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Evaluation of the attachment strength of individuals of *Asterina gibbosa* (Asteroidea, Echinodermata) during the perimetamorphic period

DELPHINE HAESAERTS[1], JOHN A. FINLAY[2], MAUREEN E. CALLOW[2], JAMES A. CALLOW[2], PHILIPPE GROSJEAN[3], MICHEL JANGOUX[1,4] & PATRICK FLAMMANG[4]

[1]*Université Libre de Bruxelles, Laboratoire de Biologie Marine, Académie universitaire Wallonie-Bruxelles, Brussels, Belgium,* [2]*University of Birmingham, School of Biosciences, Birmingham, UK,* [3]*Université de Mons-Hainaut, Laboratoire d'Ecologie numérique des milieux aquatiques, Académie universitaire Wallonie-Bruxelles, Mons, Belgium, and* [4]*Université de Mons-Hainaut, Laboratoire de Biologie Marine, Académie universitaire Wallonie-Bruxelles, Mons, Belgium*

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Abstract
A turbulent channel flow apparatus was used to determine the adhesion strength of the three perimetamorphic stages of the asteroid *Asterina gibbosa*, i.e. the brachiolaria larvae, the metamorphic individuals and the juveniles. The mean critical wall shear stresses (wall shear stress required to dislodge 50% of the attached individuals) necessary to detach larvae attached by the brachiolar arms (1.2 Pa) and juveniles attached by the tube feet (7.1 Pa) were one order of magnitude lower than the stress required to dislodge metamorphic individuals attached by the adhesive disc (41 Pa). This variability in adhesion strength reflects differences in the functioning of the adhesive organs for these different life stages of sea stars. Brachiolar arms and tube feet function as temporary adhesion organs, allowing repetitive cycles of attachment to and detachment from the substratum, whereas the adhesive disc is used only once, at the onset of metamorphosis, and is responsible for the strong attachment of the metamorphic individual, which can be described as permanent adhesion. The results confirm that the turbulent water channel apparatus is a powerful tool to investigate the adhesion mechanisms of minute organisms.

Keywords: *Shear stress, adhesion, perimetamorphic period, benthic boundary layer, larva*

Introduction

All marine benthic invertebrates going through a larval stage and attaching to the substratum as adults use adhesion mechanisms during their perimetamorphic period. Three successive attachment phases may usually be distinguished during this period: i) premetamorphic attachment allowing competent larvae to search for a favourable site for metamorphosis; ii) metamorphic attachment (sometimes referred to as fixation) securing the organism during this crucial step of its development; and iii) postmetamorphic attachment, the attachment mechanism of the future adult (Crisp, 1984). Among echinoderms, sea stars have developed the widest range of adhesive organs used during this period. Competent larvae in asteroids are called brachiolariae because they possess a specialised attachment complex on their anterior part, comprising two to three brachiolar arms and an adhesive disc (Flammang et al. 2005). The former are used by the larva before metamorphosis and provide temporary adhesion enabling the larvae to combine exploration and contact to the substratum. The latter is responsible for larval attachment by secreting a permanent adhesive ('cement'). After metamorphosis, temporary adhesion to the substratum is provided by the newly formed tube feet (Flammang et al. 2005).

Despite the importance of adhesion phenomena during the perimetamorphic period, the process of larval adhesion has rarely been quantified in marine benthic invertebrates. So far, two approaches have been used to measure adhesive strength of minute organisms: i) direct measurement with a force transducer connected to the organisms (e.g. diatoms, Arce et al. 2004; barnacle larvae, Yule & Walker, 1984); and ii) measurement of the water velocity or wall shear stress required to dislodge the organisms (e.g. diatoms, Holland et al. 2004; algal spores, Finlay et al. 2002; invertebrate larvae, Eckman et al. 1990,

Correspondence: Patrick Flammang, Université de Mons-Hainaut, Laboratoire de Biologie Marine, Académie universitaire Wallonie-Bruxelles, Mons, Belgium. Fax: +32-6537334. E-mail: Patrick.Flammang@umh.ac.be

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Ackerman et al. 1995, Koehl & Hadfield, 2004). Since very small organisms cannot be easily connected to conventional testing devices, the use of a water flow seems the best way to evaluate their attachment strength. Moreover, a water flow system better mimics the natural conditions an organism living in the wave-swept environment has to face (Ackerman et al. 1995).

To the authors' best knowledge, no information is available on larval attachment strength in echinoderms. The aim of the present study was therefore to quantify the strength of premetamorphic, metamorphic and postmetamorphic attachments in the sea star *Asterina gibbosa*. This species was chosen because it has a lecithotrophic development and is thus easily reared in laboratory conditions. Moreover, as the development is also entirely benthic, every life stages attach to the sea bottom with well-developed adhesive organs for which the fine structure and the functioning are known (Haesaerts et al. 2006).

Material and methods

Larval rearing

Adults of *Asterina gibbosa* (Pennant, 1777) were collected intertidally in Roscoff (Brittany, France) in April 2003, and thereafter transported with care, at cool temperature (10–15°C), to the University of Birmingham (UK), where they were transferred into a 10 l tank filled with natural seawater, at room temperature (about 21°C). This treatment induced spawning (Haesaerts et al. 2006) and fertilised eggs were collected the day they were laid. The eggs were deposited on clean microscope glass slides (*ca* 30 eggs per slide) immersed in compartmentalised 'Quadriperm' culture dishes (Greiner Bio-one) filled with filtered seawater (0.22 μm) which was changed daily. On average, 50% of the eggs developed to hatching, the others being removed from the compartments. After hatching, however, most of the individuals developed up to the juvenile stage in the culture dishes.

Evaluation of attachment strength

A turbulent channel flow apparatus was used to evaluate the attachment strength of the different developmental stages of *A. gibbosa*. This apparatus was designed to mimic turbulent flow conditions found along ship hulls in order to measure the attachment strength of microfouling organisms, such as diatoms and algal spores (Schultz et al. 2000; 2003). The apparatus has also been used in a number of studies to determine the attachment strength of *Ulva* spores and sporelings (young plants) to experimental surfaces designed to minimise biofouling (Youngblood et al. 2003; Hoipkemeier-Wilson et al. 2004; Holland et al. 2004; Pettitt et al. 2004; Chaudhury et al. 2005; Tang et al. 2005). The wall shear stress necessary to dislodge organisms attached to test surfaces is measured in an enclosed water channel in which a fully-developed turbulent flow is produced, the wall shear stress directly depending on the volume flow rate of seawater moving through the channel. The wall shear stress in the test section of the water channel was calculated from measurements of the pressure drop along the length of the channel, as described by Schultz et al. (2000). Calibration of this apparatus showed that the repeatability of the wall shear stresses produced in the test section of the channel was within 4% across the range of flow rates used (Schultz et al. 2000). When not exposed to the flow, individuals of *A. gibbosa* were about 600 μm high for brachiolaria larvae and metamorphic individuals, and about 300 μm in height for juveniles. When exposed to the flow, both brachiolaria larvae and metamorphic individuals bent over, the degree of bending being to some extent proportional to the velocity. At low flow rates, the height of these individuals was then roughly considered as their diameter ($\sim$300 μm and 400 μm, respectively). These heights were greater than the thickness of the viscous sublayer in the test section of the channel (6–35 μm over the design velocity range; Schultz et al. 2000) but remained within the limit of the inner boundary layer where viscous forces are still important ($\sim$525 μm; Schultz et al. 2003). The wall shear stress was thus used as an estimation of the attachment strength of the different asteroid life stages tested. However, the term ''nominal wall shear stress'' is used when reporting the results, since it represents the stress determined during calibration of a smooth test section rather than the actual stresses encountered by individual organisms (see also Abelson & Denny, 1997; Koehl & Hadfield, 2004).

Before each experiment, the glass slides were gently washed in filtered seawater to remove unattached specimens. They were then positioned flush with the channel wall and held in place by the vacuum system integral to the water channel design. The water channel was then filled before the flow was turned on, so specimens started the experiment completely immersed in seawater. For each developmental stage investigated, five flow rates were chosen to evaluate the attachment strength of the individuals. Three replicate slides were used for each flow rate, attached individuals being counted before and after the experiment in order to represent data in terms of percentage of removal. An effect of time was observed during the pilot experiments; the longer the time during which the flow was applied, the higher the percentage of individuals detached. A time of exposure of 2 min was thus chosen for all experiments.

Statistical analysis

Data were compared and analysed by logistic regression (General Linear Model) using the statistical software R (R Development Core Team, 2005). This method consists of logit transformation in order to linearise the data before using linear regression. Values of critical nominal wall shear stress corresponding to 50% of removal as well as both the slope and intercept of the regression were estimated using the maximum likelihood method.

Morphological observations

Specimens of *A. gibbosa* were observed and photographed *in vivo* with a Zeiss Stemi 2000-C binocular microscope equipped with a Sony Cybershot 3.3 megapixel digital camera. For scanning electron microscopy (SEM), specimens were fixed in Bouin's fluid for 12 h. They were dehydrated in graded ethanol series, dried by the critical point method (with CO_2 as transition fluid), mounted on aluminium stubs, coated with gold in a sputter coater, and observed with a JEOL JSM-6100 scanning electron microscope. Images were digitised with the SEMafore 3.0 Pro® software.

Results

Attachment strength was evaluated for the three successive developmental stages of *A. gibbosa* included in the perimetamorphic period, i.e. the brachiolaria stage, the metamorphic stage and the juvenile stage. At 21°C, the brachiolaria larvae hatched 4 d after fertilisation. Three days later, their attachment complex was fully-developed, comprising two asymmetrical brachiolar arms and an adhesive disc (see Figure 1A). At that time, the brachiolaria

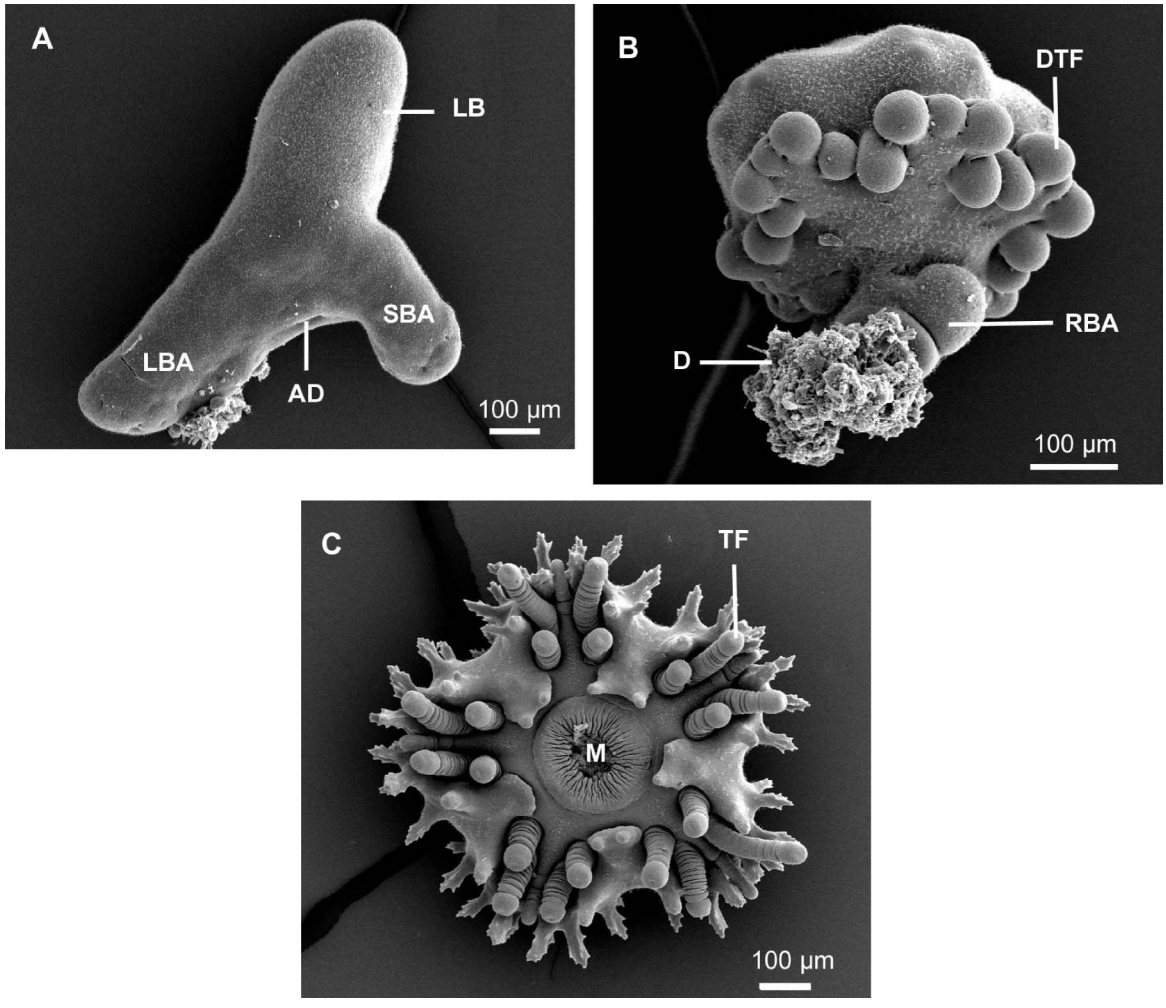

Figure 1. Illustration of the three developmental stages included in the perimetamorphic period of *A. gibbosa* (SEM). A = brachiolaria larva; B = metamorphic individual attached by the adhesive disc to a detritus particle; C = oral side of a juvenile. AD = adhesive disc; D = detritus particles; DTF = developing tube foot; LB = larval body; LBA = long brachiolar arm; M = mouth; RBA = regressing brachiolar arm; SBA = short brachiolar arm; TF = tube foot.

larvae, attached to the glass microscope slides by their brachiolar arms, were tested in the water channel. After 8 d development, the first larvae entered metamorphosis (Figure 1B). The next tests were carried out on the tenth day, when most of the individuals were anchored by the disc. The third set of assays was performed on juveniles, after 17 d development, when they were attached to the slides by their tube feet (Figure 1C).

Results are presented in Figure 2 in terms of the removed fraction of attached individuals as a function of the nominal wall shear stress. For each developmental stage, the removed fraction was positively correlated with the nominal wall shear stress, following a sigmoïdal curve. However, when comparing the logit-transformed data (following linear regression), the three straight lines showed different elevations (intercept values) and/or slopes. The elevation of the A line (larvae attached by the brachiolar arms) was significantly higher ($p = 0.004$) than that of the T line (juveniles attached by the tube feet). However, their slopes were not significantly different from each other (0.46 and 0.31 for the A and T lines respectively, $p = 0.192$). Moreover, the range of nominal wall shear stresses needed to dislodge 100% of both brachiolaria larvae and juveniles was similar (10 – 15 Pa; Figure 2). On the other hand, the range of nominal wall shear stresses recorded for the detachment of metamorphic individuals was substantially different from the range of values observed for brachiolaria larvae and juveniles. The slope of the D line (metamorphic individuals attached by the disc) was highly significantly lower than the slopes of the A and T lines (0.04 for the D line, $p < 0.001$). However, the maximum wall shear stress metamorphic individuals can withstand

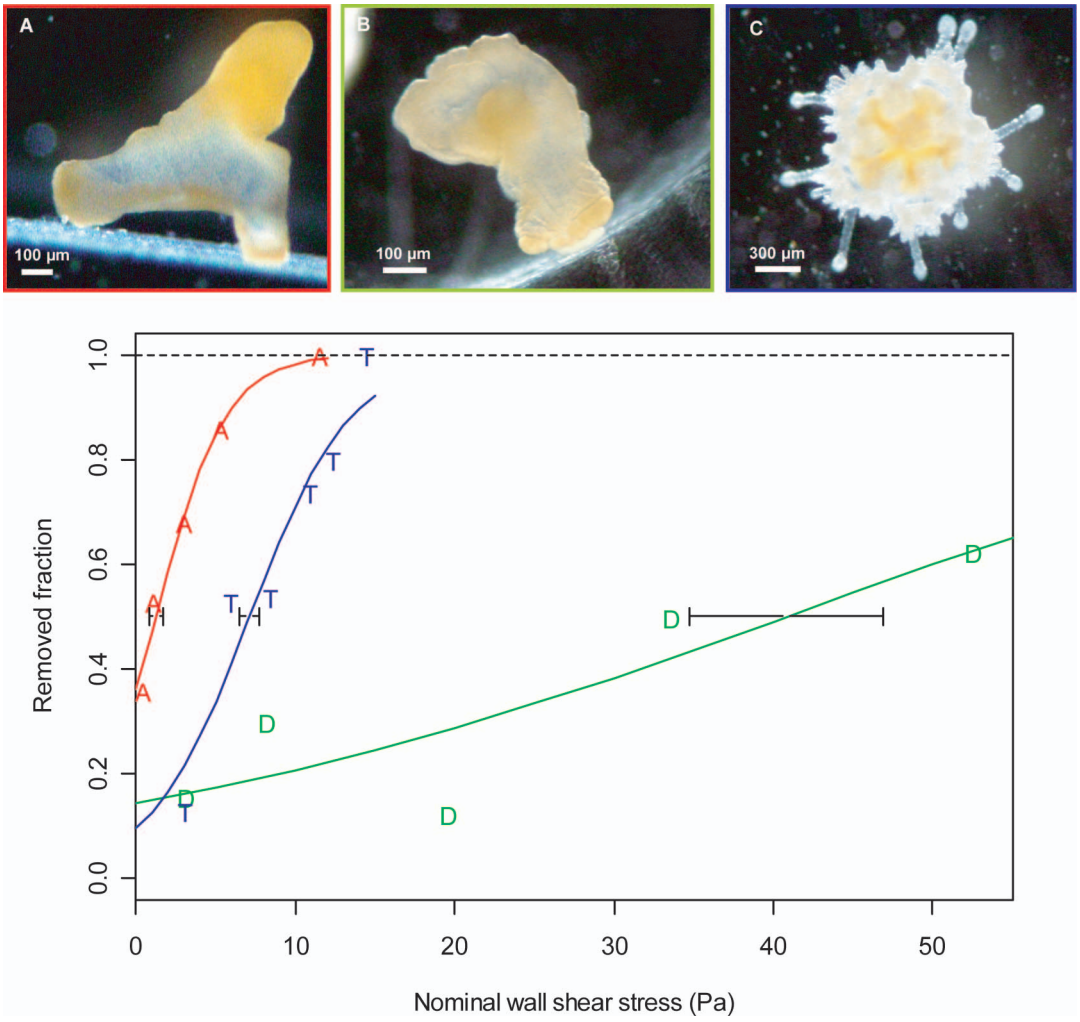

Figure 2. The effect of wall shear stress on the adhesion of the three perimetamorphic stages of *A. gibbosa* (A and B = lateral views; C = top view) to glass, as determined in the turbulent channel flow apparatus. Each character represents the removed fraction of brachiolaria larvae attached by the brachiolar arms (A; red), of metamorphic individuals attached by the adhesive disc (D; green), and of juveniles attached by the tube feet (T; blue) at a particular wall shear stress; and is derived from three replicate slides. The data (presented as untransformed) were fitted by logistic regression and critical nominal wall shear stresses ± standard errors (horizontal bars) were calculated from the curves ($n = 179$, 119 and 131 for the A, D, and T curves, respectively).

remains unknown since only 60% of the individuals were removed when the highest flow rate of the water channel was reached. Moreover, at that flow rate, approximately 30% of the metamorphic individuals became detached by rupture of the tissues, the disc remaining glued to the glass slide. These individuals were counted as removed, though they reflected tissue cohesive strength rather than the disc attachment strength.

From the regression curves, mean values of critical wall shear stress (i.e. the wall shear stress required to dislodge 50% of the attached individuals) were calculated. The critical wall shear stress ($\pm$ standard error) necessary to dislodge metamorphic individuals (40.8 ± 6.1 Pa) was one order of magnitude higher than the stresses required to detach brachiolaria larvae (1.2 ± 0.4 Pa) and juveniles (7.1 ± 0.6 Pa).

Discussion

An organism in a turbulent flow experiences numerous hydrodynamic forces associated with fluid motion, including drag, lift, skin friction, and fluid acceleration reaction forces (Denny, 1988). A small organism attached to the substratum is also subject to important forces due to a shear stress (Abelson & Denny, 1997; Crimaldi et al. 2002). This is because, within a turbulent benthic boundary layer, a velocity gradient perpendicular to the substratum is formed, resulting from the no-slip condition at the interface of the moving water and the substratum (Denny, 1988; Crimaldi et al. 2002). The faster the flow is, the higher the shear stress generated. In turbulent boundary layer flows, the shear stress is typically large (Crimaldi et al. 2002). At the scale of a larva, it has been shown that all the hydrodynamic forces exerted on the organism are proportional to the local fluid velocities (close to the substratum) and thus to the shear stress (Ackerman et al. 1995; Crimaldi et al. 2002). Thus, although predicting all of the instantaneous constituent forces acting on a larva is complex, measuring the nominal wall shear stress necessary to dislodge a microscopic organism provides a reliable measure of its attachment strength.

In this study, a turbulent channel flow apparatus was used to determine the adhesion strength of the three perimetamorphic stages of the asteroid *A. gibbosa*, viz. the brachiolaria larvae, the metamorphic individuals and the juveniles. The critical nominal wall shear stresses required to detach the different life stages are different one from the other. However, those measured for the dislodgement of larvae attached by the brachiolar arms and of juveniles attached by the tube feet are closer to each other than to the stress needed to dislodge metamorphic individuals attached by the adhesive disc (see Table I).

This variability in adhesion strength reflects differences in the functioning and fine structure of the adhesive organs for the different perimetamorphic stages of sea stars. Brachiolar arms and tube feet rely on temporary adhesion, which involves repetitive cycles of attachment to and detachment from the substratum. They both contain a duo-gland system comprising adhesive and de-adhesive cells

Table I. Mean wall shear stresses required to dislodge various microscopic aquatic organisms from their substrata in turbulent flow conditions.

Organism Species	Life stage	Adhesive structure/ organ	Substratum	Wall shear stress (Pa)	Reference
Diatom					
Amphora coffeaeformis	–	–	Glass	10	Holland et al. 2004
Craspedostauros australis				3	
Navicula perminuta				25	
Green alga					
Ulva (Enteromorpha) linza	Spore	–	Glass	130	Finlay et al. 2002
Barnacle					
Balanus amphitrite	Competent cyprids	Antennules	PMMA	1.5	Eckman et al. 1990
Nudibranch					
Phestilla sibogae	Competent pediveliger	Foot	Glass	4.3	Koehl & Hadfield, 2004
Zebra mussel					
Dreissena bugensis	Postlarva (plantigrade)	Foot (byssus)	PVC	80	Ackerman et al. 1995
Asteroid					
Asterina gibbosa	Brachiolaria larva	Brachiolar arms	Glass	1.2	Present study
	Metamorphic individual	Adhesive disc		40.8	
	Juvenile	Tube feet		7.1	

(Haesaerts et al. 2005; Haesaerts et al. 2006). Moreover, for another sea star species, *Asterias rubens*, the adhesive of the brachiolar arms cross-reacts with antibodies raised against the adhesive of the tube feet, demonstrating that they possess common epitopes (Haesaerts et al. 2005). There is nothing surprising, therefore, about the similarity between the adhesion strength of larvae and juveniles. The adhesive disc, on the other hand, is an epidermal structure that encloses a single type of secretory cell. These cells are used only once, at the onset of metamorphosis, and are responsible for the strong adhesion of the metamorphic individual. This adhesion can be described as permanent. Indeed, although the late metamorphic individual ruptures its fixation to the substratum and becomes motile, this rupture is not a true detachment of the disc but results from the regression and failure of the stalk connecting the disc to the rest of the body, the disc itself remaining cemented to the substratum in this latter case. These breaking events occurred frequently in the turbulent flow conditions and, therefore, the critical wall shear stress reported for the dislodgement of metamorphic individuals must be considered as an underestimate. This heterogeneity in the mode of failure (adhesive vs. cohesive) is reflected by the curve slope obtained for the detachment of the adhesive discs, which is much lower than the slopes measured for the detachment of brachiolar arms and tube feet, indicating a large variability in the critical shear stresses required to dislodge the metamorphic larvae.

Table I compares the mean wall shear stresses required to detach various microscopic organisms from their substrata in turbulent flow conditions. When comparing the values, however, it has to be borne in mind that these nominal wall shear stresses were obtained under different flow conditions, on different surfaces and with different times of exposure of the organisms to the flow. Nevertheless, the table shows that organisms attaching permanently to the substratum (i.e. algal spores, mussel postlarvae, and metamorphic asteroids) present an adhesive strength about one order of magnitude higher than organisms attaching non-permanently (i.e. barnacle cyprids, nudibranch pediveligers, and asteroid larvae and juveniles). Among the latter, however, asteroid larvae and juveniles, and barnacle cyprids, which all use temporary adhesion, appear to have the same resistance to flow as nudibranch pediveligers using transitory adhesion. As for diatoms, they present very large interspecific variability in their adhesion strength, the significance of which is not clear (Holland et al. 2004).

A turbulent water channel is very useful to compare the adhesion strength of organisms to different substrata (Holland et al. 2004; Koehl & Hadfield, 2004), the adhesion strength of different species (Holland et al. 2004), or the adhesion of different life stages of a same species (Ackerman et al. 1995; present study). However, these adhesion measurements cannot be extrapolated easily to what occurs in the natural conditions. Indeed, for values of water mainstream velocity typical of the wave-swept environment (i.e. $u_\infty = 1\,m\,s^{-1}$; Denny, 1988), the wall shear stress would be about 10 Pa. This means that most organisms attached non-permanently would be dislodged in the field. It does not happen because conditions in the water channel and in the field are quite different. The water channel apparatus provides a unidirectional and steady flow creating a fully-developed turbulent boundary layer (Schultz et al. 2000; 2003), a situation that is not typical of the wave-swept environment. In waters of intermediate and shallow depth, fluid velocity at the bottom oscillates along the direction of wave propagation, and thus the water gradually accelerates and decelerates (Denny, 1988). Therefore, the durations of high wall shear stresses that small organisms experience in the field are probably much shorter (lasting seconds or less) than in the water channel where maximum flow was applied for 2 min. Short pulses of a given wall shear stress are unlikely to detach an organism while a longer exposure to the same stress might wash it away. The roughness of the substratum also influences the forces acting on small organisms. Flow near the substratum can be significantly slower than in free-stream due to local topography and neighbouring organisms (Lau & Martinez, 2003; Koehl & Hadfield, 2004). Moreover, flow velocities and therefore wall shear stresses are even lower in pits and crevices (Abelson & Denny, 1997; Koehl & Hadfield, 2004). Granhag et al. (2004) showed that spores of the green alga *Ulva* were less susceptible to detachment when they settled in pits. The asterinid *A. gibbosa* lives in intertidal rock pools, from sheltered to exposed shores, where the adults are predominantly or completely restricted to the undersides of boulders (Crump & Emson, 1983). When spawning occurs, the animals remain hidden under boulders and even tend to aggregate and to enter crevices. The larvae develop and metamorphose on the same spot where fertilised eggs are laid by the adults, and thus the perimetamorphic stages of *A. gibbosa* present a low risk of being dislodged.

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