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ADHESION AND REATTACHMENT OF COMPOUND ASCIDIANS TO VARIOUS SUBSTRATA: WEAK GLUE CAN PREVENT TISSUE DAMAGE

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Summary

Sessile, soft-bodied, compound ascidians are successful competitors for substrata in crowded benthic and epibiotic marine communities and can be effective colonists of new sites, through adult rafting and reattachment. Adhesion to the substratum is essential for these ecologically important functions; we therefore studied the material properties of colony attachment to various substrata in the rafting ascidians *Botrylloides* sp. We found that, compared with the strength of the colony tissues, the glue attaching *Botrylloides* sp. to the substratum is very weak. This relative weakness may protect the soft-bodied colonies from damage if they are ripped from their host. For sessile animals, such a weak-glue 'strategy' is only effective if the animals can later reattach to a substratum. By detaching *Botrylloides* sp. colonies from host eelgrass blades and allowing them to reattach, before measuring peel strengths, we learned that the initial reattachment of a colony depends upon rapid new growth of the colony rather than on fresh secretion of glue beneath old zooids. We also found that the propagation peel force necessary to remove *Botrylloides* sp. from different substrata (e.g. mussel shells, barnacle basal plates or eelgrass blades) depends upon the surface texture of the host. Thus, the overall tenacity of a colony is affected by the types of substrata that it overgrows.

Key words: ascidian, *Botrylloides*, biofouling, tenacity, adhesion, reattachment.

Introduction

Compound ascidians grow as sheet-like colonies of zooids embedded in a common extracellular tunic, with the underside of each colony attached to the substratum by a layer of glue. Such ascidians are effective colonists of new substrata and successful competitors in benthic marine communities. Unlike many sessile animals, compound ascidians are able to reattach and recover after dislodgement. For example, colonies of *Botrylloides* sp. carried to new habitats on floating seagrass blades can reattach, grow and reproduce; this adult rafting has been shown to be an important mode of dispersal for ascidians with short-lived larvae (Worcester, 1994). Compound ascidians frequently overgrow neighboring organisms (e.g. Birkeland *et al.* 1981; Nandakumar *et al.* 1993), and some species can move, whole or in fragments, across the substratum (e.g. Birkeland *et al.* 1981; Carlisle, 1960; Cowan, 1981). In spite of the importance of adhesion in each of the functions listed above, the adhesive strengths of compound ascidians have not been quantified.

The strength with which any attached sheet, including a living one, adheres to the substratum depends upon the mechanical properties of both the sheet and the glue, since a crack may propagate in either to cause adhesive failure (Salomon, 1967). The relative strengths of glue–substratum, glue–organism and glue–glue interfaces can also determine the level of tissue damage that occurs when a sessile organism is dislodged. Although the composition of the tunic has been described for several ascidian species (e.g. Cloney, 1990; Deck *et al.* 1966; De Leo *et al.* 1977; Hirose *et al.* 1994, 1995; Smith and Dehnel, 1971; Van Daele *et al.* 1992), the mechanical properties of colonies are poorly characterized. Similarly, while the chemical and mechanical properties of marine adhesives are known for a number of organisms, including mussels, barnacles and limpets (e.g. Ackerman *et al.* 1996; Cook, 1970; Dougherty, 1990; Naldrett, 1993; Papov and Waite, 1995; Rzepecki *et al.* 1991; Smith, 1992; Waite, 1990; Young and Crisp, 1982), such information is lacking for ascidian adhesives.

We have studied adhesion by the rafting ascidians, *Botrylloides* sp. (Worcester, 1994), a species abundant off the coast of San Francisco, CA, USA. The purposes of this study were (1) to compare the strength of *Botrylloides* sp. colony tissues with that of their glue; (2) to measure the adhesive strength (standard peel strength) of *Botrylloides* sp. to different substrata on which they commonly occur, and (3) to assess the adhesive strength of colonies that have reattached to a substratum. Our focus was on the material properties of both

the tissue and glue of a representative soft-bodied, sessile organism that reattaches after dislodgement from the substratum.

Materials and methods

Animals

Colonies of *Botrylloides* sp. were collected during April and June 1994 and May 1996. Eelgrass (*Zostera marina*) blades with colonies attached were collected in Tomales Bay, CA, USA. Mussels (*Mytilus* sp.) covered by colonies were collected from docks at the Berkeley Marina, San Francisco Bay, CA, USA, as were colonies of *Botrylloides* sp. growing directly on the docks. Mechanical tests were conducted on the day of collection. For the few hours before use, animals were kept in aerated sea water at 11 °C and were removed only for immediate testing in air.

Material properties

The material properties of colonies were measured for a strip of tissue (tunic plus zooids) cut from each colony using parallel razor blades glued 9 mm apart on a handle. The width (W) and thickness (T) of each strip were measured using vernier calipers, width to the nearest 0.1 mm and length to the nearest 1 mm, and the initial cross-sectional area (A_0) was calculated ($A_0=WT$). Strips were gripped in clamps lined with rubber padding, and the unstretched specimen length between the grips (L_0) was measured to the nearest 0.1 mm using calipers. Strips were then pulled uniaxially in an Instron universal testing instrument (model 1122) that simultaneously measured force (F) and extension (ΔL). When stress ($\sigma=F/A_0$) was plotted as a function of extension ratio [$\lambda=(\Delta L+L_0)/L_0$], the slope of the straight portion of each curve was taken to be the modulus of elasticity (E, a measure of stiffness) for that specimen (Fig. 1). The strength of each specimen was the stress (σ) at which it fractured ($\sigma_{BRK}=F_{BRK}/A_0$).

The mechanical properties of pliable biological tissues often depend upon the rate at which they are deformed, so we conducted stress–strain tests on *Botrylloides* sp. tissues pulled at a variety of strain rates (strain rate=$\Delta L/\Delta t L_0$, where ΔL is the increase in specimen length in the time interval Δt). The strain rates experienced by *Botrylloides* sp. in the field were too low to be measured directly, so we estimated an upper limit to the strain rates they might experience. Although there was little water motion at our Berkeley Marina site, peak water current velocities in the Tomales Bay seagrass beds ranged between 10^{-2} and 6×10^{-2} m s^{-1} (Worcester, 1995); therefore, if a loose flap (of the order of 10^{-1} m in length) of an attached ascidian colony were pulled at exactly the rate at which the water was moving, it would experience strain rates of approximately 0.1–0.6 s^{-1}. Strain rates must be lower than these estimates because observations of water marked with fluorescein in Tomales Bay showed that the water moved past the tips of colony flaps. Therefore, we used strain rates in the range of the estimated maximum (0.2–0.3 s^{-1}), as well as one and two orders of magnitude lower (0.02–0.03 s^{-1} and 0.002–0.003 s^{-1}).

Adhesive strengths

Colony adhesive strengths to two different substrata, eelgrass blades and mussel shells, were measured using peel tests (e.g. Salomon, 1967) conducted on the Instron (Figs 2A, 3A). Every test geometry gives a characteristic curve of peeling force as a function of the distance a strip is peeled. These curves often include initiation or conclusion peaks that are disregarded when calculating propagation peel force per strip width (F/W; Benson, 1967). To determine the characteristic curves for the test geometries used here, control peels were performed using Sellotape strips of uniform stickiness. The curves for tape T-peels (in which the sticky side of one piece of tape was stuck to the non-sticky side of another, to simulate a colony stuck to a grass blade) contained initiation peaks (Fig. 2D), while those for tape peels from mussel shells contained final peaks (Fig. 3C). On the basis of the Sellotape controls, both initiation and final peaks were disregarded, and only the first 1 cm of each mussel-shell peel was digitized.

Colonies of *Botrylloides* sp. were peeled from grass blades (Fig. 2A) at peel rates spanning three orders of magnitude (length of colony peeled off the substratum per unit time 2.5, 25 and 250 mm min^{-1}), chosen to correspond to the Instron head speeds used in the stress–strain tests described above. We found no trend in peel propagation force per width (F/W) with peel rate, although the F/W values measured at the intermediate peel rate were slightly higher (by approximately 1 N m^{-1}) than the F/W values at the faster and slower rates (Kruskall–Wallis test, P=0.008, N=25). The F/W data reported below were therefore all gathered at peel rates of 25 mm min^{-1} for grass T-peels and at 20 mm min^{-1} for mussel peels (to correspond to the strain

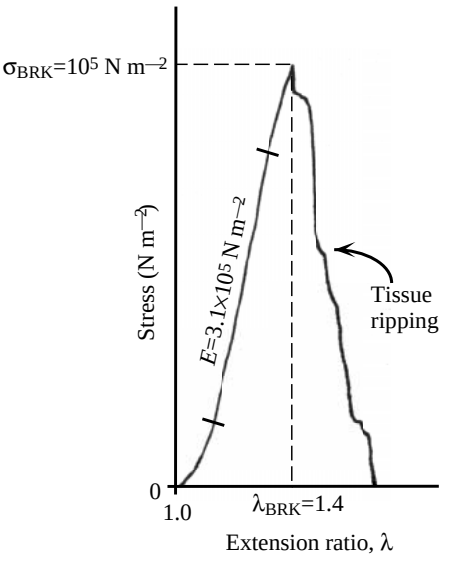

Fig. 1. A typical stress–strain curve for a strip of colony tissue (tunic plus zooids, 9.0 mm wide × 2.0 mm thick) pulled uniaxially at a strain rate of 0.02–0.03 s^{-1} in an Instron universal testing instrument. The section of the curve that was used to calculate the elastic modulus (E) is shown between the tick marks. Dashed lines indicate the breaking stress (σ_{BRK}) and breaking extension ratio (λ_{BRK}) at the time that the tissue began to rip.

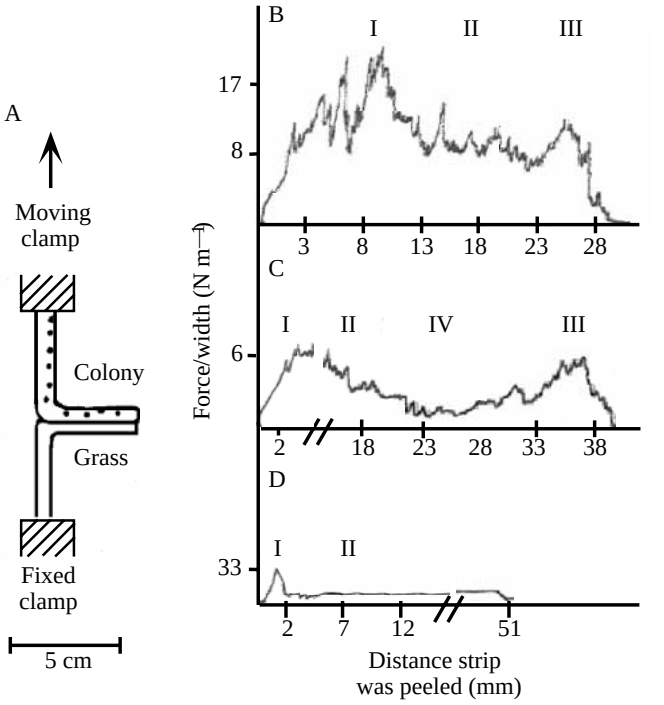

Fig. 2. (A) Geometry of a T-peel test, in which a seagrass blade was held in a fixed clamp and the ascidian colony was attached to a moving clamp that peeled it from the grass. (B–D) Examples of peel force/width measured as a function of distance a colony was peeled for an undisturbed colony on seagrass (B), a pre-peeled colony allowed to reattach to seagrass (C) and two pieces of Sellotape in a T-peel test (D). I, initiation peak; II, undisturbed old growth; III, new growth; IV, pre-peeled old growth. Old and new growth designations are explained in the text.

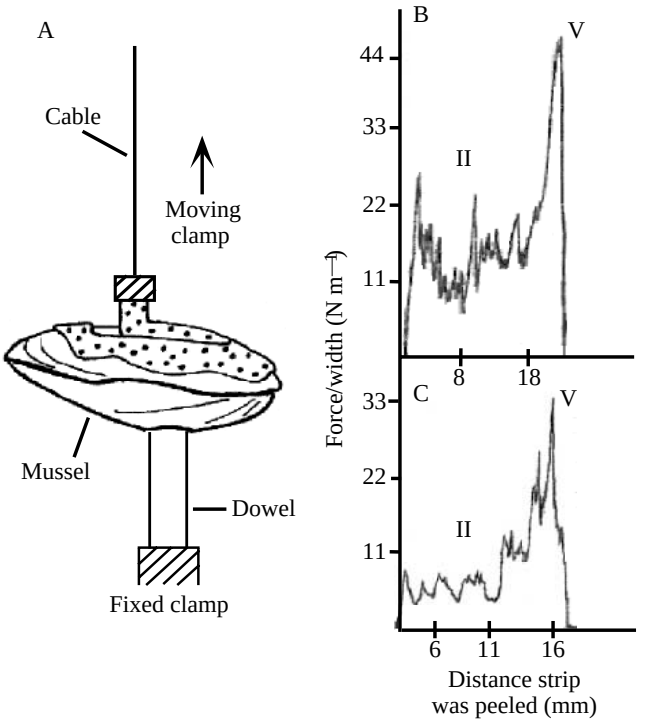

Fig. 3. (A) Geometry of a mussel peel test, for which a strip of tissue was cut from the colony using razorblades fixed 9 mm apart on a handle; the tip of this strip was held in a padded grip suspended from the upper clamp of the Instron. The mussel shells were glued to wooden dowels, which were held in the lower clamp of the Instron. Final peaks were due to the changing peel angle during a test and to the curve of the mussel shells, since both of these variables increase the force component acting to shear the glue, thereby increasing the total measured force (Lake and Stevenson, 1982). In an effort to reduce the change of peel angle during a test, the upper clamp in mussel-shell peels was suspended from an essentially inextensible cable, 25 cm long. (B–D) Examples of peel force/width measured as a function of distance a strip was peeled for an undisturbed colony on a mussel (B) and for Sellotape on a mussel (C). II, undisturbed old growth; V, final peak. Old and new growth designations are explained in the text.

rates of 0.02–0.03 s^{-1} at which we measured the tissue material properties). This was the closest match of mussel-peel and grass-peel rates possible, given the head-speed settings available on our Instron; in a mussel peel, the distance traveled by the head equaled the length of colony peeled off the shell, whereas in a grass T-peel, the distance traveled by the head was twice the length of colony peeled off the grass (Figs 2A, 3A).

Adhesive peel F/W can depend on the angle between the surfaces being peeled apart. Because the focus of this study was on the material properties of the adhesive, we chose to use the standard peel-test angle of 90°. Evaluation of experimental peels at higher or lower angles is difficult: at peel angles of less than 90°, the force component acting to shear the glue increases, thereby increasing the total necessary peel force; at angles greater than 90°, force must be exerted to bend the colony as well as to peel the glue (Lake and Stevenson, 1982). Observation of loose colony flaps in the field revealed that the angle of the flap (with respect to the substratum and rest of the colony) depended on the direction of ambient water flow, the shape of the substratum and the shape and thickness of the colony. The 90° angle used in our peel tests certainly fell within the range of flap angles observed in the field.

Reattachment

To study the reattachment capabilities of adult *Botrylloides* sp., colonies were gently detached from eelgrass substrata and allowed to reattach over a period of 5 or 7 days, before they were peeled using the Instron. On the day of collection, a butter knife was used to detach approximately 50 % of the length of each strip-shaped colony. Grass blades were then suspended from a styrofoam float at the Berkeley Marina, with the attached half of each colony uppermost, so that the loose flap hung down flat against the grass. Control colonies, which had not been detached from their grass blades, were hung among the pre-peeled colonies.

The colonies grew quickly, on average more than 20 mm in 5 days, and we looked for possible relationships between growth rate, attachment strength and glue age. Colonies were the width of the grass blade, strip-shaped and grew primarily

in length along the grass blade. After 5 days in culture below a styrofoam float, the lower half of a suspended experimental colony contained a region of old pre-peeled zooids and a tip region of new freshly grown zooids. To compare adhesive strengths in areas of new growth with those in older regions, we calculated the full increase in colony length (Δl) after 5 days; colony lengths (l_{Dayi}) were measured parallel to the axis of the grass blade, using vernier calipers, to the nearest 1 mm: $\Delta l = l_{Day5} - l_{Day1}$. The 'old' colony was then conservatively identified as beginning at least this distance (Δl) from the lower end of the strip-shaped colony. 'New growth' was identified as the portions of the colony at distances of less than $\Delta l/2$ from the growing ends of the colony. Eight evenly spaced force records from the colony were digitized within each of the regions ('new growth' and 'old'; see Fig. 2B).

Results

Material properties

We found that increasing strain rate had no significant effect on the extension ratio (λ_{BRK}) at which specimens broke, but did lead to a significant, but small, increase in strength and stiffness (σ_{BRK} increased twofold and E increased fourfold for a 100-fold increase in strain rate; Kruskall–Wallis test, $P<0.05$ for significance, $N=14$) (Table 1). Since strain rate had only a small effect on mechanical properties, the data reported below were gathered at strain rates of 0.02–0.03 s^{-1}, which we deemed to be both biologically relevant and sufficiently slow to permit careful observation of the specimen during a test.

A typical stress–strain curve for tissue from *Botrylloides* sp. is shown in Fig. 1. Colony material properties did not differ significantly between the two collection sites or between 1994 and 1996 (Mann–Whitney U-test, $P>0.05$ for all comparisons); hence, results from tests on colonies collected during both years and from both eelgrass blades and marina walls were averaged. The mean elastic modulus (E) of *Botrylloides* sp. colony tissue was $2.9\times10^5\pm0.96\times10^5$ N m^{-2} (±S.D., $N=22$), similar to that of the mesoglea of the sea anemone *Metridium senile* (Koehl, 1977), but not as stiff as the tunic wall of the leathery solitary ascidian *Pyura haustor* (compressive E approximately 10^6 N m^{-2}; R. Tabachnick, personal communication). The mean strength (σ_{BRK}) of *Botrylloides* sp. tissue was $1.4\times10^5\pm0.18\times10^5$ N m^{-2} ($N=20$), similar to that of *M. senile* mesoglea, but lower than that of the spiculated coenenchyme of various species of soft corals (10^6 to 2×10^6 N m^{-2}) (Koehl, 1997). The tissue of *Botrylloides* sp. was very extensible, with breaking extensions (mean $\lambda_{BRK}=1.5\pm0.17$, $N=14$) as high as those of the stretchy stipes of the kelp *Nereocystis luetkeana* (Koehl and Wainwright, 1977).

Adhesive strengths

The peel strength of *Botrylloides* sp. glue could be affected by the type of substratum to which the colonies attached. For example, the peel forces of *Botrylloides* sp. over old barnacle basal plates on mussel shells were significantly higher than those over an immediately adjacent area of clean shell (Mann–Whitney U-test, $P=0.005$, $N=6$). When we removed the sections of peel force records that occurred when the colonies were being peeled from barnacle basal plates, we found that the mean propagation F/W for colonies peeled from mussel shells was 6.9 ± 3.42 N m^{-1} ($N=7$). This mean mussel F/W did not differ significantly from the mean F/W of 4.7 ± 0.64 N m^{-1} ($N=9$) for colonies peeled from eelgrass blades (Mann–Whitney U-test, $P=0.49$, $N=16$). However, the variance of F/W values between colonies pulled from mussel shells was significantly greater than the variance between colonies pulled from grass blades (test of equal variance, $P<0.01$) (Fig. 4). Furthermore, there was more variation in the peel forces measured across a single colony on a mussel shell than across a comparable colony on a grass blade (e.g. compare Fig. 2B with Fig. 3B). Because they supported small regions of very tight or very loose ascidian attachment, other epibionts growing on the mussel shells were a major source of peel-force variability. Dips in F/W during a peel test often occurred when the colony was being peeled from a region of shell surface covered by a brown slime, apparently the decayed tissue of another epibiont over which the ascidian had grown.

The forces required to peel colonies from their substrata were quite low relative to the loads necessary to break colony tissue. The calculated stresses [$\sigma=(F/W)(1/T)$] in each colony peeled at these F/W values were only 3 %, on average, of the colony breaking stress. Such stresses in peeling colonies correspond to extension ratios (λ) of only 1.08 (range 1.05–1.19). Colonies therefore stretch by only 8 % before beginning to peel, but require extensions of 50 % before beginning to break. Even the highest peel forces we measured (e.g. initiation peaks and peaks over barnacle basal plate substrata) were substantially lower than the colony breaking strength. For example, the mean F/W measured in initiation

Table 1. *Material properties of* Botrylloides *sp. tissue when pulled at different strain rates*

	Strain rate (s^{-1})		
	0.002–0.003	0.02–0.03	0.2–0.3
E (N m^{-2})	$1.2\times10^5\pm0.45\times10^5$ (5)	$2.9\times10^5\pm0.96\times10^5$ (22)	$4.5\times10^5\pm1.3\times10^5$ (8)
λ_{BRK}	1.7±0.08 (5)	1.5±0.17 (14)	1.5±0.18 (5)
σ_{BRK} (N m^{-2})	$0.6\times10^5\pm0.16\times10^5$ (5)	$1.4\times10^5\pm0.18\times10^5$ (20)	$1.4\times10^5\pm0.53\times10^5$ (5)

Values are means ± S.D. (N)
E, elastic modulus; λ_{BRK}, breaking extension ratio; σ_{BRK}, breaking stress.

peaks was 7.5 N m^{-1} (range 2.5–17.5 N m^{-1}, N=25), corresponding to a mean stress (σ) of only 3.8×10^3 N m^{-2}, which is two orders of magnitude smaller than the tissue breaking stress. Even the highest peel stress we recorded in this study (4.4×10^4 N m^{-2}, measured over a barnacle basal plate) was an order of magnitude lower than tissue breaking stress and permitted the colony to peel intact.

Reattachment

The linear growth rates of *Botrylloides* sp. colonies on eelgrass blades were very high, with a mean growth rate of 4.2 mm day^{-1} (range 0.4–11.8 mm day^{-1}, N=34). There was a direct association between growth rate and original colony size (Kendall τ, P<0.05, d.f.=12), with larger colonies growing faster than small ones. Experimentally detached colonies reattached and had similar linear growth rates to those of the controls (Mann–Whitney U-test, P=0.16, N=34). There was no association between F/W and growth rate (Kendall τ, P>0.05, d.f.=7); hence, there appeared to be no adhesive penalty for rapid growth. There was also no association between F/W and final size (Kendall τ, P>0.05, d.f.=7). Furthermore, peel strengths after 5 days were no different from those after 7 days (Mann–Whitney U-test, P>0.8, for all comparisons).

Experimental colonies had significantly lower peel strengths in all comparisons with controls (Mann–Whitney U-test, P<0.02 for all; Fig. 4), indicating that pre-peeled colonies suffered a decrease in adhesive strength, in both 'new growth' and reattached 'old' regions. However, in control undisturbed colonies, 'old' and 'new growth' regions had indistinguishable peel strengths (Mann–Whitney U-test, P=0.32, N=16), while in reattached, experimental colonies, the peel strengths of 'old' regions were significantly lower than those of the 'new growth' regions (Mann–Whitney U-test, P=0.004, N=16). We interpret this to mean that, although the original zooids did reattach loosely in 5 days, regions of new growth were principally responsible for holding the flaps in place (Figs 2C, 4).

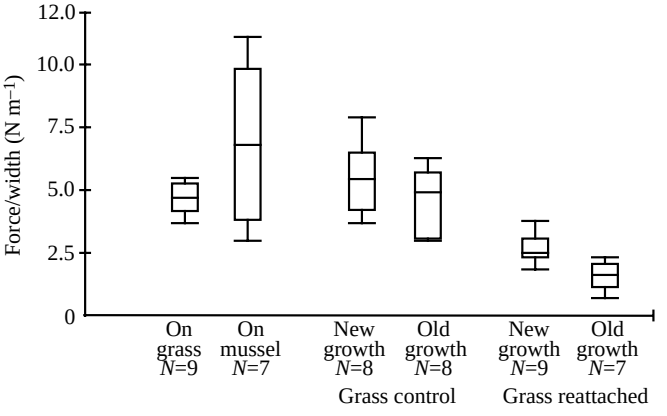

Fig. 4. Peel force/width (F/W) for four colony types in 1994: (1) first harvest colonies on eelgrass, tested on the day of collection, (2) first harvest colonies on mussels, tested on the day of collection, (3) cultured, control colonies on eelgrass, tested after a 5 or 7 day suspension from styrofoam floats, (4) cultured, pre-peeled colonies on eelgrass, tested after a 5 or 7 day suspension from styrofoam floats. Box plots are used to show patterns in F/W value and variability. The outlined central box depicts the middle half of the data between the twenty-fifth and the seventy-fifth percentiles. The horizontal line across the box marks the median F/W. Top and bottom lines depict extreme F/W values. Old and new growth designations are explained in the text.

Discussion

This study has revealed that the glue of the ascidian *Botrylloides* sp. is much weaker than its tissues, that the peel strength of the glue can be affected by the host surface and that detached colonies initially reattach *via* rapid growth, rather than by secretion of fresh glue beneath old zooids. In combination, the weak glue, the ability to reattach and the rapid growth rates of these ascidians no doubt contribute to their success in both colonization of new sites (by adult rafting and reattachment to new substrata) and competition for space (by overgrowth of neighbors).

Weak glue protects colony tissue

The peel forces we measured for the glue of *Botrylloides* sp. are very low relative to the loads required to stretch or break colony tissue. One consequence of this combination of material properties is that these ascidian colonies, while composed of deformable weak tissue, are in little danger of stretching by more than a few per cent, or of breaking, before they peel from the substratum. By permitting dislodgement without tissue damage, weak glues may enhance the survivorship of soft-bodied sessile organisms, such as ascidians, if the organisms can later reattach to the substratum.

Glue strength and colony tenacity

The peel strengths (F/W) we report, which represent the material properties of ascidian glue attached to particular host surfaces, should not be confused with whole-colony 'tenacity' (the force required to detach an entire colony per total area of attachment). Tenacity measurements have proved valuable for assessing the probability that sessile marine organisms will be swept away by hydrodynamic forces (e.g. Denny *et al.* 1985); a number of features of ascidian–substratum geometry could affect whole-colony tenacity without changing mean peel strength. For example, if the tenacity of the whole colony is determined by its point of strongest attachment, then mussel shells bearing barnacle basal plates could permit more secure overall attachment for colonial ascidians than the mean peel strengths would reveal. Furthermore, as our peel curves illustrate (Figs 2, 3), the F/W required to initiate a peel can be higher than the propagation F/W, depending upon the peel geometry when the colony is pulled off the host; such initiation peaks could certainly increase whole-colony tenacity. The site of peel initiation within a colony could also affect the tenacity of that colony; although we did not have the opportunity to observe the events that separated colonies from the substratum in the field, we did see a number of naturally occurring, loosely attached or detached regions within or on the edges of colonies. These

regions often appeared to have come free because an underlying, overgrown and decomposing organism had detached from the original substratum. The importance of the angle at which a colony is pulled from the substratum, and its possible effect on tenacity, was mentioned in Materials and methods. Differences in colony shape may further affect the tenacity achieved for a given peel strength. Although the colonies and strips of colonies used in these peel tests were selected for their flatness, it was common to find colonies that had grown flaps around the organism to which they were attached. Tenacity may be greatly enhanced if a colony is wrapped around curves or edges in the substratum, as this geometry limits the distance a colony can be peeled by a force acting in any one direction. In other words, peel strength is the proper measure for the material properties of the glue of an organism on a particular type of substratum, whereas tenacity (which depends not only on the peel strength, but also on the geometry of the organism and the substratum) is the proper measure for assessing the danger that an organism will detach in nature.

Regardless of the magnitude of whole-colony tenacity, the combination of glue and tissue material properties used by *Botrylloides* sp. permits them to be detached from the substratum without tissue damage, while their rapid growth permits them to reattach and overgrow other organisms.

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