

**Exploration and metamorphosis in Balanus amphitrite Darwin ( Cirripedia ;  
Thoracica ) cyprids: significance of sugars and adult extract**

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**Abstract**

Earlier observations have shown that sugars in solution adsorb electrostatically through –OH groups to polar groups associated with the cypris larva temporary adhesive (CTA). Lens culinaris agglutinin (LCA)-binding sugar chains of the adult extract (AE) have been suggested to be involved in the settlement of Balanus amphitrite. In the present study experiments were carried out to assess how cypris larvae would explore and metamorphose when treated with LCA specific sugars (i.e. D-glucose and D-mannose). The influence of D-galactose was also assessed similarly. Evaluation of sugar-treated as well as non-treated cyprids was carried out with AE-coated and non-coated multiwells containing filtered sea water (FSW). D-mannose-treated cyprids resulted in higher metamorphosis than the non-treated cyprids at  $10^{-8}$ M, whereas cyprids treated with the other LCA specific sugar (D-glucose) showed an inhibitory effect in the absence of AE. This result implies the involvement of D-mannose moieties of AE in the settlement promotion of Balanus amphitrite. The barnacle cyprid, while exploring some surfaces, leaves behind 'footprints' of temporary adhesive. In the absence of AE, sugar-treated cyprids did not deposit footprints. Concurrently, the sugar-treated cyprids deposited footprints when exposed to multiwells coated with AE. A comparison of observations

between single cyprid and multiple cyprid assays showed a similar trend, thus indicating that larva-larva interaction may not play an important role, when such pretreated larvae are subjected to assays. The third antennular segment with its attachment disc is the most obvious point of contact between the cyprid and the substratum during exploration. The detection of AE, even after blockage of polar groups of CTA on the third antennular segment, suggests availability of alternate sites for pheromone reception.

*Keywords:* Balanus amphitrite ; cyprid; metamorphosis; footprints; sugars

## **Introduction**

Most marine invertebrate larvae select certain environments by metamorphosing in response to cues associated with them (Pechenik, 1990; Pawlik, 1992). The identification of the exact nature of these cues remains an active field of study. The life cycle of Balanus amphitrite includes planktotrophic larval development consisting of six naupliar instars and a non-feeding cypris instar. The first instar nauplii do not feed and molt into the second instar within a few hours. Instars II to VI are phytoplanktotrophic. The cyprid, which is the settlement stage larva of the barnacle Balanus amphitrite, has been used to study the cues influencing settlement and metamorphosis (Clare et al., 1992; Crisp, 1990; Holm, 1990; Maki et al., 1994; Pechenik et al., 1993; Rittschof et al., 1992; Wieczorek et al., 1995; Yamamoto et al., 1995).

Many barnacle species show a gregarious response towards adult and juvenile conspecifics. Arthropodin or settlement factor, a glycoprotein present in the adults is thought to be responsible for this behavior (Knight-Jones, 1953; Knight-Jones and Crisp,

1953; Crisp and Meadows, 1963). Clare et al. (1995) reported the involvement of cyclic AMP in the pheromonal modulation of barnacle settlement.

The barnacle cyprid is discriminating in its choice of settlement site and in order to exercise its power of discrimination it has to explore the surface. While exploring some surfaces, cyprids leave behind 'footprints' of temporary adhesive (Walker and Yule, 1984), which are believed to be secreted by antennular glands that open out onto the antennular attachment disc (Nott and Foster, 1969). This temporary adhesive serves to hold the cyprid onto the substratum while it searches for a suitable place to settle. The cyprid footprints have also been reported to induce settlement of other cyprids, even in the absence of conspecific adults (Walker and Yule, 1984; Yule and Walker, 1985; Clare et al., 1994).

Chemical cues such as bacterial exopolymers have also been shown to be involved, the composition of which can influence subsequent settlement by invertebrate larvae (Maki et al., 1988, 2000). Three neutral sugars, D-mannose, D-glucose and D-galactose, form the most common constituents of bacterial exopolysaccharides from both marine and freshwater environments (Sutherland, 1980). The interactions between bacterial exopolymers and cypris temporary adhesive are most likely to be effected via polar groups. The strength of these interactions will determine how well a cyprid adheres to a filmed surface, which in turn will provide a further cue in determining the settlement potential of that surface (Yule and Crisp, 1983; Neal and Yule, 1992).

Neal and Yule (1996) while studying the effects of dissolved sugars upon the temporary adhesion of cypris larvae of five barnacle species from four families reported D-glucose to show a common, concentration-dependent, inhibitory effect for the five species;

maximum inhibition occurred at  $10^{-8}$ M glucose. D-mannose and D-galactose showed similar activity to D-glucose. Recently, Lens culinaris agglutinin (LCA)-binding sugar chains of the adult extract (AE) have been implicated in the settlement of Balanus amphitrite (Matsumura et al., 1998a). LCA binds to glucose and mannose. The settlement-inducing activity is associated with 3 major subunits with estimated molecular masses of 76 (often present as a possible dimer), 88 and 98 kDa. Moreover, three LCA-binding subunits of settlement-inducing protein complex (SIPC) were isolated by SDS-PAGE and found that each individual subunit also induced larval settlement, suggesting an important role of specific sugar chain of SIPC in the settlement of Balanus amphitrite (Matsumura et al., 1998b). Immunostaining studies indicated that the SIPC was present in the footprints of the cyprids (Matsumura et al., 1998c).

Taking into consideration the above perspectives, experiments were carried out in order to assess how cypris larvae would explore and metamorphose when treated with LCA specific sugars (i.e. D-glucose and D-mannose). The influence of D-galactose was also assessed similarly. Evaluation of sugar-treated cyprids was carried out with AE-coated and non-coated multiwells containing filtered seawater (FSW). This was carried out in order to observe how a cyprid would behave when the polar groups associated with CTA are blocked by cues such as sugars and under such conditions how AE influences the search behavior and metamorphosis response. The settlement assays were conducted using single as well as multiple cyprids.

In this investigation, we present the first study on the exploratory behavior and subsequent metamorphosis response of cyprids when subjected simultaneously to sugars and AE.

## Materials and methods

### Preparation of adult extract (AE)

The adult extract (AE), which stimulates the settlement of barnacle cyprids, was prepared by following the method of Larman et al. (1982). Adults of Balanus amphitrite, collected from the intertidal area of Dona Paula (15° 27.5' N, 73° 48' E), were brought to the laboratory and cleaned by brushing off the epibiotic growth on their shells using a nylon brush. These animals were then washed and ~100-g wet wt. of whole adults were crushed with a mortar and pestle using 100 ml of deionised water. The supernatant of the crushed mixture was decanted, centrifuged (12,000 x g for 5 min) and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged (12,000 x g for 5 min) and then frozen at -20°C until further use. The protein content of the extract was estimated following the method described by Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard. A protein concentration of 50 µg ml<sup>-1</sup> of AE was used for all assays.

### Rearing of Balanus amphitrite larvae

Balanus amphitrite nauplii were mass reared in 2-liter glass beakers, using filtered seawater of 35 ‰ salinity, on a diet of Chaetoceros calcitrans, at a concentration of  $2 \times 10^5$  cells ml<sup>-1</sup>. The feed organism was replenished every day while changing the water. After 5-6 days the cyprids obtained were siphoned out and stored at 5°C prior to settlement assays. Two-day-old cyprids were used to carry out the assays. Rittschof et al. (1984) have described these methods in detail.

### Treatment with sugars

The cyprids were immersed in seawater containing different concentrations ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-5}$  or  $10^{-3}$ M) of D-glucose, D-galactose or D-mannose for 5 min. In the case of single cyprid assays, cyprids were immersed individually into the sugar solutions and after removal were transferred each to a separate multiwell for the assessment of footprint deposition and metamorphosis. On the other hand, in case of multiple cyprid assays, approximately 25-30 cyprids were used and treated similarly for the assessment of metamorphosis. This method has been described by Neal and Yule (1996). All the sugar solutions were made up in millipore-filtered (0.22  $\mu$ m), UV- irradiated seawater.

### Visualization of footprints

The sugar-treated, as well as non-treated cyprids, were siphoned out and introduced individually into six-well plates (Corning- 430343) coated with 50  $\mu$ g ml<sup>-1</sup> AE and to non-coated wells, each containing 5 ml of millipore- filtered, autoclaved seawater at 35 ‰ salinity. The experiments were carried out with single cyprids in order to prevent larva-larva interactions. The experiments were repeated three times using three different batches of larvae with six replicates at each trial (n=6, with batch as an additional factor). The cyprids were allowed to explore the wells for 2 hours at 20°C (there was no settlement during this time), after which the wells were emptied and stained for footprints with a protein dye reagent (Bradford, 1976) as described by Walker and Yule (1984).

### Assay protocol for evaluation of metamorphosis

About 25-30 sugar-treated or non-treated cyprids were introduced into wells of 24-well plates coated with AE and to non-coated multiwells (Corning-430262) along with 2 ml of autoclaved, filtered seawater at 35‰ salinity (Maki et al., 1990). The above treatments were repeated employing a single cyprid (single cyprid assay). The AE-coated surfaces were prepared by inoculating the multiwells with AE at a protein concentration of  $50 \mu\text{g ml}^{-1}$ . After 3 hours, the multiwells were washed 3 times with autoclaved filtered seawater after which the cyprids were introduced.

The assays were repeated four times using four different batches of larvae with four replicates at each trial ( $n=4$ , with batch as an additional factor) and were maintained at  $26 \pm 1^\circ\text{C}$  on a 12 h Light: 12 h Dark cycle. They were monitored every 24 hours for a period of 4 days and metamorphosed cyprids were counted at the end of each day. Fresh wells were used each time while repeating the assays.

### Statistical analysis

The influence of different concentrations of D-glucose, D-galactose and D-mannose and larval batch on footprint deposition by the cyprids was evaluated by three-way ANOVA (Sokal and Rohlf, 1981). Two-way ANOVA was performed to evaluate the differences in metamorphosis with respect to cyprids treated with different concentrations of three sugars and the non-treated cyprids in presence or absence of AE. A three-way ANOVA was also carried out to evaluate the differences in metamorphosis with respect to sugar type, concentration and age of the cyprids exposed to AE-coated or non-coated

surfaces. The data on metamorphosis (%) was arcsine transformed to ensure normality and homogeneity of variances before subjecting to statistical analysis, whereas the raw data on footprint deposition was square-root transformed. The influence of sugar-treated and non-treated cyprids in the presence of AE on the deposition of footprints was evaluated by one-way ANOVA. A post-ANOVA Scheffe's test was done to test the difference between the treatments on footprint deposition (Sokal and Rohlf, 1981).

## Results

The footprints were densely stained and roughly oval in shape making them easily distinguishable from adsorbed glycoprotein of the adult extract; the footprints measured about 30-37 $\mu$ m across. The numbers of footprints deposited by sugar-treated cyprids in the presence of AE in the assay wells at different concentrations of D-glucose, D-galactose and D-mannose are shown in Figure 1. The number of footprints deposited by a cyprid increased with increasing concentration of sugars. In the absence of AE, footprints were not deposited by the cyprids treated with sugars. One-way ANOVA indicated that the number of footprints deposited by non-treated cyprids in the presence of AE were significantly different from the sugar-treated cyprids ( $p \leq 0.001$ , One-way ANOVA;  $p \leq 0.05$ , Scheffe's test). Three-way ANOVA performed between all three sugars, larval batch, and different sugar concentrations revealed a significant difference in the footprint deposition with respect to sugar type at different concentrations (Table 1).

The metamorphosis response of the cyprids after 24 hours is shown in Figures 2a and 2c. The cyprids treated with D-mannose resulted in maximum metamorphosis at a concentration of  $10^{-8}$ M when exposed to wells devoid of AE and the metamorphosis rate was almost twice that observed with the non-treated cyprids (Fig. 2a). Such a response



was not given by cyprids treated with D-glucose or D-galactose. At  $10^{-8}$ M, D-glucose showed an reduced effect. Two-way ANOVA also indicated significant differences in the metamorphosis rates at  $10^{-8}$ M concentration with treatments (sugar-treated and non-treated) and with sugars in the presence and absence of AE ( $p \leq 0.025$  &  $p \leq 0.001$ ).

D-mannose-treated cyprids metamorphosed in higher percentages in the presence of AE but less than the non-treated cyprids (Fig. 2c). The results on day 4, which reflect the response of ageing of cyprids, showed a substantial increase in the metamorphosis rates (Figs. 2b and 2d). Three-way ANOVA revealed that the metamorphosis differed significantly with respect to sugar type and cyprid age and with sugar type and concentration when exposed to AE-coated or non-coated surfaces (Table 2).

When all the treatments were assessed using single cyprids (Fig. 3), a trend similar to that observed with the assays employing multiple cyprids (Figs. 2 and 3) was obtained. The metamorphosis rates were higher in the presence of AE and D-mannose facilitated maximum metamorphosis at  $10^{-8}$ M when exposed to wells without AE.

## Discussion

It has been hypothesized that sugars in solution adsorb electrostatically through  $-OH$  groups to polar groups associated with the cypris temporary adhesive (CTA) (Yule and Walker, 1987). Higher sugar concentrations block more polar groups, thus nullifying their contribution to adhesion resulting in lower adhesion thresholds below those for cohesive failure (Neal and Yule, 1996). Many of the extracellular signalling molecules act at very low concentrations (typically  $\leq 10^{-8}$ M) and the receptors that recognize them usually bind to them with high affinity (affinity constant  $K_a \geq 10^8 \text{ l mol}^{-1}$ ) (Bruce et al., 1994).

The results of the present investigation showed that D-mannose triggered metamorphosis significantly at a concentration of  $10^{-8}$  M and the metamorphosis rate was almost twice that observed with the non-treated cyprids when assessed in the absence of AE. However, the other LCA specific sugar (D-glucose) showed an reduced effect, which suggests the involvement of D-mannose moieties of AE in the promotion of Balanus amphitrite Settlement. Earlier investigations have reported stimulation, inhibition or no effect of bacterial films on the attachment of barnacle cyprids (Visscher, 1928; Harris, 1946; Crisp and Meadows, 1962; Tighe-Ford et al., 1970). Previous studies on the effect of bacterial films on cypris larvae (Maki et al., 1988, 1990; Holmström et al., 1992; Avelin Mary et al., 1993; Neal and Yule, 1994a,b) have generally found such films to reduce either settlement or adhesion. Neal and Yule (1996) consider that the structure of bacterial exopolymers is capable of either determining the effectiveness of the cypris temporary adhesive or affecting the cyprid's 'willingness to detach' (Yule and Walker, 1984). Thus the results of the present investigation suggest that exopolysaccharides, rich in D-mannose, would be most effective in triggering metamorphosis. However, larvae are likely to respond to more than one sensory stimulus when searching for a settlement location, and some factors, such as naturally produced bacterial metabolites, may override the importance of others (Maki et al., 1989).

In the absence of AE, sugar-treated cyprids did not deposit footprints suggesting that the response of cyprids towards sugars was quick thus resulting in either metamorphosis promotion or reduction without further exploration. During search behavior the most obvious point of contact between the cyprid and the substratum is the attachment disc of the third antennular segment (Nott 1969; Nott and Foster, 1969). However, settlement

factor could also be detected in solution which directs attention to the fourth antennular segment with its array of sensory setae (Gibson and Nott, 1971; Clare and Nott, 1994). Flicking of the fourth antennular segment with its associated setae is evident while a cyprid explores a substratum (Balanus balanoides, Gibson and Nott, 1971; Balanus amphitrite, Clare and Nott, 1994) and suggests an analogy to the flicking action of decapod antennules (Schmidt and Ache, 1979). Secondly, recent evidence has been obtained in support of the role of cAMP in cyprid settlement (Clare et al., 1995). A laser ablation technique to evaluate the role of sensory setae of cyprid antennules was also advocated for identifying the sites of pheromone reception (Clare et al., 1994). Clare and Matsumura (2000) suggested that barnacle settlement induction involves receptor-ligand interactions and a signal transduction pathway(s) that translates into permanent attachment and metamorphosis. The detection of AE even after blockage of polar groups of CTA on the third antennular segment with its attachment disc, suggests the availability of alternate sites for pheromone reception. It is possible that the settlement proteins of AE are detected by the receptors on the fourth antennular segment via olfaction. The absence of AE rendered these sites non-functional, thus the cyprids responded to sugars in either the promotion or reduction of metamorphosis without further search. This aspect needs attention and will be helpful in identifying the role of alternate pheromone reception sites. Clare et al. (1994) recommended that whenever feasible, barnacle settlement assays should employ a single larva. However, a comparison of the results of single cyprid and multiple cyprid assays showed a similar trend, thus indicating that larva-larva interaction may not play an important role, when such pretreated larvae are subjected to assays.

Although D-mannose proved to be an effective cue in eliciting metamorphosis, all the cyprids did not show a similar response. The cyprids that metamorphosed successfully may be the ones that were physiologically fit. The non-feeding cypris larvae have to depend upon the energy reserves incorporated during planktotrophic naupliar development. The nutritional as well as environmental conditions seem to jointly determine the energy status of the larvae (Anil and Kurian, 1996; Anil et al., 2001). Older larvae had increased rates of metamorphosis. Earlier research has indicated that larval age is known to affect settlement. In the laboratory, the older cyprids responded more readily to external cues than the recently formed ones due to the decrease in the response threshold with larval age (Rittschof et al., 1984). A possible explanation for this fact could be that young cyprids which are more discriminating during settlement than older cyprids become less discriminating with age (Rittschof et al., 1984; Crisp, 1988) presumably due to the decline in their energy reserves and physiological quality.

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### **Legends to figures**

Fig. 1 Mean number of footprints deposited by sugar-treated cyprids exposed to AE-coated surface. (C\*- non-treated cyprids exposed to AE-coated surface; C - non-treated cyprids exposed to non-coated surface).

*Please note:* Footprints were not deposited by sugar-treated cyprids when exposed to non-coated surface.

Fig. 2 Percentage metamorphosis of cyprids (multiple cyprid assay).

(a) & (b) sugar-treated and non-treated cyprids (control) exposed to non-coated surface.  
(c) & (d) sugar-treated and non-treated cyprids exposed to AE-coated surface. Vertical lines indicate the standard deviation from mean.

Fig. 3 Percentage metamorphosis of cyprids (single cyprid assay).

(a) & (b) sugar-treated and non-treated cyprids (control) exposed to non-coated surface.  
(c) & (d) sugar-treated and non-treated cyprids exposed to AE-coated surface. Vertical lines indicate the standard deviation from mean.

C\* - Non-treated cyprids

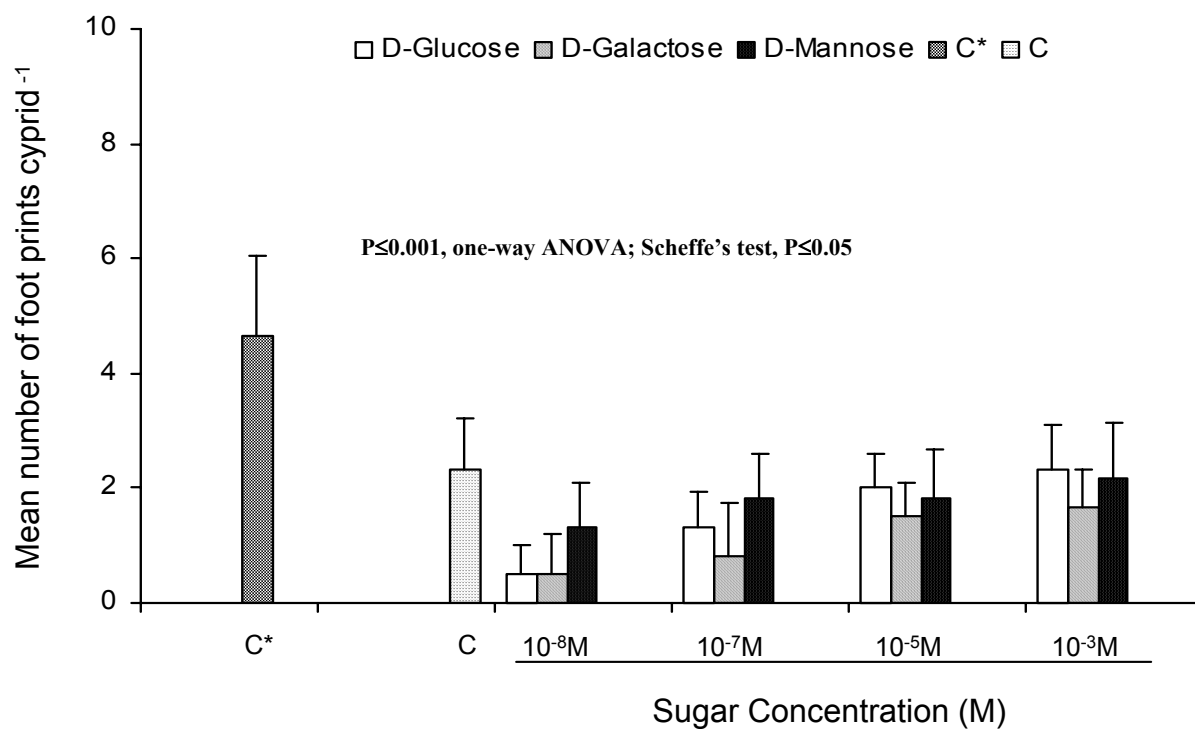
Table 1. Three-way ANOVA : The influence of D-glucose, D-galactose or D-mannose (sugar type), larval batch and different concentrations of all the three sugars on the deposition of footprints by the sugar-treated cyprids exposed to AE-coated surfaces. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor	df	SS	MS	Fs
A (sugar type)	2	0.83	0.41	
B (larval batch)	2	0.08	0.04	
C (Conc.)	3	2.8	0.93	
A*B	4	0.04	0.01	1.75ns
A*C	6	0.63	0.10	18****
B*C	6	0.033	0.005	0.94ns
A*B*C*	12	0.070	0.006	
Total	35	4.48		

Table 2. Three-way ANOVA: The influence of D-glucose, D-galactose or D-mannose (sugar type), cyprid age and different concentrations of all the three sugars on the metamorphosis of cyprids exposed to AE-coated or non-coated surfaces in a multiple cyprid assay. (df. degree of freedom; SS. sum of the squares; MS. Mean of squares; Fs. Fischer constant).

Factor	AE-coated surfaces				Non-coated surfaces		
	df	SS	MS	Fs	SS	MS	Fs
A (Sugar type)	2	5.2	2.6		1.2	0.6	
B (Cyprid age)	1	2.2	2.2		4.2	4.2	
C (Conc.)	3	0.4	0.13		1	0.3	
A*B	2	0.9	0.5	4.85**	0.9	0.4	6.03**
A*C	6	2.8	0.5	4.89**	2.7	0.4	6.07***
B*C	3	0.8	0.3	2.95 ns	0.2	0.08	1.1 ns
A*B*C*	6	0.6	0.09		0.4	0.07	
Total	23	12.9			10.6		

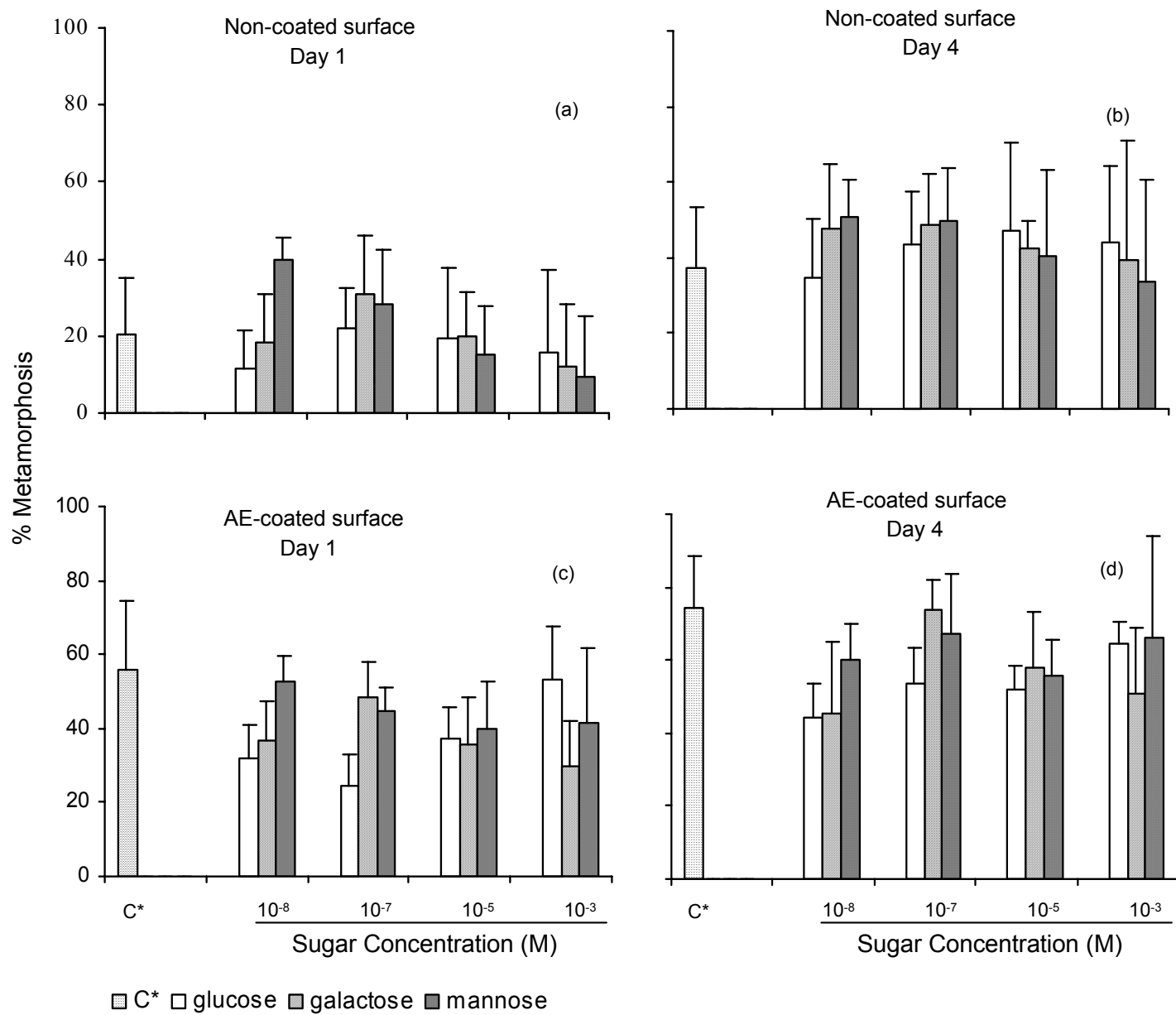
(\*\*\*\*p≤0.001, \*\*\*\*≤0.005, \*\*\* p≤0.025, \*\*p≤0.05, \* p≤0.01 ns - Not significant)



(C\*- non-treated cyprids exposed to AE-coated surface, C- non-treated cyprids exposed to non-coated surface).

**# Footprints were not deposited by sugar-treated cyprids when exposed to non-coated surface.**

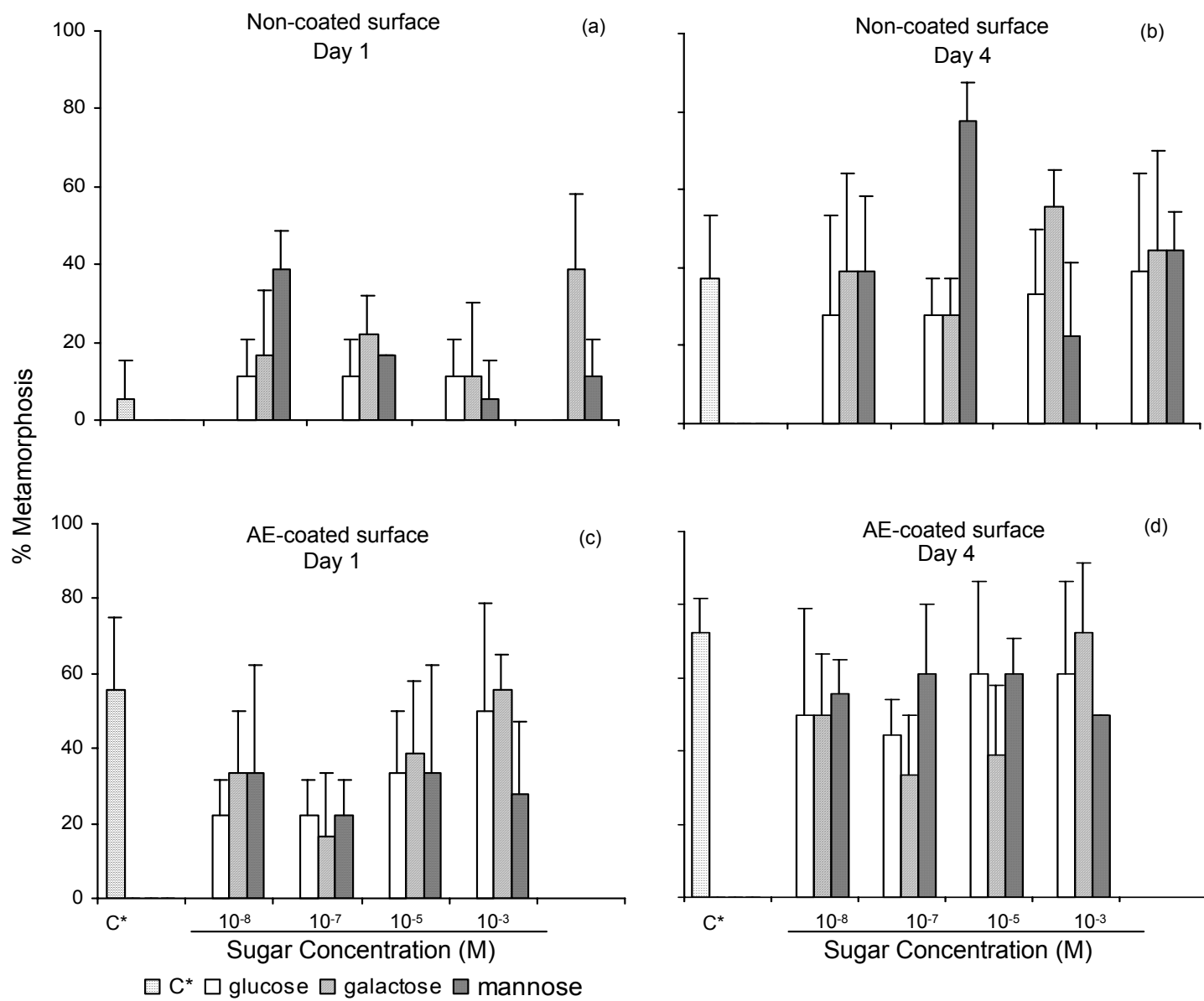
Fig. 1



**C\*- Non-treated cyprids**

**Multiple cyprid assay**

Fig. 2



C\*- Non-treated cyprids

Single cyprid assay

Fig. 3