Journal of Experimental Marine Biology and Ecology 372 (2009) 82–90

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Measurement of the attachment strength of brachiolaria larvae and metamorphic individuals of the sea star *Asterina gibbosa* by a centrifugation method

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ARTICLE INFO

Article history:
Received 5 September 2008
Received in revised form 6 February 2009
Accepted 9 February 2009

Keywords:
Adhesion
Asteroidea
Centrifugation
Echinodermata
Larva
Metamorphosis
Settlement

ABSTRACT

Two methods are generally used to measure the adhesive strength of invertebrate larvae: direct measurement with a force transducer connected to the organisms and indirect measurement with a water flow used to dislodge the organisms. Each of these methods, however, has its drawbacks. The present study aimed to design a simple and straightforward method to measure the adhesion strength of marine invertebrate larvae based on centrifugation. This centrifuge technique works in immersed conditions and applies forces acting at 45° to the substratum, therefore mimicking natural conditions. It was tested with three different substrata on two developmental stages of the sea star *Asterina gibbosa*: the brachiolaria larvae, which use temporary adhesion, and the metamorphic individuals which use permanent adhesion. Measurements were completed by SEM and TEM observations of the larval adhesive organs. The critical detachment force (force required to detach 50% of the larvae) of brachiolaria larvae attached to glass (36 ± 9 μN) and rough PMMA (43 ± 16 μN) were equivalent and both significantly higher than the critical detachment force measured on smooth PMMA (11 ± 8 μN). Most metamorphic individuals, on the other hand, resisted to the highest centrifugation speed used, corresponding to a force of 2.13 mN. For the hydrodynamics of larval settlement and metamorphosis, force is the ecologically relevant factor, and adhesion forces obtained by centrifugation are strikingly similar to forces measured for other marine invertebrate larvae with other methods. This indicates the usefulness of the centrifugation technique to compare adhesion of larvae between different species or development stages, or between different treatments.

1. Introduction

Many marine benthic organisms have an indirect development with pelagic or benthic larvae. Settlement and metamorphosis of these larvae is usually associated with different attachment processes that may include initial contact with the hard substratum, temporary adhesion and permanent adhesion (Chia and Rice, 1978; Crisp, 1984; Railkin, 2004). Although the importance of the adhesive strength of larvae in limiting where they are able to settle in complex habitats exposed to turbulent water flow has been discussed (e.g. Abelson and Denny, 1997; Crimaldi et al., 2002; Koehl, 2007), the adhesive strengths of the larvae of only a few species have been measured (see Koehl, 2007, for review). Yet, adhesion force measurements and their variations under different conditions may give precious information about the attachment mechanisms taking place during settlement and metamorphosis (see e.g., Zardus et al., 2008).

Two approaches have been used to measure adhesive strength of invertebrate larvae: i) direct measurement with a force transducer connected to the organisms (e.g. barnacle cyprids, Yule and Walker, 1984); and ii) measurement of the water velocity or wall shear stress required to dislodge the organisms (e.g. barnacle cyprids, Eckman et al., 1990; mussel postlarvae: Ackerman et al., 1995; nudibranch pediveligers: Koehl and Hadfield, 2004; sea star brachiolaria larvae and metamorphic individuals: Haesaerts et al., 2005). Each of these methods, however, has its drawbacks. Direct force measurement is a straightforward approach but, for microscopic organisms, it requires very sensitive transducers. Moreover, soft-bodied organisms like many invertebrate larvae cannot be easily connected to such testing devices. On the other hand, laboratory flumes used for the measurement of the wall shear stress required to dislodge organisms are usually complex devices requiring careful calibration to ensure the formation of precisely-controlled flows (see e.g., Schultz et al., 2000, 2003). In these experiments, larvae are placed inside a channel or a tube and are subjected to increasing flow until dislodgement. However, to generate shape-independent measurements, the maximum size of the organism has to be lower than the thickness of the viscous sublayer inside the channel (Schultz et al., 2000). This is

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doi:10.1016/j.jembe.2009.02.011

usually not the case for large invertebrate larvae (see, e.g., Koehl and Hadfield, 2004; Haesaerts et al., 2005).

The aim of the present study was to design a simple and straightforward method to measure the adhesion strength of marine invertebrate larvae based on centrifugation. Centrifugation techniques have already been used successfully to measure the adhesion of small insects (Federle et al., 2000; Gorb et al., 2001; Gorb and Gorb, 2004) and of adult barnacles, though in emerged conditions (Dougherty, 1990). The centrifugation method was then used measure the attachment strength of larvae of the sea star *Asterina gibbosa*. This species possesses an entirely benthic development comprising two main larval stages, the brachiolaria and the metamorphic individual (for a more detailed description of the development of *A. gibbosa*, see Haesaerts et al., 2006). Brachiolaria larvae are composed of a posterior ovoid body and an anterior attachment complex made up of two brachiolar arms and a central adhesive disc. Brachiolar arms are used for temporary adhesion to the substratum. The adhesive is synthesized and secreted by specialized adhesive cells containing electron-dense secretory granules (Haesaerts et al., 2006). Metamorphosis starts with the fixation of the competent larva by its adhesive disc. This adhesion, which is achieved by the release of cement from disc secretory cells packed with granules having a fibrous content, can be described as permanent (Haesaerts et al., 2006). Indeed, although the newly metamorphosed postlarva detaches from the substratum and becomes motile, detachment occurs at the level of the stalk connecting the disc to the rest of the body, the disc itself remaining cemented to the substratum. Using the centrifugation technique, it was possible to measure the adhesion force of both brachiolaria larvae and metamorphic individuals on different substrata in immersed conditions. Moreover, some specific morphological observations in SEM and TEM were done on the attachment complex of the larvae, attached or not, and correlated with the adhesion strength measurements.

2. Materials and methods

2.1. Larval rearing

Adult specimens of *A. gibbosa* (Pennant, 1777) were collected in March 2006 and 2007 from the rocky intertidal zone near Roscoff (Brittany, France). They were brought back to the marine biology laboratory of the University of Mons (Belgium) where they were kept in re-circulating marine aquaria (14 °C, 33 psu). When maintained in such conditions, individuals spawned spontaneously but not synchronously, and laid small egg masses. Fertilised eggs were collected and placed in large Petri dishes with filtered sea water (FSW 0.22 µm pore size). Rearing was done at room temperature (20–22 °C) and the FSW was changed every 2–3 days.

2.2. Adhesion measurements

Adhesion was investigated in two successive larval stages, the brachiolariae and the metamorphic individuals, on three substrata: glass, smooth poly(methylmethacrylate) (PMMA) and rough PMMA.

The different substrata were cut into 9×30 mm rectangular pieces (size required to fit tightly in a 2 ml microtube), either from a 1 mm thick PMMA plate or from microscope glass slides. Rough PMMA was manufactured from smooth PMMA: microtextured surfaces were created by squeezing a nylon filter (mesh size 11 µm, Millipore) between two PMMA slides for 3 h in an oven at 160 °C (Granhag et al., 2004). All substrata were thoroughly rinsed in fresh water for 3 days (2 water changes per day) before use.

Brachiolaria larvae were selected under binocular microscope and placed on different substrata with a Pasteur pipette. The larvae usually re-attached immediately to the substrata, which were then transferred delicately into 2 ml Eppendorff microtubes filled with FSW. Competent brachiolariae were isolated in six-well culture plates filled with FSW containing streptomycin sulphate (50 mg/l, Sigma), and whose bottom was covered with the different substrata. Plates were checked regularly and when a larva was fixed and had started to metamorphose, the substratum was transferred in a microtube.

For both brachiolariae and metamorphic individuals, the position of the larva was marked on the tube external surface in order to check easily when it was detached and to measure the centrifugation radius. Tubes were placed in a fixed-angle microtube rotor (Heraeus #3332, angle of 45°) with the larvae facing outwards (Fig. 1), and centrifuged in a Heraeus Biofuge Stratos centrifuge (acceleration and deceleration set to 8 and 5, respectively). Different centrifugal steps, ranging from 500 to 17,000 rpm (rotor limit), were applied successively. Larvae were centrifuged for 3 s at the selected speed; after which the centrifuge was stopped and the position of each larva was recorded. The first centrifugation step (500 rpm) was used as a control for larval adhesion: larvae detached at this step were not taken into account. The following centrifugation steps were 1000 rpm, from 2000 rpm to 10,000 rpm by steps of 500 rpm, and from 10,000 rpm to 17,000 rpm by steps of 1000 rpm. When a larva was detached, the distance between its initial position and centrifugation axis was measured. This centrifugation radius together with the centrifugation speed which allowed to detach the larva were used to calculate its detachment force. The centrifugation frequency f_c (rpm) was converted to angular velocity ω (rad s^{-1}) according to the following equation.

$$\omega = f_c \, \pi / 30 \tag{1}$$

The centrifugal acceleration a_c was then calculated as the product of the centrifugation radius r (ranging from 0.052 to 0.073 m according to the position of the larva in the microtube; Fig. 1) and the square of the angular velocity;

$$a_c = r \, \omega^2 \tag{2}$$

and the detachment force F (N) as the product of the immersed weight of the larva m (kg) and of the acceleration.

$$F = m \, a_c \tag{3}$$

Different ways were explored to estimate the immersed weight of the larvae. Firstly, the density of brachiolariae and metamorphic individuals was estimated by density gradient centrifugation. A solution of Percoll (GE Healthcare) was prepared with 1 M solution of NaCl to a final osmolarity of 1000 mOsm l^{-1}. This solution was poured in 2 ml microtubes with Density Marker Beads (GE Healthcare), and a few larvae were added. After centrifugation for 30 min at 15,000 *g*, the position of larvae relative to the Density Marker Beads gave an

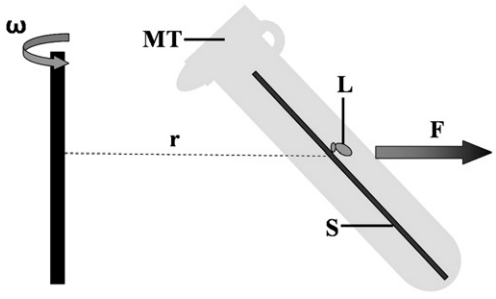

Fig. 1. Diagram showing the experimental setup used to centrifuge larvae. A piece of substratum (S) with a larva (L) attached on its surface is placed in a microtube (MT) filled with sea water. The microtube is placed in the rotor of the centrifuge with the larva facing outward, the position of the larva on the substratum determining the radius of centrifugation (r). During centrifugation, a force (F), proportional to the angular velocity (ω), will pull on the larva with an angle of 45°.