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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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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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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

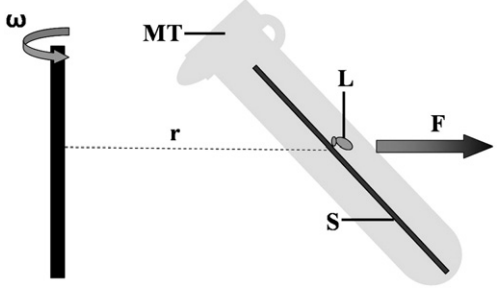

Fig. 1. Diagram showing the experimental setup used to centrifuge larvae. A piece of substratum (S) with a larva (L) attached on its surface is placed in a microtube (MT) filled with sea water. The microtube is placed in the rotor of the centrifuge with the larva facing outward, the position of the larva 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°.

estimation of their density. Approximate calculations of larval volume were based on light microscopy measurements of live individuals. The immersed weight was obtained by multiplying the larval volume (V_1) by the difference between larval density and sea water density.

$$m = V_1(\rho_1 - \rho_{SW}) \quad (4)$$

Secondly, the larval immersed weight was followed by a daily weighting of a group of 150 larvae reared inside an immersed weighting basket in FSW containing streptomycin sulphate (50 mg/l). The weighting basket was made from one well of a six-well culture plate and fishing line. An empty weighting basket was also followed to correct for the possible effect of an algal biofilm development. Larvae were weighted by hanging the basket immersed in FSW to the hook of the Unimatic CL5D balance (precision 0.01 mg). Six replicate measurements were taken every day.

Data were analysed as typical survival curves by the Cox proportional hazards model, time being replaced by centrifugal force in *N*, in R software (R Development Core Team, 2005). A logistic model was also fitted by least squares on the same data and was used to calculate confidence intervals for the centrifugal force required to dislodge 50% of the organisms (critical detachment force).

2.3. Morphological observations

For scanning electron microscopy (SEM), larvae were fixed in Bouin's fluid for 2 h at room temperature. They were then dehydrated in a graded ethanol series, dried by the critical point method, mounted on aluminium stubs and coated with gold in a sputter-coater.

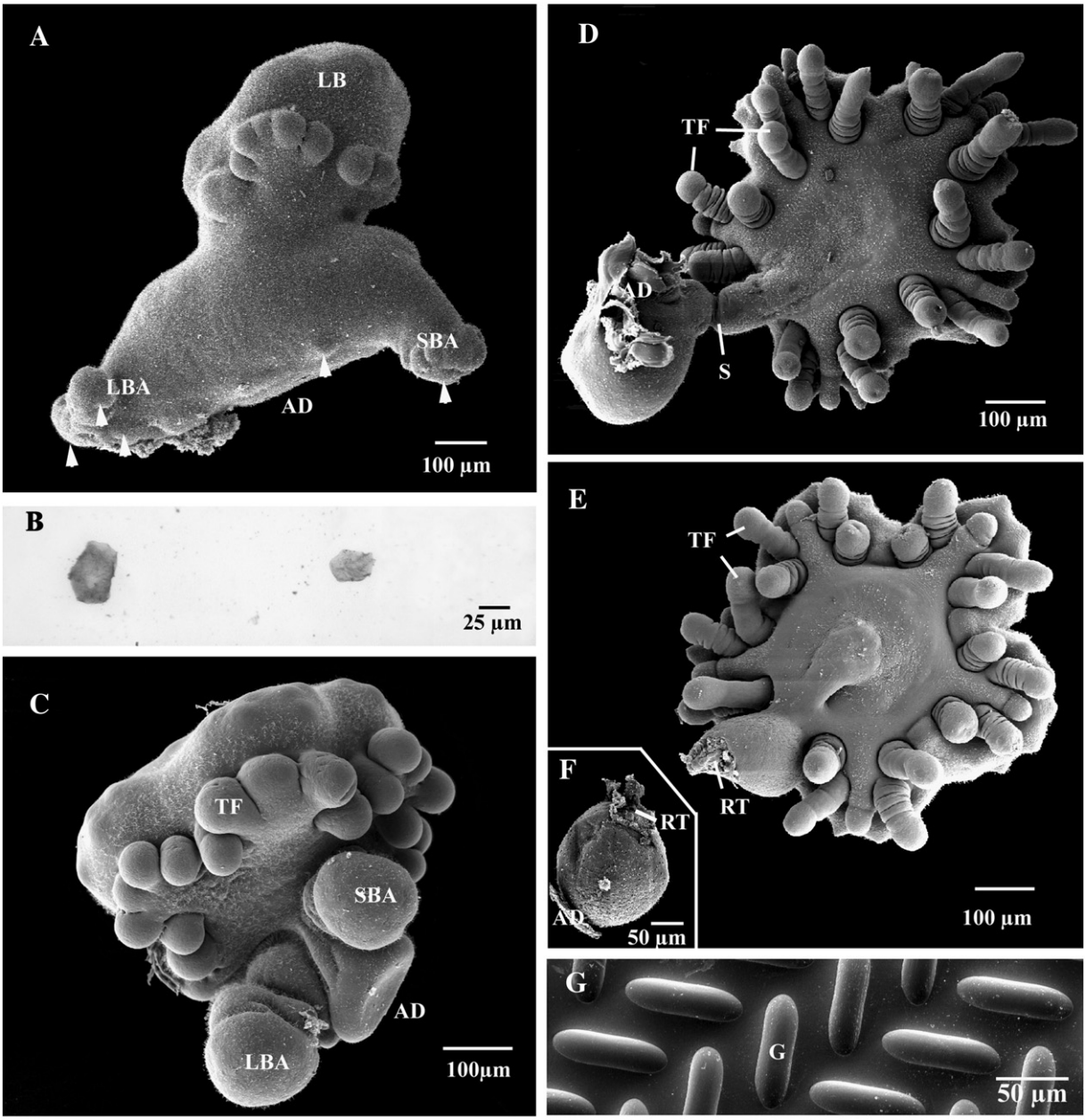

Fig. 2. Scanning electron microscopy of the different stages taking place successively during the perimetamorphic period of *Asterina gibbosa*. A) Competent brachiolaria larvae (arrowheads indicate the adhesive areas). B) LM view of the adhesive footprints left on a glass substratum by a competent brachiolaria larva and stained with Crystal Violet. C) Early metamorphic individual with contracted brachiolar arms. D–E) Late metamorphic individuals with intact and broken regressing attachment complex, respectively. F) Remnants of the attachment complex left on the substratum after stalk rupture. G) View of the surface of the rough PMMA showing the grooves (G) imprinted by the mesh components of the filter. AD: adhesive disc; LB: larval body; LBA long brachiolar arm; RT: ripped tissues; S: stalk; SBA: short brachiolar arm; TF: tube foot.

Observations were done with a Jeol JSM 6100 scanning electron microscope.

For transmission electron microscopy (TEM), larvae were fixed for 30 min in a 3% solution of glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.8, 1030 mOsm l^{-1}) at 4 °C, rinsed in cacodylate buffer, and post-fixed for 1 h with 1% osmium tetroxide in the same buffer. Larvae were embedded in Spurr resin and ultrathin sections were cut with a Leica Ultracut UCT ultramicrotome. Sections were collected on copper grids and contrasted with uranyl acetate and lead citrate. Observations were done with a Zeiss LEO 906E transmission electron microscope equipped with a Keen View Slow Scan CCD digital camera.

Footprints left by brachiolaria larvae on microscope glass slides were stained for 1 min in a 0.05% aqueous solution of the cationic dye Crystal Violet. They were observed and photographed with a Leitz Orthoplan light microscope equipped with a Leica DC 300F digital camera.

3. Results

3.1. Fine structure of the developmental stages used in the centrifugation experiments

Competent brachiolariae possess a fully-developed attachment complex comprising two asymmetrical brachiolar arms and an adhesive disc (Fig. 2A). The arms are used for temporary attachment to the substratum, the adhesive being released at the level of patches of secretory pores (arrowheads in Fig. 2A). When the arms detach, the adhesive material remains on the substratum as footprints (Fig. 2B).

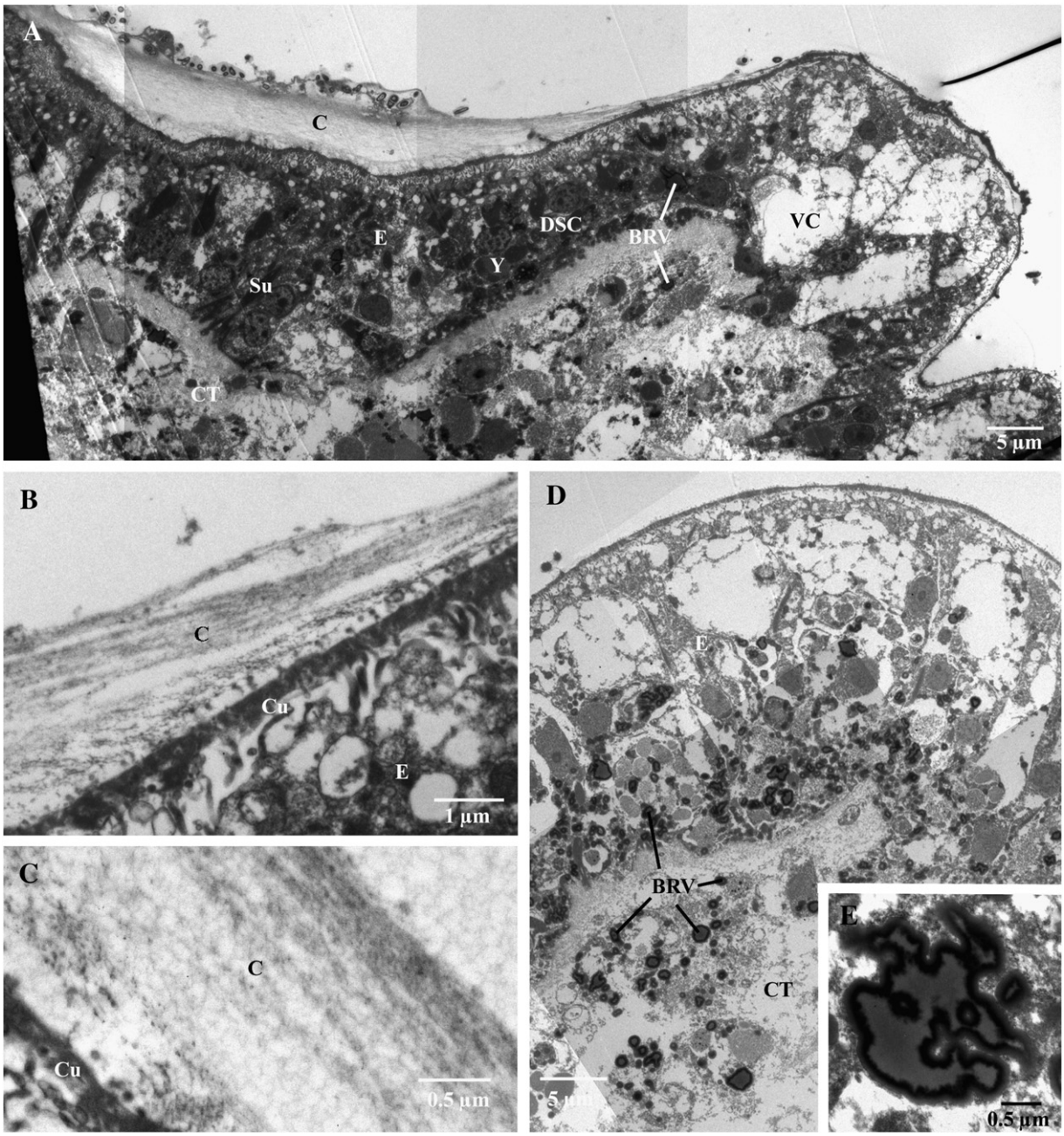

Fig. 3. Ultrastructure of the attachment complex in metamorphic individuals of *Asterina gibbosa* (TEM). A) Longitudinal section through the adhesive disc of an early metamorphic individual fixed to a resin block. B–C) Details of the cement layer. D) Longitudinal section through the disc of a late metamorphic individual. E) Detail of a black-rimmed vesicle. BRV: black-rimed vesicle; DSC: type D secretory cell; C: cement; Cu: cuticle; CT: connective tissue; E: epidermis; Su: support cell; Y: yolk granule; VC: vacuolated cell.

From these footprints, the adhesive surface area of a brachiolaria larva can be estimated to about 1×10^{-9} m^2. On the other hand, measurement of the surface area of the brachiolar arms covered by secretory patches on SEM images gives a figure for the adhesive surface area of approximately 1.5×10^{-8} m^2.

Fixation of the larva with the adhesive disc marks the onset of metamorphosis. Early metamorphic individuals are morphologically very similar to competent brachiolaria larvae (compare Fig. 2A and C), except that, in the former, the arms are retracted making the disc clearly visible. Its adhesive surface area, estimated from several detached individuals, is about 1.5×10^{-8} m^2. Sections through attached early metamorphic individuals reveal the cement layer between the disc epidermis and the substratum (Fig. 3A). This fibrillar cement shows a lamellar aspect with several superposed layers (Fig. 3B,C). During metamorphosis, the attachment complex regresses progressively in parallel to the development of post-metamorphic structures (Fig. 2D–F). In late metamorphic individuals, the attachment complex is reduced to a thin stalk connecting the individual to the still attached adhesive disc (Fig. 2D). In several of our centrifugation measurements (see below), this stalk broke, leaving the disc cemented to the substratum (Fig. 2E,F). Sections through the disc of late metamorphic individuals show looser and more degraded tissues (Fig. 3D), presenting numerous irregular black-rimmed vesicles (Fig. 3E). These vesicles, which were already present in the disc of early metamorphic individuals though in lower numbers (compare Fig. 3A and D), could be lipid droplets accumulating in the tissues due to the resorption process (Byrne, 1994).

3.2. Adhesion measurements

The detachment force of brachiolaria larvae and metamorphic individuals was measured on the 3 substrata. Two substrata (glass and

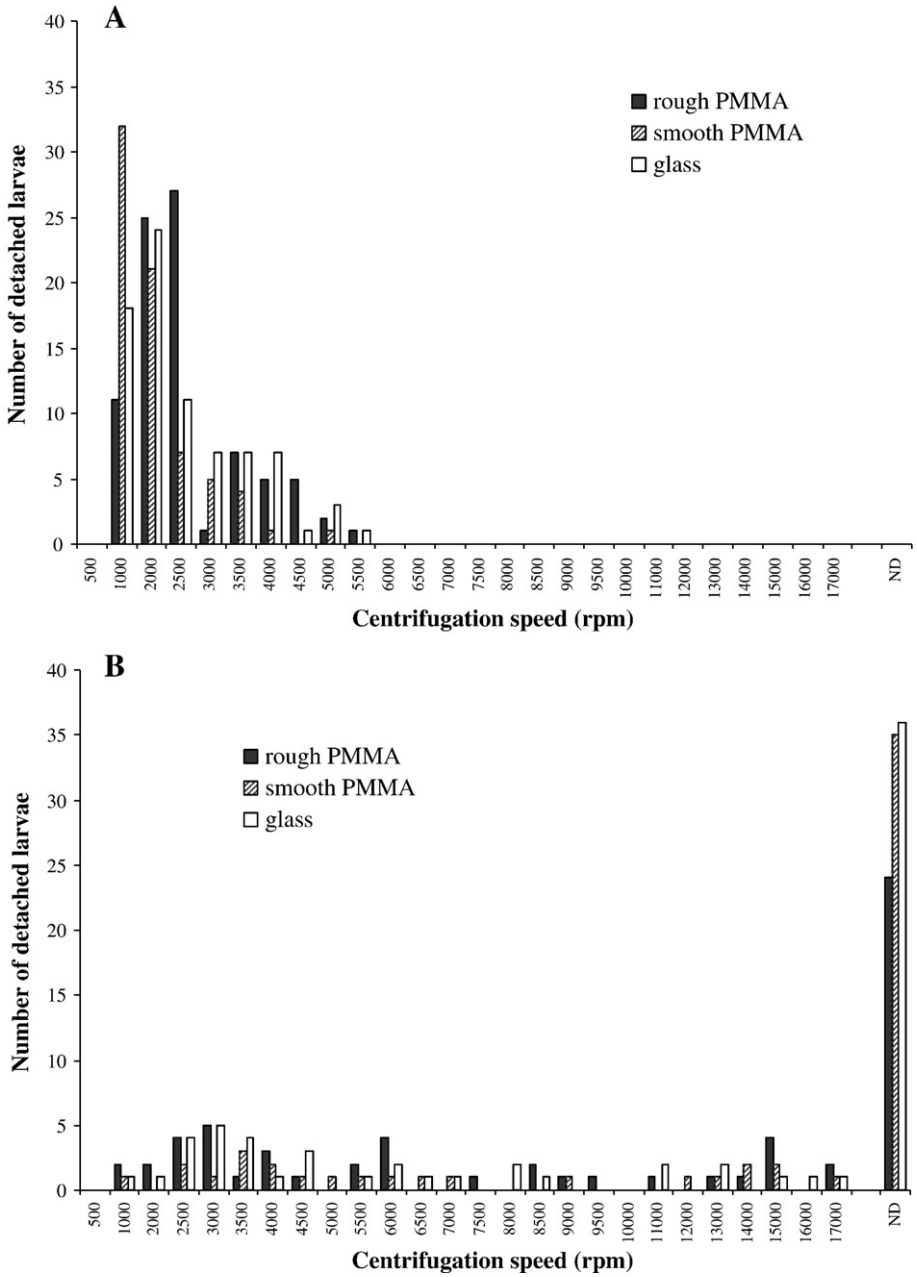

Fig. 4. Graphs showing the number of brachiolaria larvae (A) and metamorphic individuals (B) of *Asterina gibbosa* detached from the different substrata at each centrifugation step. The last step corresponds to a relative centrifugal acceleration of 28,110 ×*g*. For the other steps, relative centrifugal acceleration can be calculated, for the rotor used in this study (Heraeus #3332), as RCA = 65.96 × (centrifugation speed / 1000)^2. ND: number of larvae having resisted the last centrifugal step (not detached).

smooth PMMA) present a completely smooth surface and the third one (rough PMMA) have a regularly micro-patterned surface, consisting of parallel rows of small elliptical grooves (about 25×85 µm) which are oriented perpendicularly to each other from one row to another (Fig. 2G). A total of 234 brachiolaria larvae were tested: 79 on glass, 71 on smooth PMMA and 84 on rough PMMA. For metamorphic individuals, on the other hand, 203 specimens were tested: 75 on glass, 62 on smooth PMMA and 66 on rough PMMA.

The distribution of the number of detached brachiolaria larvae and metamorphic individuals for each centrifugation speed shows that differences are clearly more important between developmental stages than between substrata (Fig. 4). Indeed, whatever the substratum considered, all brachiolaria larvae were detached before 6000 rpm (Fig. 4A). The distribution of detached metamorphic individuals, on the other hand, suggests the presence of two groups of measurements: one comprising individuals detached at speeds similar to those observed for brachiolaria larvae, and one composed principally of individuals that resisted to a centrifugation speed of 17,000 rpm (speed limit of the centrifuge with the rotor used) and were never detached (Fig. 4B). In view of this distribution, it is assumed that individuals from the first group are probably late brachiolaria larvae whose development stage was not correctly identified. To avoid mixing measurements from the two stages, presumed metamorphic individuals removed at speeds below 6000 rpm were not taken into account. In addition, in 13 metamorphic individuals, dislodgement was due to the rupture of the stalk, leaving the disc attached to the substratum. These stalk failures occurred at centrifugation speeds ranging from as low as 2000 up to 16,000 rpm independently of the type of substratum used. These measurements, which express tissue strength and not adhesion strength, were also removed from the data set.

Different ways were explored to estimate the immersed weight of brachiolaria larvae and metamorphic individuals. In a first experiment, the density of both brachiolaria larvae and metamorphic individuals was measured by density gradient centrifugation, giving a similar value of about 1085 kg m^{-3} for both stages. Using estimated volumes of 0.90×10^{-10} m^3 for brachiolaria larvae and of 1.43×10^{-10} m^3 for metamorphic individuals, and 1025 kg m^{-3} for the density of seawater (at 20 °C), the immersed weights were calculated as 5.40 µg and 8.58 µg, respectively. On the other hand, the daily weighting, underwater, of a group consisting of 150 individuals from the brachiolaria stage to the metamorphic stage gave immersed weights of 13 µg and 9.60 µg for these two developmental stages. In view of these different results, we used the value of 10 µg as the immersed weight for both brachiolaria larvae and metamorphic individuals.

Knowing the immersed weight and centrifuge speed, it was possible to calculate the force acting on each larva and metamorphic individual. Results were plotted as fraction of individuals remaining on the substratum against force, and were analyzed as survival curves with the Cox proportional hazards model (Fig. 5). A first analysis showed that adhesion of metamorphic individuals is significantly higher than that of brachiolaria larvae ($p = 1.2 \times 10^{-15}$).

Among brachiolaria larvae, adhesion on glass was not significantly different from that on rough PMMA ($p = 0.54$), but was significantly higher than on smooth PMMA ($p = 6.3 \times 10^{-4}$). The data were then fitted with a logistic model (Fig. 5A), which allowed calculation of critical detachment forces (i.e. the force required to dislodge 50% of the attached individuals). The critical detachment force (±95% CI) necessary to dislodge brachiolaria larvae from glass, smooth PMMA and rough PMMA were $3.59 \times 10^{-5} \pm 8.77 \times 10^{-6}$ N, $1.08 \times 10^{-5} \pm 7.66 \times 10^{-6}$ N and $4.32 \times 10^{-5} \pm 1.61 \times 10^{-5}$ N, respectively. Using the adhesive surface areas measured in the morphological approach, tenacity (the adhesion force per unit surface area) can be calculated. It ranges from 11 to 43 kPa if footprint surface area is taken into account and from 0.7 to 2.9 kPa if brachiolar arm secretory surface area is considered.

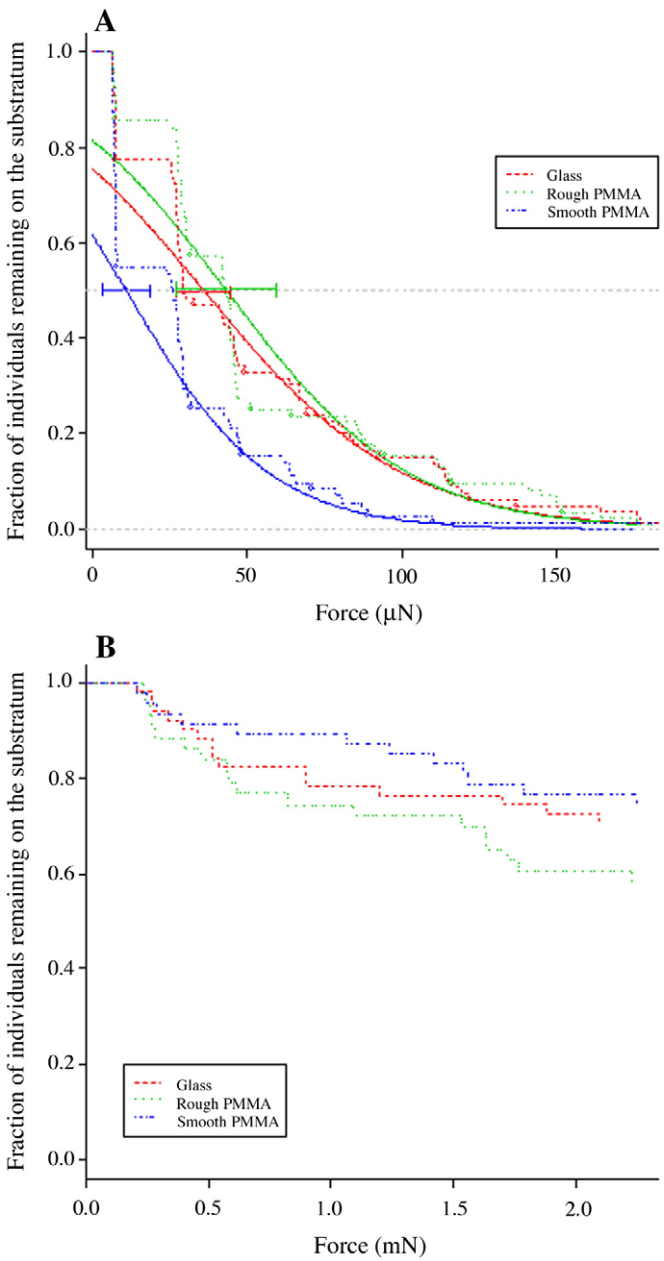

Fig. 5. Graphs illustrating the resistance to dislodgement of brachiolaria larvae (A) and metamorphic individuals (B) of *Asterina gibbosa* attached to different substrata. For brachiolaria larvae, the data were fitted by logistic regression and critical detachment forces ±95% confidence intervals (horizontal bars) were calculated from the curves.

For metamorphic individuals, the Cox model did not detect any difference between adhesion on glass and rough PMMA ($p = 0.21$) but demonstrated a significant difference between adhesion on glass and on smooth PMMA ($p = 0.041$). In this case, it was not possible to fit the data with the logistic model and calculate the critical detachment forces because of the insufficient number of detached individuals (less than 50%). Mean centrifugal force for metamorphic larvae which have resisted to the step at 17,000 rpm all substrata together is 2.13×10^{-3} N, corresponding to a tenacity of 142 kPa.

4. Discussion

A settling larva on the substratum in a turbulent flow experiences numerous hydrodynamic forces associated with fluid motion, including drag, lift, skin friction, and fluid acceleration reaction forces

(Denny, 1988; Koehl, 2007). The relative magnitudes of these forces depend on the size and shape of the larva and the water velocities and accelerations it encounters. The resultant instantaneous force experienced by the larva can be calculated as the vector sum of the different forces involved and, as a consequence, its direction varies from parallel (when drag predominates) to perpendicular (when lift predominates) to the substratum. When this net force exceeds the attachment force, the larva is dislodged from the substratum. We developed a centrifuge method to measure detachment forces of larvae. This technique allows to work under immersed conditions and to apply forces acting at 45° to the substratum, thus mimicking natural conditions. It is also a direct method which, contrary to systems relying on water flows, is simple to model and independent from the size and shape of the organism.

The centrifugation method was used to measure the attachment strength of 2 successive larval stages from the sea star *A. gibbosa*, a species characterised by a lecithotrophic and completely benthic larval development (Marthy, 1980; Crump and Emson, 1983; Haesaerts et al., 2006). In *A. gibbosa*, hatching occurs directly at the brachiolaria stage and the larva immediately adheres to the substratum with its two well-developed brachiolar arms. During this stage, individuals are attached by one or two arms and are still able to move over short distances. When metamorphosis occurs, the larva cements itself more strongly with the adhesive disc. In our TEM sections through attached metamorphic individuals, the lamellar aspect of the cement suggests a fairly progressive secretion rather than a massive granule release as it is the case in some forcipulatid sea stars (Barker, 1978). Regression of the attachment complex during the metamorphic stage was confirmed by TEM. Degraded tissues presumably explain why dislodgement occurs through stalk rupture in late metamorphic individuals. In early metamorphic individuals, the attachment complex is still cohesively strong and dislodgement occurs mostly through disc detachment. To measure disc adhesion, only early metamorphic individuals were therefore used in our centrifugation experiments.

Adhesion of brachiolaria larvae was compared on 3 different substrata, glass, smooth PMMA and rough PMMA. The critical detachment force measured on glass (35.9 ± 8.8 μN) was significantly higher than the force measured on smooth PMMA (10.8 ± 7.7 μN), but not different from that measured on rough PMMA (43.2 ± 16.1 μN). For smooth surfaces, larvae therefore stick better on glass than on PMMA. It is well documented in several adult marine organisms such as barnacles, limpets, mussels and sea urchins that substratum surface characteristics influence adhesion force and tenacity (see e.g., Grenon and Walker, 1981; Crisp et al., 1985; Yule and Walker, 1987; Santos and Flammang, 2006). As a general rule, there is a positive correlation between tenacity and the polarity (usually estimated by water-based contact angle) of the substratum. Substrata with high-energy, polar surfaces increase the spreading and molecular adhesion of marine bioadhesives (adhesiveness), but also influence their bulk properties (cohesiveness) (Waite, 1987; Aldred et al., 2006; Santos and Flammang, 2006). This may explain the difference in the attachment strength of brachiolaria larvae observed between glass and smooth PMMA although there is another possible explanation. In competent cyprids of barnacles (a larva using temporary adhesion for substratum exploration), voluntary detachment from non-attractive surfaces can lead to reduced detachment force or tenacity measurements (Crisp et al., 1985; Yule and Walker, 1987; Neal and Yule, 1994). Although they are exclusively benthic, the brachiolaria larvae of *A. gibbosa* appeared to be reluctant to attach to smooth PMMA and thus, in this species too, some degree of voluntary control may be involved in the force of detachment. The same behavioural effect may explain the better adhesion of brachiolaria larvae on rough PMMA than on its smooth counterpart. The two explanations generally proposed for increased adhesion on rough surfaces are mechanical interlocking for organisms in which the cement, initially fluid, flows into the interstices of the substratum before curing (e.g., adult barnacles; Yule and Walker, 1987); and contact increase for organisms whose deformable adhesive pads replicate the surface profile therefore increasing real contact area and hence adhesion force (e.g., echinoderm tube feet; Santos et al., 2005). However, in the case of *A. gibbosa*, the relatively large dimensions of the grooves imprinted in the substratum suggest a predominant effect of the brachiolaria larva behavior rather than any of the two other explanations. It is well-known that the larvae of many marine benthic invertebrates are influenced by substratum roughness. For example, a number of studies have shown higher larval settlement on rough surfaces than on smooth surfaces, although cases of higher larval settlement on smooth surfaces than on rough surfaces have also been reported (see Koehl, 2007, for review).

The centrifugation experiment was also tested with metamorphic individuals on the three substrata. The Cox proportional hazards model indicates that metamorphic individual adhesion is much higher than brachiolaria adhesion. It also suggests that, within metamorphic individuals, the detachment force is significantly higher on smooth PMMA than on the other two substrata. This result

Table 1
Comparison between adhesion measurements obtained by different methods for the competent and metamorphic larvae of barnacles and sea stars.

Organism / *Species*	Life stage	Adhesive structure/organ	Method	Substratum	Detachment force (N)	Tenacity (KPa)	Wall shear stress (Pa)	Ref.
Barnacle								
Balanus amphitrite	Competent cyprids	Antennules	Flow channel	PMMA	0.55×10^{-5}[a]	13.2	1.5	1
Balanus improvisus	Settled cyprids	Cement gland	Microbalance	PMMA	1.9×10^{-3}	–	–	2
Balanus perforatus	Competent cyprids	Antennules	Microbalance	Glass	6.5×10^{-5}	67.5	–	3
Elminius modestus					4.3×10^{-5}	81.9	–	
Semibalanus balanoides	Competent cyprids	Antennules	Microbalance	Glass	–	98	–	4
				PMMA	–	76	–	
	Settled cyprids	Cement gland		Glass	–	1250	–	
				PMMA	–	160	–	
Sea star								
Asterina gibbosa	Brachiolaria larvae	Brachiolar arms	Flow channel	Glass	–	–	1.2	5
	Metamorphic individuals	Adhesive disc			–	–	40.8	
	Brachiolaria larvae	Brachiolar arms	Centrifuge	Glass	3.59×10^{-5}	2.4 (36)[b]	–	6
				PMMA	1.08×10^{-5}	0.7 (11)[b]	–	
	Metamorphic individuals	Adhesive disc		Glass and PMMA	$>2.13 \times 10^{-3}$	>142	–	

[1]Eckman et al., 1990, [2]Berglin et al., 2001, [3]Neal and Yule 1994, [4]Yule and Walker 1987, [5]Haesaerts et al., 2005, [6]present study.
[a] Calculated as a drag force from the mean water velocity at which larvae were dislodged.
[b] Tenacity calculated using the brachiolar arm adhesive surface area or using footprint surface area (value between brackets), see text for details.

has to be taken with caution because of the low numbers of detached individuals. Indeed, more than half of the tested individuals have resisted to the maximal centrifugation speed (17,000 rpm for the rotor used) and were never detached. Consequently, it was not possible to calculate critical detachment forces for the metamorphic individuals; and only the centrifugation force value corresponding to 17,000 rpm, i.e. 2.13 mN, gives an estimation of the adhesion strength of this developmental stage. As discussed above, only early metamorphic individuals were chosen in order to avoid stalk failures. This is the reason why several late brachiolaria larvae were presumably inadvertently included with the metamorphic individuals. On the other hand, it allowed use of the same immersed weight (1×10^{-5} g) for the two stages as they both take place within a very short time frame.

To compare larval adhesion in *A. gibbosa* to values reported for adult sea stars, we have to calculate the tenacity of larvae. For brachiolaria larvae on glass, the tenacity range from 2.4 to 36 kPa according to whether arm surface area or footprint surface area is used (Table 1). For PMMA, the range is 0.7–11 kPa. These values are much lower than the tenacity reported for tube foot temporary adhesion in the species *Asterias rubens* on the same substrata (198 and 180 kPa, respectively; Flammang and Walker, 1997; Santos et al., 2005). Tenacity of metamorphic individuals, estimated with our method to be superior to 142 kPa, is closer to tube foot tenacity. So far, barnacle cyprids were the only larvae for which values of detachment force and/or tenacity were reported in the literature. In terms of tenacity, barnacle and sea star larvae differ greatly, with the former possessing tenacity up to two orders of magnitude higher than the latter for both the competent and metamorphic stages (Table 1). In terms of detachment force, however, both organisms are strikingly similar despite the different methods used. The forces for temporary adhering competent larvae are in μN range while the forces for permanently attached metamorphic larvae are in the mN range (Table 1). To avoid dislodgement, adhesion force must counterbalance hydrodynamic force, and force is therefore the ecologically relevant factor. The difference in tenacity only reflects the fact that, in cyprids, these forces are achieved with much smaller adhesive surface areas. For barnacle and sea star larvae, attachment strength has also been evaluated as critical wall shear stresses in turbulent flow channels (Eckman et al., 1990; Haesaerts et al., 2005). Table 1 shows that, for these larvae which have similar size and shape, critical wall shear stress, although expressed in Pa, does not correlate with tenacity but rather with detachment force.

The centrifugation method appears to be a relatively simple and straightforward method to measure adhesion forces of small organisms like larvae in immersed conditions. It can indeed be set up easily in most laboratories, with eventual modifications of the experimental design according to the organism considered and the number of individuals available. The centrifugation forces mimic flow-induced forces without the complexity linked to work with flows (e.g. their modification by surface roughness). The method, however, requires knowledge of immersed weight of tested organisms, which may be tricky to measure for very small buoyant larvae. In *A. gibbosa*, we had to combine two approaches to estimate this immersed weight. For larvae combining low immersed weight and high adhesion, the method can also be limited by the possible maximal centrifugation speeds, as for metamorphic individuals of *A. gibbosa*. Increasing centrifugation time could however remove this limitation. In the context of settlement and metamorphosis, data obtained by centrifugation can be very interesting to compare adhesion of larvae between different species or development stages, or between different treatments.

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References

Abelson, A., Denny, M., 1997. Settlement of marine organisms in flow. Ann. Rev. Ecolog. Syst. 28, 317–339.
Ackerman, J.D., Cottrell, C.M., Ethier, C.R., Allen, D.G., Spelt, J.K., 1995. A wall jet to measure the attachment strength of zebra mussels. J. Fish Aquat. Sci. 52, 126–135.
Aldred, N., Ista, K.L., Callow, M.E., Callow, J.A., Lopez, G.P., Clare, A.S., 2006. Mussel (*Mytilus edulis*) byssus deposition in response to variations in surface wettability. J. R. Soc. Interface 3, 37–43.
Barker, M.F., 1978. Structure of the organs of attachment of brachiolaria larvae of *Stichaster australis* (Verrill) and *Coscinasterias calamaria* (Gray) (Echinodermata: Asteroidea). J. Exp. Mar. Biol. Ecol. 33, 1–36.
Berglin, M., Larsson, A., Jonsson, P.R., Gatenholm, P., 2001. The adhesion of the barnacle, *Balanus improvisus*, to poly(dimethylsiloxane) fouling-release coatings and poly (methyl methacrylate) panels: the effect of barnacle size on strength and failure mode. J. Adhes. Sci. Technol. 15, 1485–1502.
Byrne, M., 1994. Ophiuroidea. In: Harrison, F.W., Chia, F.S. (Eds.), Microscopic Anatomy of Invertebrates, vol 14: Echinodermata. Wiley-Liss, New York, pp. 247–343.
Chia, F.-S., Rice, M., 1978. Settlement and Metamorphosis of Marine Invertebrate Larvae. Elsevier, New York. 290 pp.
Crimaldi, J.P., Thompson, J.K., Rosman, J.H., Lowe, R.J., Koseff, J.R., 2002. Hydrodynamics of larval settlement: the structure of turbulent stress events at model recruitment sites. Limnol. Oceanogr. 47, 1137–1151.
Crisp, D.J., 1984. Overview of research on marine invertebrate larvae, 1940–1980. In: Costlow, J.D., Tipper, R.C. (Eds.), Marine Biodeterioration: An Interdisciplinary Study. E. and F.N. Spon, London, pp. 103–126.
Crisp, D.J., Walker, G., Young, G.A., Yule, A.B., 1985. Adhesion and substrate choice in mussels and barnacles. J. Colloid Interface Sci. 104, 40–50.
Crump, R.G., Emson, R.H., 1983. The natural history, life history and ecology of the two British species of *Asterina*. Field Stud. 5, 867–882.
Denny, M.W., 1988. *Biology* and the Mechanics of the Wave-swept Environment. Princeton University Press, Princeton, NJ.. 329 pp.
Dougherty, W.J., 1990. Barnacle adhesion: reattachment of the adult barnacle *Chtamalus fragilis* Darwin to polystyrene surfaces followed by centrifugational shearing. J. Crustac. Biol. 10, 469–478.
Eckman, J.E., Savidge, W.B., Gross, T.F., 1990. Relationship between duration of cyprid attachment and drag forces associated with detachment of *Balanus amphitrite* cyprids. Mar. Biol. 107, 111–118.
Federle, W., Rohrseitz, K., Hölldobler, B., 2000. Attachment forces of ants measured with a centrifuge: better 'wax-runners' have a poorer attachment to a smooth surface. J. Exp. Biol. 203, 505–512.
Flammang, P., Walker, G., 1997. Measurement of the adhesion of the podia in the asteroid *Asterias rubens* (Echinodermata). J. Mar. Biol. Assoc. U.K. 77, 1251–1254.
Gorb, S., Gorb, E., Kastner, V., 2001. Scale effects on the attachment pads and friction forces in syrphid flies (Diptera, Syrphidae). J. Exp. Biol. 204, 1421–1431.
Gorb, S.N., Gorb, E.V., 2004. Ontogenesis of the attachment ability in the bug *Coreus marginatus* (Heteroptera, Insecta). J. Exp. Biol. 207, 2917–2924.
Granhag, L.M., Finlay, J.A., Jonsson, P.R., Callow, J.A., Callow, M.E., 2004. Roughness-dependent removal settled spores of the green alga *Ulva* (syn. *Enteromorpha*) exposed to hydrodynamic forces from a water jet. Biofouling 20, 117–122.
Grenon, J.-F., Walker, G., 1981. The tenacity of the limpet, *Patella vulgata* L. an experimental approach. J. Exp. Mar. Biol. Ecol. 54, 277–308.
Haesaerts, D., Finlay, J.A., Callow, M.E., Callow, J.A., Grosjean, Ph., Jangoux, M., Flammang, P., 2005. Evaluation of the attachment strength of *Asterina gibbosa* (Asteroidea, Echinodermata) during the perimetamorphic period. Biofouling 21, 229–235.
Haesaerts, D., Jangoux, M., Flammang, P., 2006. Adaptations to benthic development: functional morphology of the attachment complex of the brachiolaria larva in the sea star *Asterina gibbosa*. Biol. Bull. 211, 172–182.
Koehl, M.A.R., Hadfield, M.G., 2004. Soluble settlement cue in slowly-moving water within coral reefs induces larval adhesion to surfaces. J. Mar. Syst. 49, 75–88.
Koehl, M.A.R., 2007. Hydrodynamics of larval settlement into fouling communities. Biofouling 23, 357–368.
Marthy, H.J., 1980. Etude descriptive du développement de l'oeuf d'*Asterina gibbosa* (Echinoderme, Astéride) et son intérêt en embryologie expérimentale. Vie Milieu 30, 75–80.
Neal, A.L., Yule, A.B., 1994. The tenacity of *Elminius modestus* and *Balanus perforatus* cyprids to bacterial films grown under different shear regimes. J. Mar. Biol. Assoc. U.K. 74, 251–257.
R Development Core Team, 2005. R. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 3-900051-07-0. http://www.R-project.org.
Railkin, A.I., 2004. Marine biofouling. Colonization Processes and Defenses. CRC Press, Boca Raton. 303 pp.
Santos, R., Flammang, P., 2006. Morphology and tenacity of the tube foot disc of three common European sea urchin species: a comparative study. Biofouling 22, 187–200.
Santos, Gorb, S., Jamar, V., Flammang, P., 2005. Adhesion of echinoderm tube feet to rough surfaces. J. Exp. Biol. 208, 2555–2567.
Schultz, M.P., Finlay, J.A., Callow, M.E., Callow, J.A., 2000. A turbulent channel flow apparatus for the determination of the adhesion strength of microfouling organisms. Biofouling 15, 243–251.

Schultz, M.P., Finlay, J.A., Callow, M.E., Callow, J.A., 2003. Three models to relate detachment of low form fouling at laboratory and ship scale. Biofouling 21, 59–71.
Waite, J.H., 1987. Nature's underwater adhesive specialist. Int. J. Adhes. Adhes. 7, 9–14.
Yule, A.B., Walker, G., 1984. The temporary adhesion of barnacle cyprids: effects of some differing surface characteristics. J. Mar. Biol. Assoc. U.K. 64, 429–439.
Yule, A.B., Walker, G., 1987. Adhesion in barnacles. In: Southward, A.J. (Ed.), Crustacean Issues. Biology of Barnacles, vol. 5. Balkema, Rotterdam, pp. 389–402.
Zardus, J.D., Nedved, B.T., Huang, Y., Tran, C., Hadfield, M.G., 2008. Microbial biofilms facilitate adhesion in biofouling invertebrates. Biol. Bull. 214, 91–98.