 seasons based on the influence of monsoons i.e. pre-monsoon (February-May), monsoon (June-September) and post-monsoon (October-January). Larval samples were collected during post-monsoon and pre-monsoon seasons (samples were collected every alternate month in each season). Samples representing post-monsoon season were collected during October (12th, 14th and 16th) and December (14th, 16th and 18th) 2009. Samples representing pre-monsoon season were collected during March (15th, 17th and 19th) and May (17th, 19th and 21st) 2010. Five replicate samples were analysed from each sampling events.

2.5. Data analysis

Gut fluorescence estimations of larvae obtained from different sampling months were subjected to one-way analysis of variance (ANOVA) (Sokal and Rohlf, 1981) to evaluate the variance between different sampling months (temporal variations). Data were log transformed before being subjected to ANOVA to ensure normality of means and homogeneity of variance.

3. Results and Discussion

Fluorescence analysis of the laboratory reared larvae indicated that the mean chlorophyll *a* content of the larval gut is 370 (± 12) nanogram larva⁻¹ and the faecal pellets defaecated by laboratory reared larvae had 224 (± 63) nanogram chlorophyll *a* larva⁻¹ (Fig. 1). A pheopigment concentration in the larval gut was found to be 311 (± 13) nanogram larva⁻¹ and the faecal pellets had 172 (± 61) nanogram pheopigment larva⁻¹ (Fig. 2).

As could be observed, gut fluorescence values obtained from the laboratory reared larvae was much higher when compared to field collected larvae. Standardization of gut fluorescence method utilising laboratory reared larvae provided a basis for further investigation of larval feeding in the field and their temporal variations. Application of gut fluorescence method to field collected larvae offered some useful insights into the food web dynamics of this region.

During post-monsoon season (October and December 2009) chlorophyll *a* content of the larvae was in the range of 27.64-35.70 nanogram larva⁻¹, whereas during pre-monsoon season (March and May 2010) it was in the range of 1.89-13.02 nanogram larva⁻¹. Chlorophyll *a* content of the larvae collected during post-monsoon season was found to be consistently higher when compared to pre-monsoon season (Fig. 3). Analysis of variance (ANOVA) also showed a significant variation in gut fluorescence of larvae obtained during different months ($p \leq 0.0005$). Chlorophyll *a* content of the larvae collected during post-monsoon season was found to be eleven times less compared to laboratory reared larvae whereas chlorophyll *a* content of the larvae collected during pre-monsoon season was found to be seventy times less compared to laboratory reared larvae (Fig. 4).

Gut fluorescence of the field collected larvae during post-monsoon season was significantly higher than the pre-monsoon season. Such differences in the feeding behaviour could be due to differential availability of food for the larvae. Probably during the post-monsoon season larval food

consisted of mostly autotrophic forms, whereas during the pre-monsoon season, larval food consisted of sources other than autotrophs.

Generally the pre-monsoon season, which is mostly warmer, is known to be characterized by lower chlorophyll *a* concentration in the water column. Microzooplanktons such as protozoans, ciliates and heterotrophic dinoflagellates are generally known to be dominant during the pre-monsoon season. The summer months (i.e. pre-monsoon season) are also reported to have higher numbers of bacteria in the water column (Thakur and Anil, 2000). The area is dominated by diatoms during the post-monsoon season, whereas during the pre-monsoon season dinoflagellates also contribute substantially along with diatoms (Patil and Anil, 2008).

Microzooplanktonic forms which are known to be abundant during the pre-monsoon season can successfully feed on bacteria which lead to the possibilities of alternative food chain when there are less of autotrophic forms. Such an alternative food chain can have greater implications from the point of biological production potential in an area which eventually determines the success of higher trophic level organisms.

Seasonal variations in natural community assemblages and the adaptability of larvae to such variations were reflected in gut fluorescence analysis of larvae. Earlier studies carried out in the region to quantify the food available for larvae also demonstrated that the feeding rate of larvae does not change during different seasons although the phytoplankton community composition is influenced by temporal changes (Gaonkar and Anil, 2010). Successful metamorphosis of larvae in the field during pre-monsoon season, as observed in settlement and recruitment studies of barnacles carried out in the region (Gaonkar, 2012) also revealed the capability of larvae to survive on wide range of food sources. Besides, studies by Turner et al. (2001) and Vargas et al. (2006) have shown the potential of barnacle larvae to feed on small flagellates and autotrophic picoplankton at relatively high rates along with diatoms. Based on these observations it is possible to infer successful larval development in barnacles on varying forms of food sources.

The results of gut fluorescence analysis in this study might have also been affected by size and morphology of potential phytoplankton food available in the area, since phytoplankton cells that are too large cannot be ingested by the larvae. Other than this, the degree of degradation and pigment loss in the guts of larvae can also vary under different circumstances as shown in case of copepods (Dagg and Walser, 1987; Penry and Frost, 1991; Head, 1992; Head and Harris, 1996), although we cannot directly compare the results of our studies with copepods. The laboratory

experiments in this study showed pheopigments in the guts of larvae to an extent of 46%. In faecal pellets, the pheopigment concentration was found to be around 43% out of the total proportion of pigments found. Further experiments are needed in order to estimate the degree of degradation and pigment loss in the guts of barnacle larvae collected from the field during different seasons. Nevertheless the method provided promising results and is in agreement with earlier studies carried out in the region using different means (Gaonkar and Anil, 2010).

Acknowledgements

We are grateful to Dr. S.R. Shetye, Director, National Institute of Oceanography for his support and constant encouragements. The Ballast Water Management Programme, India (Directorate General of Shipping, Government of India) supported this work. We thank our colleagues from the division for their ever-willing help and cooperation during this study. We also thank the anonymous reviewers for their valuable comments on the earlier version of this manuscript. C.A.G. acknowledges the Research Fellowship provided by Council of Scientific and Industrial Research (CSIR), India. This is a NIO contribution #####.

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Legends to figures

Fig. 1. Chlorophyll *a* content of the laboratory reared larvae and their faecal pellets.

Fig. 2. Pheopigment concentrations of the laboratory reared larvae and their faecal pellets.

Fig. 3. Chlorophyll *a* content of the larvae collected from field during post-monsoon and pre-monsoon seasons (Error bar indicates standard deviation from the means of 5 replicates).

Fig. 4. Comparison of chlorophyll *a* content of the laboratory reared larvae and field collected larvae during post-monsoon and pre-monsoon seasons.

Fig. 1. **Fig. 2.**

Fig. 3.

Fig. 4.