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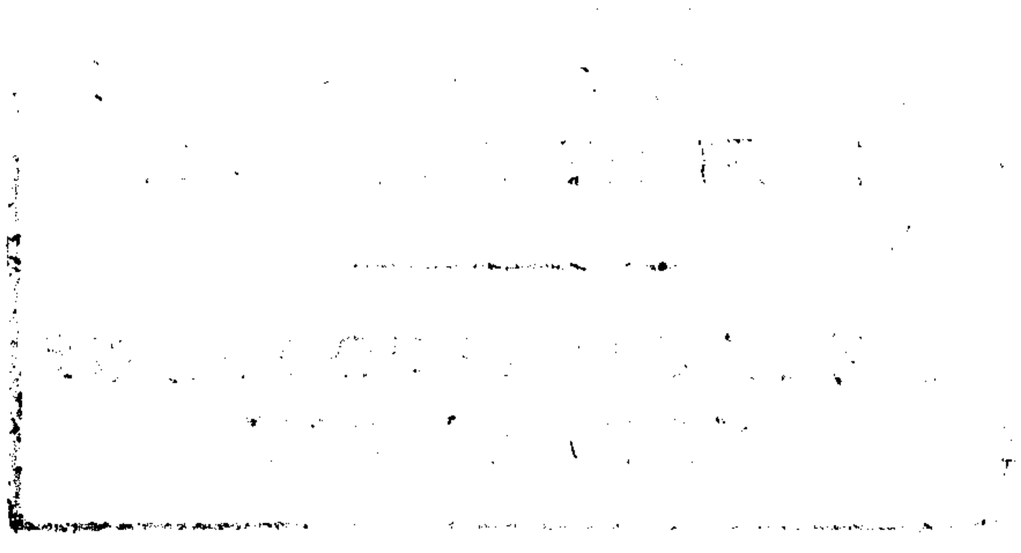
ADHESION IN LEPADOMORPH BARNACLES

A thesis submitted to the University of Wales, Bangor for the degree of *philosophiae doctor* in the School of Ocean Sciences

by

Michael Kugele B.Sc. (Wales)



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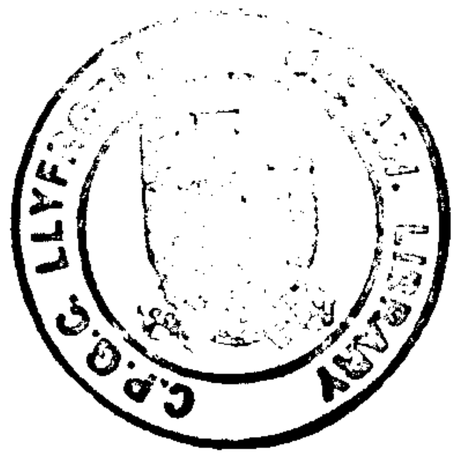

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SUMMARY

The larvae of *Pollicipes pollicipes* were succesfully reared in the laboratory and their morphological characteristics described and compared to the previously described *P. polymerus*. Attempts to induce apparently healthy cyprids to settle in quantity, using methodology commonly employed for balanomorph barnacles, were unsuccessful indicating the lack of some major settlement cue(s).

The scalpellids *P. pollicipes* and *Capitulum mitella* were shown able to voluntarily relocate, with measured speeds of up to 50 μm d^{-1}, but the lepadid *Lepas anatifera* cannot do so. The scalpellids used different mechanisms for relocation although both involved growth and sloughing of basal integument. A stimulus for directed travel was not found but gravity and unidirectional flow were rejected.

The cement of lepadomorphs was shown to dissolve very slowly in sterile seawater. Cement in flowing seawater tanks, or in the presence of bacterial isolates collected from the cement, or in the presence of protease concentrated from bacterial cultures, did not dissolve at faster rates, to that of sterile cement, than could be explained by the sample sizes.

The proteinaceous cement of *P. pollicipes* was delivered as a liquid in nl quantities over a period of 5-20 minutes before curing which took around 2 hours. Cement masses cured in seawater were found to be zoned due to a variable volume of space within, whilst cement delivered and cured in air or nitrogen was homogeneous. It was determined that the more porous inner zone of cement masses was inhibited from curing fully as a result of an inability to displace water. The partially cured zone could be induced to cure fully, by heating to dryness from a minimal volume of water. The presence of water was determined to be essential for curing. Differential degrees of curing of cement masses allowed for various physical and histochemical treatments which support both the rejection of disulphide bonding and phenol tanning and the growing evidence of hydrophobic complexing as central to the solidification mechanism of barnacle cement.

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CHAPTER 1

General Introduction

Organisms in the sea are subject to drag (pressure and friction), lift and inertial forces (which are resultant from the viscosity and density of seawater) which are often greater in magnitude than those experienced by terrestrial organisms. Many organisms resist these forces in order to maintain position which may be achieved mechanically (e.g. fish, crabs), passively, although only in one dimension (e.g. vertically migrating zooplankton and *Physalia*), or by adhesion. Adhesion may be either indirect with the organism living freely inside a structure cemented to the substratum (e.g. *Pomatoceros*, *Sabella* and corals) or direct with the organism itself cemented to the substratum (e.g. macro algae, anemones and barnacles).

Direct adhesion can be generally described as a continuum ranging from short term temporary adhesion to long term permanent adhesion. Temporary adhesion may include that caused by a pressure difference through suction (e.g. lumpsucker) but this is not common in the sea where applied forces result in pressures much greater than ambient. Thus the area of the sucker would need to be prohibitively large (in comparison to the organism's size). Temporary adhesion allows for mobility (e.g. gastropods, Miller, 1974, Grenon and Walker, 1981 and barnacle cyprids, Yule and Walker, 1987). Permanent adhesion does not necessarily prohibit mobility (e.g. anemones, Young *et al.*, 1988) or the ability to reattach (e.g. bacteria, barnacles, mussels), but organisms utilising permanent adhesion are not generally mobile (e.g. barnacles, lichens, algae, bacteria).

Direct adhesion in the sea, other than mechanical or suction, is achieved with glues. Close contact adhesion between solids (e.g. geckos, G. Walker, pers. com.) resultant from van der Waals and other electrostatic forces is not a realistic prospect in the sea. Even when sufficient area of an organism could get close enough to a surface there

would be an attached layer of water which would act as an adhesive by virtue of its viscosity (see Denny, 1988).

Marine glues are fluid when delivered with viscosity and surface tension such that natural substrata are sufficently wetted for an effective adhesive bond (e.g. see Baier *et al.*, 1968). Grenon and Walker (1980, 1981) demonstrated that limpets use such a glue. These workers also found that the adhesive was tacky, i.e. viscoelastic, reacting like an elastic solid (Grenon, 1979, Denny, 1988, Grenon and Walker, 1980, 1981) at high rates of stress due to randomly coiled long chain molecules of the mucopolysaccharide and protein in the glue. At low rates of stress the glue responded as a viscous fluid (Stéfan adhesion), essential for mobility. The response of viscoelastic glues has been termed non-Stéfan or ultra-Stéfan adhesion (e.g. see Grenon and Walker, 1981 and references therein). Young *et al.* (1988) showed that the glues employed by the anemones *Actinia equina* L. and *Metridium senile* (L.) were predominantly protein and probably operated through an ultra-Stéfan type adhesion. Protein adhesives in the marine environment appear to be commonly employed (e.g. mussels, Tamarin *et al.* 1976 and barnacles, Walker and Yule, 1984) rather than the essentially mucopolysaccharide adhesive used by gastropods (Miller, 1974, Grenon and Walker, 1980) or lipid which is far too hydrophobic for such an environment. Fluid glues are particularly useful for temporary adhesion in mobile organisms since they retain their ability to stick after they have been ruptured. Such glues are generally less energetically expensive to produce, comprising mostly water and, since their cohesive strengths are generally low, less energy is required by the organism to break the adhesive joint in order to move. The loss of material, which is inevitable as the organism moves, is of minor consequence again due to the low cost of production.

Once delivered and having wetted the adherands a glue may solidify thereby increasing its adhesive strength, particularly to shear forces. Though hard or rubbery such a cement must have sufficient strength to resist yielding or breaking under the stresses experienced in the environment. Barnacles employ both fluid and solid glues for adhesion at different stages of their life (Yule and Walker, 1987).

Yule and Crisp (1983) noted that the ability of *Semibalamus balanoides* (L.) cyprids to explore a surface by walking with comparable cohesive forces of adhesion to those of limpets indicated a high viscosity adhesive operating through Stéfan type adhesion. Nott and Foster (1969) had earlier found unicellular glands in the second segment of the antennule and suggested that they might produce a secretion. Walker and Yule (1984) demonstrated that the exploring cyprid of *S. balanoides* secretes a proteinaceous temporary adhesive over the antennular disc (third segment) and suggested that the glue may be similar in composition to integumentary proteins.

Yule and Walker (1984a) clearly demonstrated that cyprids exhibited disparate adhesion to surfaces differing only in colour which they interpreted as the cyprids' willingness to detach, concluding that adhesion force measurements were likely to be a combination of cypris election and the cohesive/adhesive strength of the adhesive. Yule and Crisp (1983) had determined that the adhesive organ of dead cyprids or amputated antennules would not stick to a surface. It would be very difficult to collect a sufficient quantity of cypris temporary adhesive to measure its true strength. Thus much research utilises the tenacity of cyprids on a variety of substrata to distinguish adhesion promoting and inhibiting properties resultant from a combination of cypris behaviour and adhesive strength to evaluate settlement inducement or inhibition (e.g. Yule and Crisp, 1983, Goodrick, 1994, Neal and Yule, 1992, 1996).

There has been little work on the temporary adhesion of lepadomorph cyprids. However, Goodrick (1994), measured the effects of cypris ageing and coating surfaces with arthropodin (soluble cuticular proteins) on the tenacity of *Pollicipes pollicipes* (Gmelin, 1790) cyprids, finding the tenacities at *ca.* 2-6 x 10^4 N m^{-2} broadly similar to those of the cyprids of the balanomorphs *Chthamalus montagui* Southward, 1976 (3-7 x 10^4 N m^{-2}), *Balamus perforatus* Brugière, 1789 (5-10 x 10^4 N m^{-2}) and *B. amphitrite* Darwin, 1854 (10-40 x 10^4 N m^{-2}. Likewise, Neal and Yule (1996) demonstrated a reduction of up to 40 % on the relative temporary adhesion of *P. pollicipes* (≡ *P. cornucopia*) and 5 balanomorph species cyprids to substrata in solutions of hexoses and glucuronic acid, indicative of glues with similar physical properties.

The permanent cement used by barnacle cyprids for fixation to the selected substratum prior to metamorphosis is proteinaceous and is passed as a fluid from the cement glands (posterior to the compound eyes) through ducts in the antennules to embed the attachment organs and usually the fourth antennular segment before it solidifies (Hillman and Nace, 1970, Walker, 1971, Cheung and Nigrelli, 1972, Yule and Walker, 1987). Walker (1971) studied the cement glands of *Semibalamus balanoides* cyprids finding two cell types which he termed α and β cells. Histochemical staining of α cells indicated secretions which were composed of proteins, phenols and polyphenol oxidase, whilst β cells were found to contain only proteins with free amino groups. Walker (1971) therefore suggested that permanent cement might solidify through quinone tanning. Hillman and Nace (1970), however, were earlier unable to demonstrate phenolic amino acids in the cement gland cells of *Balanus eburneus* Gould, 1841, but Saroyan *et al.* (1970a) demonstrated tyrosine in the gland of *B. crenatus* Brugière, 1789. Cheung and Nigrelli (1972) did not find β cells in the gland of *B. eburneus* or tyrosine and with the

results of other histochemical stains concluded that the cement was collagenous. Yule and Walker (1987), although suggesting the involvement of tanning and postulating, as Walker (1971) had done, that the α and β cells may serve to separate cement precursors until secretion, concluded only that molecular cross-linking of the delivered fluid results in the white rubbery cement. There is no published material concerning the cement glands of the cyprids of any lepadomorph barnacle.

Since the cement solidifies it has been possible to estimate its strength without any moderating behavioural component. Yule and Walker (1987) measured increasing tenacity with time from fixation for *S. balanoides* cyprids estimating the strength of fully cured cement at *ca.* 9×10^5 N m^{-2} around five times greater than the maximum tenacity (as defined by Yule and Walker, 1984) measured for temporary adhesion.

Following metamorphosis and before the adult cement apparatus is functional (Walker, 1971) juvenile *S. balanoides* augment their adhesion with a juvenile proteinaceous adhesive over the growing base which has a strength of 1.7×10^5 N m^{-2} (Yule and Walker, 1984). Since the adhesive had a similar measured strength to that of cypris temporary adhesive delivered from antennular glands, Yule and Walker (1987) suggested that the juvenile adhesive may be secreted from the base hypodermis noting that it could not be delivered from the re-differentiating cement glands.

Walker (1973) showed that the adult cement apparatus of *S. balanoides* was formed from the α cells and collecting duct cells of the cyprid cement apparatus. Lacombe and Ligouri (1969) found morphological differences in the cement glands of *Balanus tintinnabulum* (L.) and *Lepas anatifera L. L. anatifera* had more than one region of intracellular collecting ducts in the unicellular glands whilst those of *B. tintinnabulum* had a well defined single secretory region. Walker (1970) found differences in the glands

of balanomorph barnacles with membranous bases (*Elminius modestus* Darwin, 1854 and *S. balanoides*) and *Chirona hameri* (Ascanius, 1767) with a calcareous base. *E. modestus* and *S. balanoides* had intracellular collecting ducts whilst those for *C. hameri* (≡*Balanus hameri*) were extracellular. Walker (1970) also determined that the cement ducts of *E. modestus* and *S. balanoides* were chitin-lined whereas those of *C. hameri* were not. The cement delivery ducts at the base of balanomorph barnacles consist of radial primary ducts from which lead secondary ducts to the margin of the base where cement is delivered through terminal chambers (Darwin, 1854, Lacombe, 1967, Walker, 1970). There is little information concerning the ducts and delivery sites within the base of lepadomorph barnacles but Darwin (1851) figures ductwork within the base of *Pollicipes polymerus* Sowerby, 1833. Although morphological and cytological differences exist in the cement apparatus between barnacle species there is no evidence that the exuded cements are grossly different.

Like barnacle cypris cement the adult cement is proteinaceous and delivered as a liquid which solidifies with time to a rubbery white mass (Cook, 1970, Saroyan *et al.*, 1970a,b, Walker, 1972, Cheung and Nigrelli, 1972, Lindner and Dooley, 1973, Barnes and Blackstock, 1974, 1976, Walker and Youngson, 1975, Cheung *et al.*, 1977, Yan and Tang, 1981, Naldrett, 1993). Interest in barnacle cement, together with cypris cement has been directed for the most part by the cost of barnacle fouling and the development potential for underwater adhesives particularly in dentistry. Research has focused on balanomorph barnacles which are readily available in the field and easily maintained in the laboratory. Analyses of lepadomorph cements (Barnes and Blackstock, 1974, 1976, Walker and Youngson, 1975) has, however, indicated that they have a similar biochemical composition to those of balanomorphs.

The great stability of barnacle cement (e.g. Lindner and Dooley, 1973, Cheung *et al.*, 1977) has made determining the curing process with conventional analytical techniques difficult. It has been suggested that the cement cures through a phenolic tanning process (Harris, 1946, Shimony and Nigrelli, 1972, Walker, 1981, Yule and Walker, 1987) or by electrostatic, disulphide and hydrogen bonding and/or by hydrophobic complexing (Barnes and Blackstock, 1976, Yan and Pan, 1981, Naldrett, 1993). Recent work by Naldrett, (1993), concurring with the evidence from Walker's (1970) and Fyhn and Costlow's (1976) studies of cement gland cells indicates that disulphide bonds are made prior to the delivery of the liquid cement. Naldrett's (1993) NMR study also discounted the involvement of phenolic tanning, favouring hydrophobic complexing. However, curing through electrostatic (ionic and hydrogen) interaction between protein moieties, indicated by Barnes and Blackstock (1976) and Yan and Pan (1981) has yet to be confirmed or denied.

Crisp (1973) suggested that barnacle cement functioned as a Stéfan adhesive because balanomorph barnacles can be moved laterally on very smooth surfaces and the cement is delivered as a liquid which increases in viscosity on curing. The suggestion however, was ruled out by Yule and Walker (1987) on the basis of the biochemical similarity of balanomorph cement to the large masses of cement embedding the peduncle of lepadomorphs and the cross-linked nature of cured cement. Dougherty (1990), using *Chthamalus fragilis* Darwin, 1854, showed that the cement exhibited similar cohesive strength when stressed by both normal and shear forces thereby confirming Yule and Walker's (1987) negation of Stéfan type adhesion. After curing, barnacle cement must therefore act as a solid adhesive, albeit elastic or rubbery.

Of around 700 species of thoracican barnacles (Bowman and Abele, 1982) there are some 400 extant species of lepadomorphs (Zevina, 1981, 1982, Foster and Buckeridge, 1987) with many intertidal species (e.g. *Lithotrya*, *Pollicipes*, *Capitulum* and some *Calantica* and *Ibla* species). Nevertheless, research into barnacle adhesion, both ecologically and specifically dealing with the properties of the adhesive, has almost single mindedly addressed balanomorph species. There is therefore a clear requirement to consider the adhesion of lepadomorph barnacles for potential addition to our knowledge and to increase the weight of evidence to accepted theory. Tacit assumptions equating the similarity of composition to comparable functionality require testing.

By intuition, the muscular peduncle of lepadomorph barnacles is more predisposed to conjecture regarding mobility which would not be contemplated for balanomorph barnacles, irrispective of the nature of the cement. There is some existing evidence of mobility in pollicipid lepadomorphs (Hoffman, 1984, Chaffee and Lewis, 1988) if the distribution of spat and juveniles around adult peduncles is interpreted as indicating movement rather than a result of growth of the adult. An initial aim of the current work involves the appraisal of mobility in the lepadid *Lepas anatifera* and the scalpellids *Pollicipes pollicipes* and *Capitulum mitella (L.).*

It has long been known that lepadomorphs produce cement in much larger quantities than balanomorphs yet studies on only two such species have thus far been published (Barnes and Blackstock, 1974, 1976, Walker and Youngson, 1975). Larger volumes of cement should provide the opportunity to make observations and measurements that are much more difficult using the smaller quantities found under the bases of balanomorph barnacles and such is a major aim of the current work. An assessment of the stability of lepadomorph cement against dissolution in seawater and its potential as a substrate for

marine bacteria, hence an indication of its biodegradability have been made with the aim of both establishing an ecologically valid longevity of the cement in a chemically hostile environment and to provide indirect evidence for determining the potential curing mechanism. The results derived from determining stability led to the development of a novel technique which was used to investigate the mechanism of curing directly and address questions on the polymerisation posed by earlier literature (e.g. Harris, 1946, Lindner and Dooley, 1973, Barnes and Blackstock, 1976, Walker, 1981).

Overfishing of *P. pollicipes* is becoming evident on the coast of Portugal and Spain (Cardoso and Yule, 1995) where the muscular peduncle is considered a delicacy. Such is the market for *P. pollicipes* that their potential for aquaculture has been considered (Goldberg, 1984). Successful culture relies on an adequate understanding of the rearing of the larvae and the ability to induce fixation and metamorphosis at will. The involvement of adhesion in the selection of a fixation site for balanomorph larvae is the subject of much literature (e.g. see review by Walker, 1995) and such larvae can be routinely stimulated to settle in the laboratory in large numbers. Fixation of lepadomorph barnacle larvae has never been accomplished, repeatedly, in large numbers *in vitro*. Only one publication describes the larvae of *P. pollicipes* (Molares *et al.*, 1994) with less than adequate detail. The current work therefore fully describes all stages of *P. pollicipes* development and the conditions for repeatable culture. The study of fixation by the cypris larvae was a natural aim following successful culture.

CHAPTER 2

The larval morphology of *Pollicipes pollicipes* (Gmelin, 1790) (Cirripedia: Lepadomorpha) with notes on cypris settlement

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ABSTRACT

The larvae of the scalpellid barnacle *Pollicipes pollicipes* were reared in the laboratory on a mixed diet of *Skeletonema costatum* with *Rhinomonas reticulata*. The major morphological characteristics of the seven larval stages are described. Minor differences between such characteristics and those of the previously described *P. polymerus* support a close phylogenetic relationship. Stage I and II *P. pollicipes* nauplii reared from laboratory maintained adults were similarly sized to those reared from egg masses collected from the Algarve (Portugal). *P. pollicipes* larvae were larger than *P. polymerus* nauplii at each developmental stage, but differences in rearing conditions make it impossible to establish the cause as either genetic or environmental. Limited success was achieved from attempts to induce cyprids to settle using methodology commonly employed for balanomorph barnacles indicating the lack of some major settlement induction cue(s).

INTRODUCTION

Pollicipes pollicipes is a scalpellid lepadomorph found intertidally on the rocky coasts of Spain, Portugal, north-west Africa and the south coast of Brittany (Stubbings, 1967, Cruz, 1993, Jensen *et al.*, 1994). By virtue of a large muscular peduncle it has become an economically important species subject to intensive collection for human consumption. Nevertheless there is little published material on its biology (Bernard, 1988, Cruz, 1993).

Bernard (1988) estimated that 30-70 kg of the similar Californian species *P. polymerus* could be harvested at each low tide by one person. Harvesting of *P. pollicipes* has been restricted in Spain due to severe stock depletion, especially of larger animals (Bernard, 1988). Such overfishing is also becoming evident on the coast of Portugal (Cardoso & Yule, 1995).

Darwin (1851), Newman (1987) and Foster & Buckeridge (1987) indicate a close phylogenetic relationship between *P. pollicipes* and *P. polymerus*. Larval settlement (in significant numbers) has not yet been induced in the laboratory for either of these species. Lewis (1975a), using small numbers of *P. polymerus* cyprids in laboratory assays reported settlement (of 3 only) on or near healthy adult peduncles. Various other substrata elicited no settlement.

Bernard (1988) reported indiscriminate settling of *P. polymerus* on solid, natural substrata throughout the intertidal zone, although most spat disappeared within six weeks. He consequently reported that no cleared areas were colonized by *P. polymerus* but that adjacent clusters did encroach on the cleared area. He attributed this

recolonisation failure and the high mortality of spat/juveniles to predation by small crabs, polychaetes and exclusionary competition by mussels.

Hoffman (1988) addressed the question of how new clusters of *P. polymerus* are formed. Using terracotta tiles in a regularly cleaned seawater flume he reported aggregated settlement in and around pits, scratches and fixing bolts, with a maximum of 15 animals per aggregation. Hoffman (1989) later studied settlement and recruitment of *P. polymerus* in the intertidal zone of California. He found dense gregarious settlement (>300 cm^{-2}) limited to the proximal region of adult peduncles in sampled clusters. Evidence of new clusters was found on cleared rock with settlement directly to the rock and not to mussel valves. Large numbers of juvenile *P. polymerus* were also found attached directly to rock when sections of barnacle/mussel assemblages were lifted. Other novel cluster failure was considered to be due to exposure conditions. Solitary specimens were common but it could not be determined whether such individuals were founders of new clusters or remnants of failed clusters. Like Bernard (1988), Hoffman (1989) found high mortality following settlement with greater survival on solitary barnacles than on members of clusters.

It is obviously important to determine the factors necessary for successful recruitment for such an economically important species. Such is particularly crucial if the very substratum that is evidently responsible for the greatest spat survival is the object of an intense fishery.

The present work describes the development and major morphological characteristics of the larval stages of *P. pollicipes* and the results of attempts to induce settlement using methods that have previously proved successful for balanomorph species (e.g. see review by Gabbott & Larman, 1987).

METHODS

Adult *Pollicipes pollicipes*, collected from the south-west coast of Portugal, were maintained in flowing seawater tanks in the School of Ocean Sciences, Menai Bridge, North Wales. The adults were fed every day on *Artemia* sp. and produced many broods of viable larvae. The larvae were collected from the tank effluent using an immersed 106 µm square mesh. Daily sieving ensured collection of nauplii within a few hours of release. Larvae were reared in UV-irradiated, filtered (to 0.2 µm), aerated seawater at room temperature (15-24 °C) in 5 l glass culture vessels. The seawater was changed every 2 or 3 days. A mixture of *Skeletonema costatum* (Greville) Cleve 1873 and *Rhinomonas reticulata* (Lucas) Novarino 1991 was added to the culture vessels at each change of seawater to give total algal abundance of *ca.* 100 cells μl^{-1} (and maintaining *S. costatum* at twice the cell density of *R. reticulatum*). The diatom *S. costatum* had a modal chain length of 2 cells with a size range of 3-6 µm x 15-25 µm per chain. The size range of *R. reticulata* was 5-10 µm x 8-16 µm.

Cultures of larvae were sampled throughout development. Larvae were either relaxed in MS222 (Sandoz) or 7 % $MgCl_2$, or fixed in 5 % formalin (in seawater). Using the criteria of Bassindale (1936), total length (TL), cephalic shield width (CSW) and cephalic shield length (CSL) of nauplii in each sample were measured to the nearest 5 µm using a calibrated eyepiece graticule in a binocular microscope. Cyprids were measured along their greatest length and greatest width.

From later cultures, drawings of each larval stage were made using a drawing tube attached to a binocular microscope. Removal of limbs and labrum from the body was achieved with fine needles. All measurements of naupliar dimensions were taken without

coverslips. All drawings were made with larvae under a coverslip. It was found unnecessary to stain animals for drawing purposes but when confirmation of certain features was necessary, high power phase contrast was used with larvae mounted in polyvinyl lactophenol with incorporated lignin pink.

Various substrata were employed in an effort to effect settlement and metamorphosis of the cyprids. Light grey, dark grey and brown slate plates with a smooth, pitted or slotted finish and Perspex plates roughened on one side with aluminium oxide rubbing compound (Aloxite, N°. 50) were used initially. Later, black plastic (Darvic), sandstone, adult *P. pollicipes*, plastic mesh (80 μm square holed pvc) and models of *P. pollicipes* were used. Adult animals were gently brushed under running seawater before use to remove debris. Plastic mesh was mounted on 5 mm lengths of 30 mm diameter ABS (acrylonitrile-butadiene-styrene) pipe and around 50 mm lengths of 22 mm diameter ABS pipe. Models of adults were cast from epoxy resin (Cital 28.03; Chemical Building Products Ltd.) in moulds made from silicon moulding compound (Silform N°. 1; Stag polymers & Sealants Ltd.). Models were of fine detail such that individual peduncle plates were cast. Inanimate substrata were cleaned before use by immersion in sodium hypochlorite solution (5 % Chloros or equivalent) then washed in surfactant detergent (2.5 % Decon in tap water) followed by rinsing in tap water.

Seawater extracts of whole *Chthamalus montagui*, *P. pollicipes* and *Lepas anatifera* peduncle integument and *Mytilus edulis* L., periostracum (including valve) were adsorbed onto certain cleaned substrata to encourage settlement, as for balanomorphs (see Crisp & Meadows, 1963). Crude extracts (E_1 after Gabbott & Larman, 1971) were made from homogenised substrates (valve, integument or whole organism) in seawater, boiled for 20 minutes before filtering through filter paper (Whatman no. 3; 6 μm

retention) or vacuum filtered (Sinterglass no. 3; max pore diameter 30 µm), then autoclaved at 120 °C for 15 minutes. Concentrated protein extracts (E_2 after Gabbott & Larman, 1971) were prepared from E_1 by ammonium sulphate precipitation. Both E_1 and E_2 were kept frozen until required. The E_2 extract of *C. montagui* had a protein concentration of 0.5 mg ml^{-1} (BSA equivalent, Pierce BCA assay) and that for *P. pollicipes* E_2 of 0.1 mg ml^{-1} (BSA equivalent, Pierce BCA assay).

Substrata and cyprids (*ca.* 400-3000) were placed in 2 l of filtered, UV-irradiated, seawater in 2.5 l crystallizing dishes or in 4 l of filtered, UV-irradiated seawater in 5 l plastic tanks. Continuous mixing was accomplished by magnetic stirrer, air stone, or air jet directed onto the water surface.

RESULTS

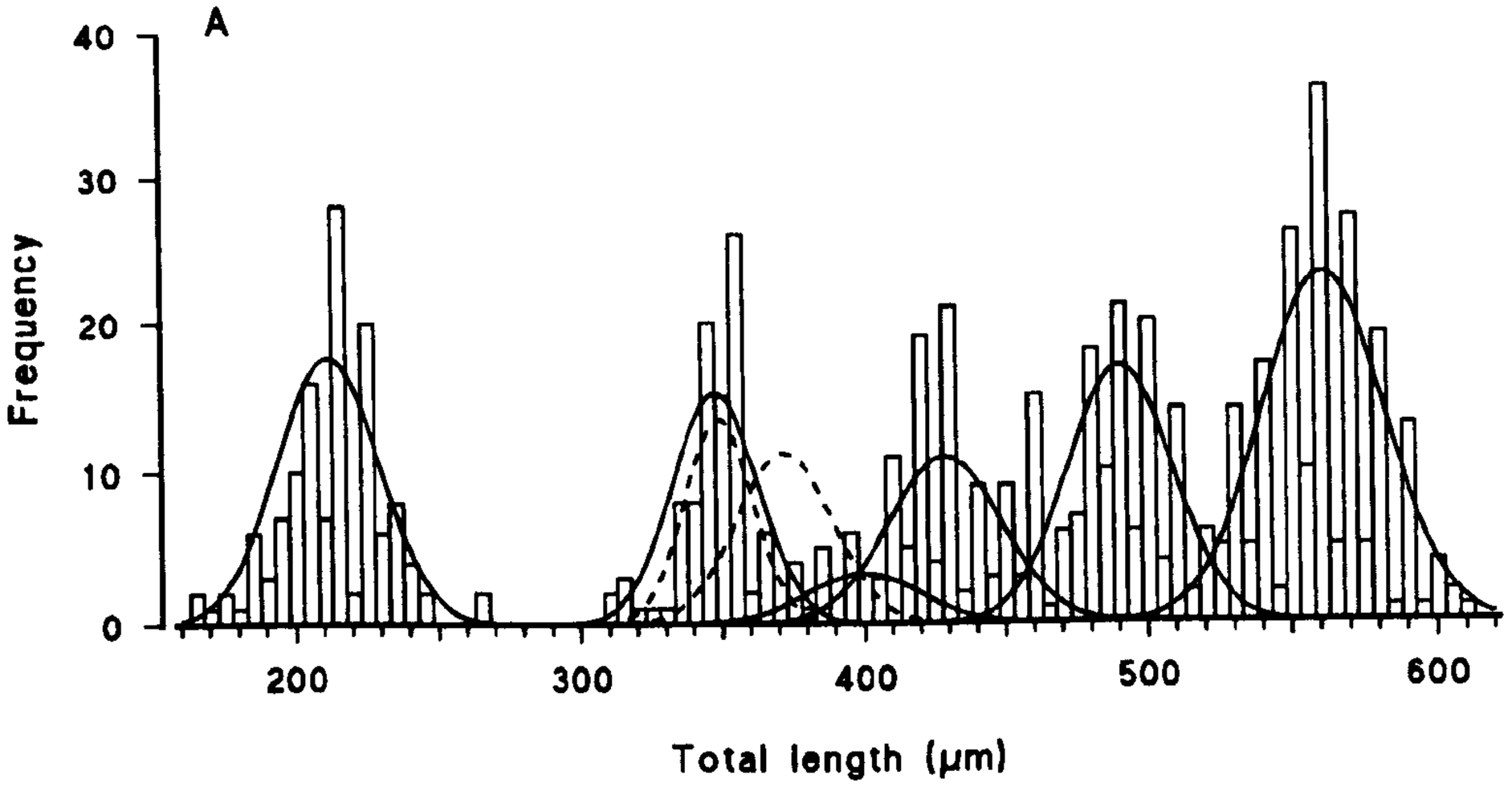
Larval sizes

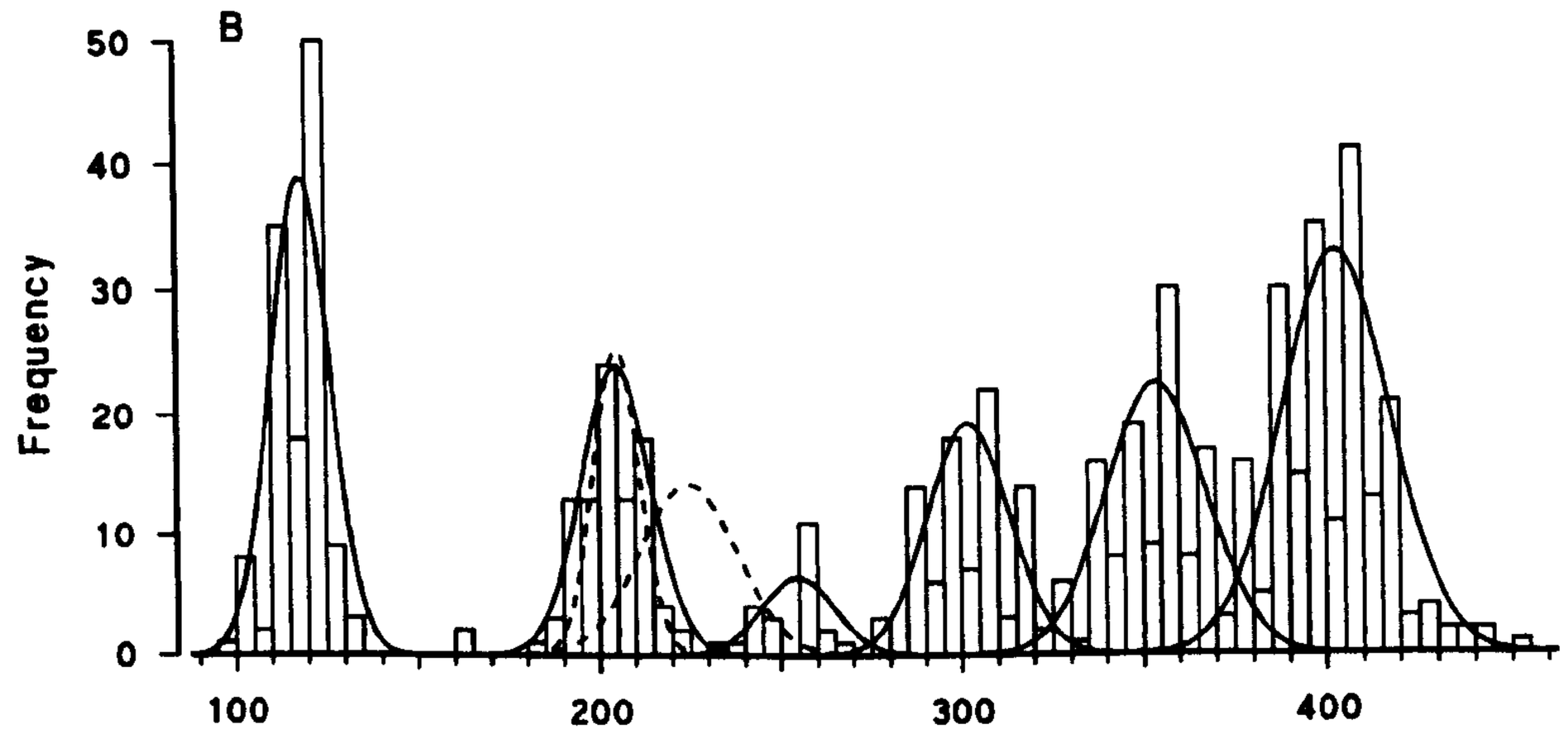
Following Knight-Jones & Waugh (1949), histograms of the measurements of TL, CSW and CSL were used to initially assign a stage to each nauplius larva (Figure 1A,B and C). Linear discriminant analysis using TL and CSW as predictors for stage was reiterated with adjustment of assigned stage until predicted stage matched assigned stage in all cases, thereby producing the most likely linear discriminant function (LDF) from the predicted distributions and squared distances between stages. The LDF for stage is shown in Table 1. A Bonferroni pairwise comparison procedure showed significant differences in the squared distances of all adjacent stages ($F_{0.05/15[2,645]} \approx 6$, $p < 0.001$ in all cases) indicating the LDF is a good predictor of stage.

The CSL of metanauplii (stages IV-VI) showed great variability within each stage (see Fig. 1C). Linear discriminant analysis for metanauplii (stages IV-VI), including CSL as a predictor, did not improve the ability of the LDF to discriminate these stages over the function employing TL and CSW only. The normal probability distribution functions (PDFs), constructed from the means and standard deviations of TL, CSW and CSL distributions of each stage predicted by the discriminant analysis, are shown in Figure 1 as solid curves.

Function parameter	Naupliar stage I	II	III	IV	V	VI
Constant	-87.529	-246.950	-351.759	-449.195	-603.849	-785.994
Total Length (μm)	0.492	0.797	0.893	0.923	1.046	1.199
Greatest Width (μm)	0.600	1.060	1.359	1.665	1.964	2.233

Table 1: Linear discriminant function for separating the naupliar stages of *Pollicipes pollicipes*.





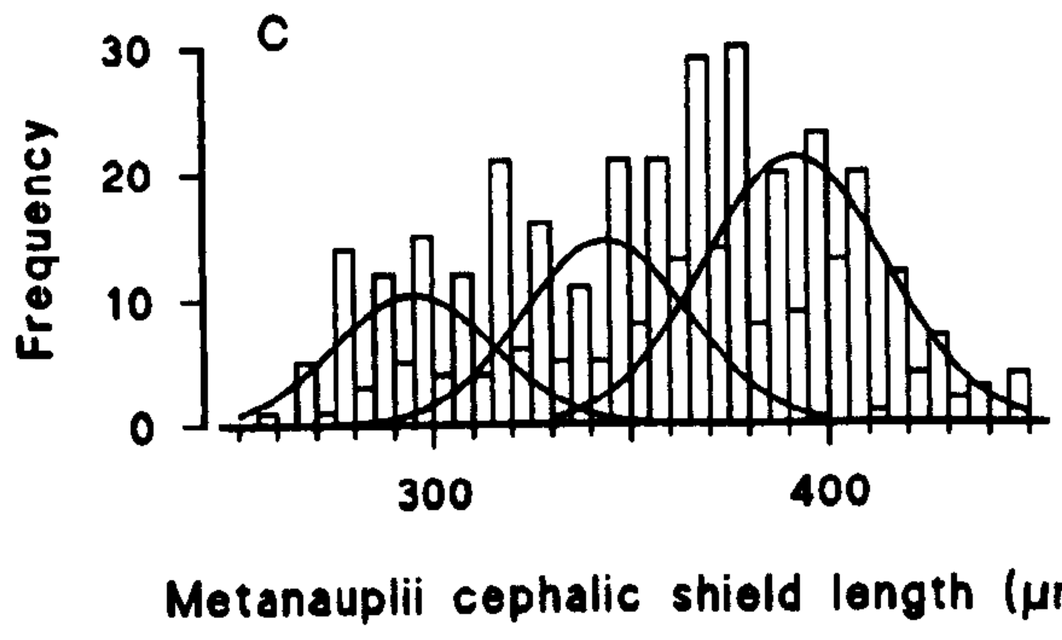


Figure 1: Size distributions and fitted distributions from discriminant analysis for *Pollicipes pollicipes* nauplii. Dotted curves in A and B are measured distributions for stage II and III nauplii identified from shape and antennule setation formulae.

Figure 1A,B highlights a difficulty in using size alone to differentiate stage II and III nauplii. It would appear, from the small frequency distributions for TL and CSW between the obvious distributions for stages II and IV nauplii, that few stage III larvae were sampled. When, however, stage II and III nauplii were later identified on the basis of shape and antennule setation formulae, it became clear that, although correctly assigning all stage IIs, the LDF could only discriminate 29 % of stage IIIs, consigning the rest to stage II. The dashed curves in Figure 1 show the PDFs for known stage II and III nauplii, with considerable overlap in both dimensions. The individuals assigned to stage III to construct the LDF were undoubtedly large stage IIIs and probably included some small stage IVs. The LDF, and hence size alone, can therefore not be used with confidence to discriminate between stage II and III nauplii.

Table 2 summarises the dimensions of stages I, IV, V and VI predicted from discriminatory analysis, and of stage II and III nauplii identified through shape and antennule setation formulae. Table 3 summarises the dimensions of stage I and II nauplii hatched from mature egg masses removed from adults on the Algarve coast (Portugal).

	TL (µm)				CSW (µm)				CSL (µm)			
Stage	n	Mean	SD	Range	n	Mean	SD	Range	n	Mean	SD	Range
I	126	212	18	163-265	127	118	8	97-163				
II	65	349	12	311-383	65	205	6	189-219				
III	76	372	17	326-408	76	224	13	194-255				
IV	87	429	20	347-469	87	302	11	286-337	85	295	20	255-367
V	123	490	18	434-531	123	354	14	316-388	123	344	21	286-393
VI	194	561	21	490-612	194	403	15	352-454	194	392	23	337-449
cyprid	55	433	14	403-455	55	215	17	170-248				

Table 2: The total length (TL), cephalic shield width (CSW) and length (CSL) of all *Pollicipes pollicipes* larval stages. Stages II, III and cyprid from direct measurement, stages I and IV-VI ascribed by LDF (see text).

Use of the LDF to predict the stage of each animal showed only four mismatches where large stage II nauplii were predicted as stage III. The coefficients of variation (CV), for TL and CSW, were the same for stage II nauplii released from tank maintained adults and those from collected egg lamellae, as were the length:width ratios. The CV for stage I nauplii for TL and CSW were slightly larger (2-4 %) for larvae produced from tank maintained adults but the length:width ratios were the same. The mean sizes of stage I and II larvae produced from tank maintained adults were consistently smaller (*ca.* 10 %) than those produced from adults in their natural environment (Tables 2 & 3).

	TL (µm)				CSW (µm)			
Stage	n	Mean	SD	Range	n	Mean	SD	Range
I	47	243	11	214-265	47	128	6	112-143
II	39	374	12	326-393	39	219	8	194-235

Table 3: Direct measurements of the total length (TL) and cephalic shield width (CSW) of *Pollicipes pollicipes* larvae hatched from field populations in the Algarve (Portugal).

Larval morphology

Figure 2 shows the ventral outline of each larval stage of *Pollicipes pollicipes*. There are no large dorsal or marginal carapace ornamentations or spines. The fronto-lateral horns, in typical fashion, point almost posteriorly in stage I and laterally or slightly forward and ventrally in stages II-VI. Similar sized stage II and III nauplii may be easily distinguished by the relatively longer fronto-lateral horns and more tapered shape of the stage II nauplii. The cypris carapace ornamentation has been described recently by Jensen *et al.* (1994) for *P. pollicipes*.

The labra of stages I-VI are not figured since they are essentially the same as those described and figured for *P. polymerus* by Lewis (1975a), but with two exceptions: Stage I nauplii have a unilobed rounded labrum with no apparent spines or setae and in Stage II the labrum bears a pair of marginal tricuspid spines which become three discrete spines in stage III.

Lateral and ventral views of the dorsal thoracic spine (DTS) and ventral thoracic process (VTP) are shown in Figure 3 for larval stages I-VI. Except in stage I where only the DTS bears spines, the DTS and VTP bear randomly distributed small spines and patterned large spines throughout development. Small setae around the maxillae are omitted from Figure 3.

The antennules of all larval stages are shown in Figure 4 and conform to the general morphology observed for numerous other species. The antennae and mandibles of stages I-VI are shown in Figures 5 and 6 respectively. For clarity, the setulation is not shown fully, although relative abundance and length of setules is figured. The numerous small setae and spinules on the dorsal or ventral limb surface are also not shown.

The setules of the antennal exopodite setae are more abundant in the proximal region of the setae. The first terminal seta of the antennal exopodite bears very long setules only on the anterior edge in stages II-VI (Fig. 5). In stage VI the second terminal seta of the antennal exopodite bears only a few setules to the posterior edge, and longer setules to the anterior edge. The terminal seta of the mandibular exopodite in stages II-VI bears only one row of long setules which appear to follow a curve up the seta, but the setules

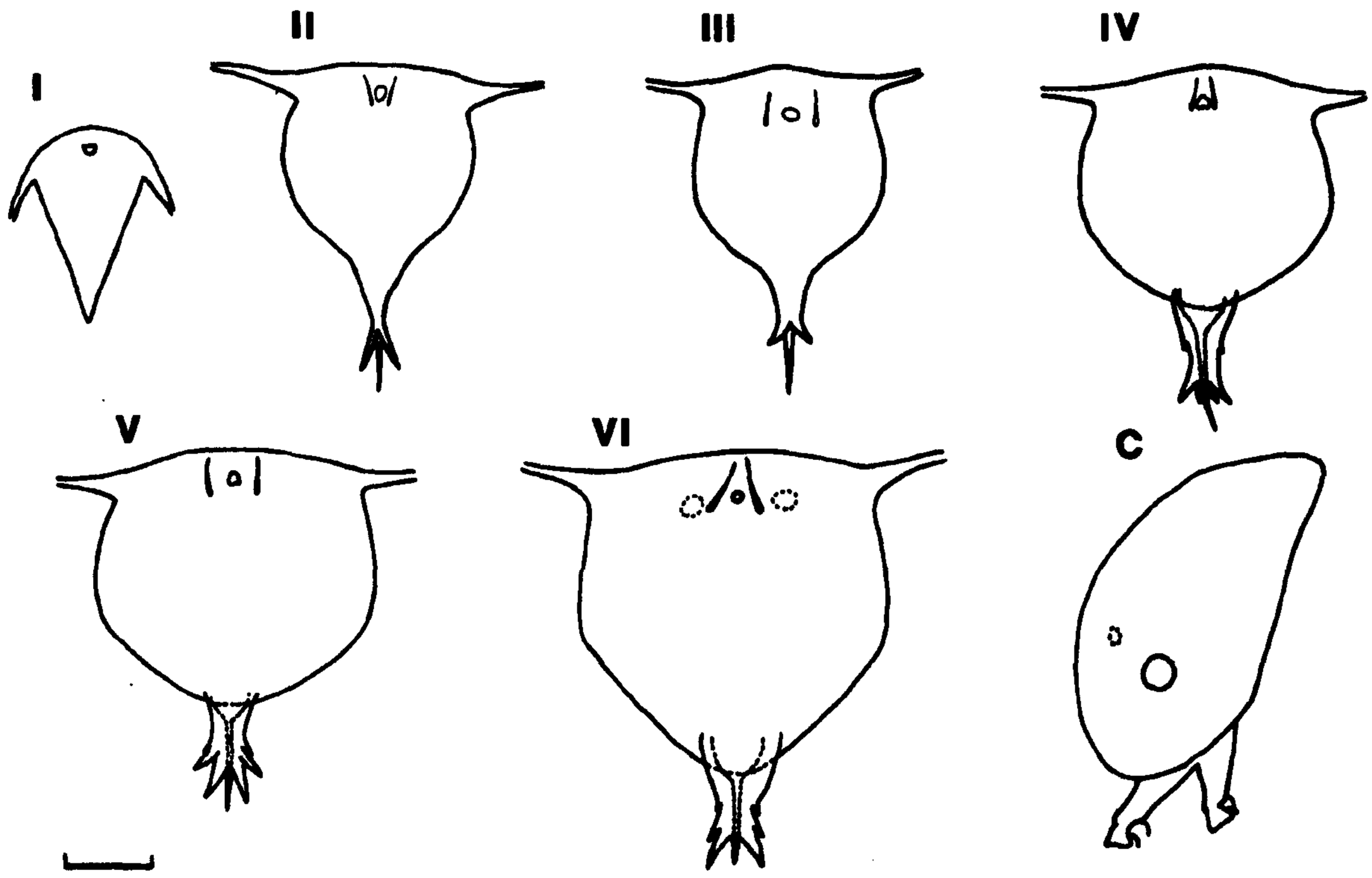


Figure 2: Ventral view of outlines of the naupliar stages (I-VI) and side view of the cypris stage (C) of *Pollicipes pollicipes*. Scale bar is 100 µm.

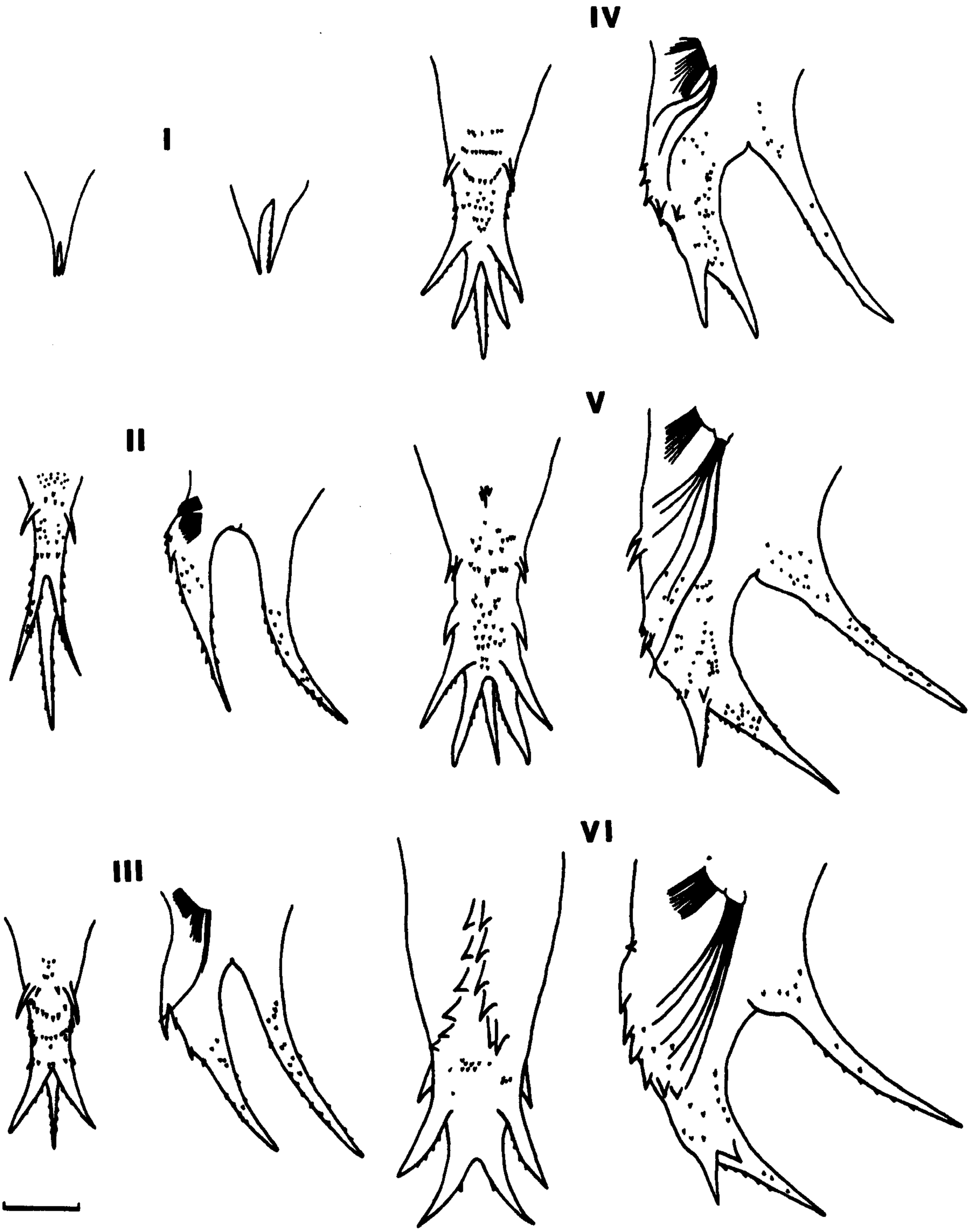


Figure 3: Ventral and side views of the caudal thoracic spine and ventral thoracic process of *Pollicipes pollicipes* nauplii. Scale bar is 50 µm.

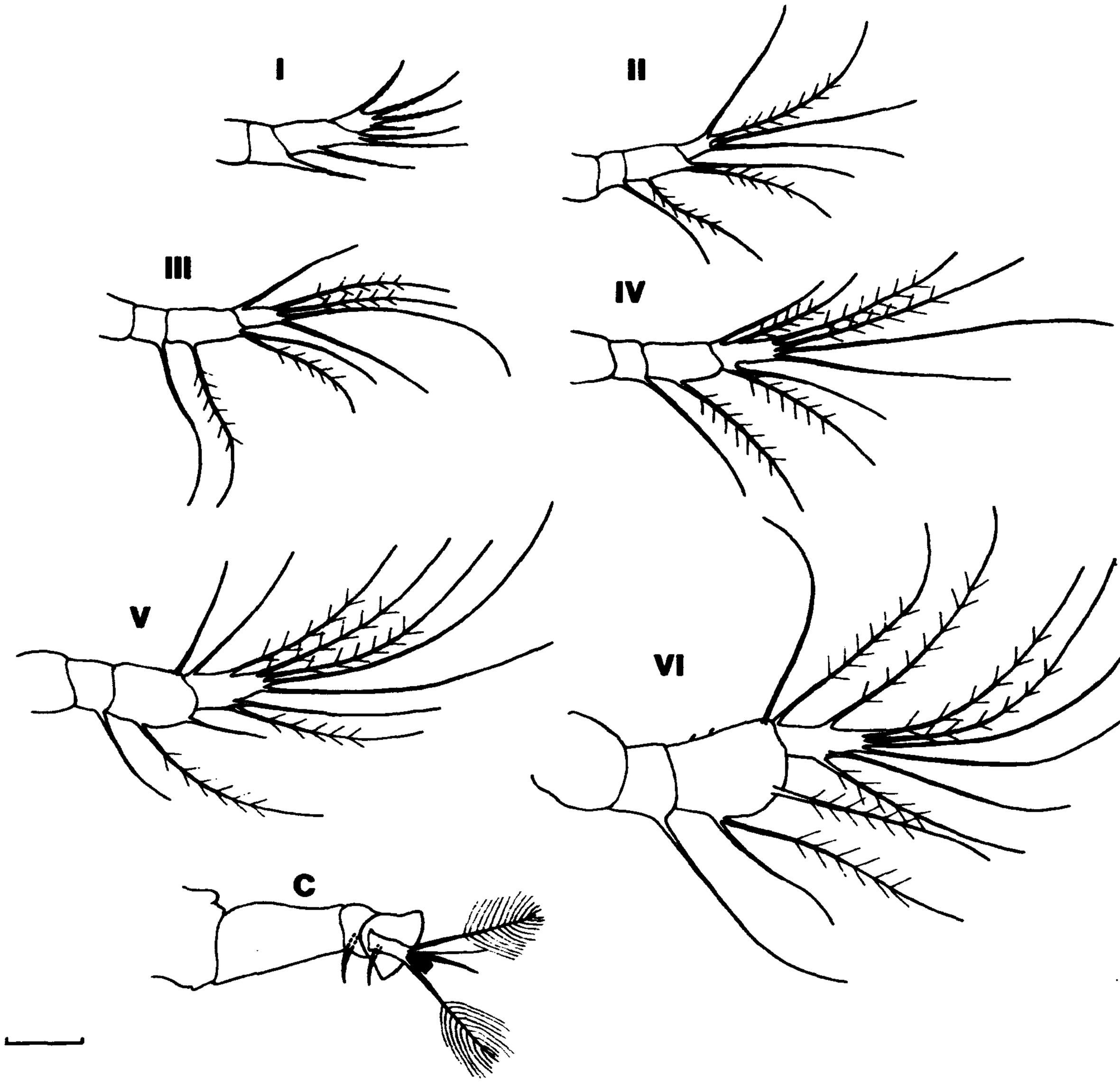


Figure 4: Dorsal view of the right antennule of *Pollicipes pollicipes* nauplii (I-VI) and the cyprid (C). Scale bar is 50 µm.

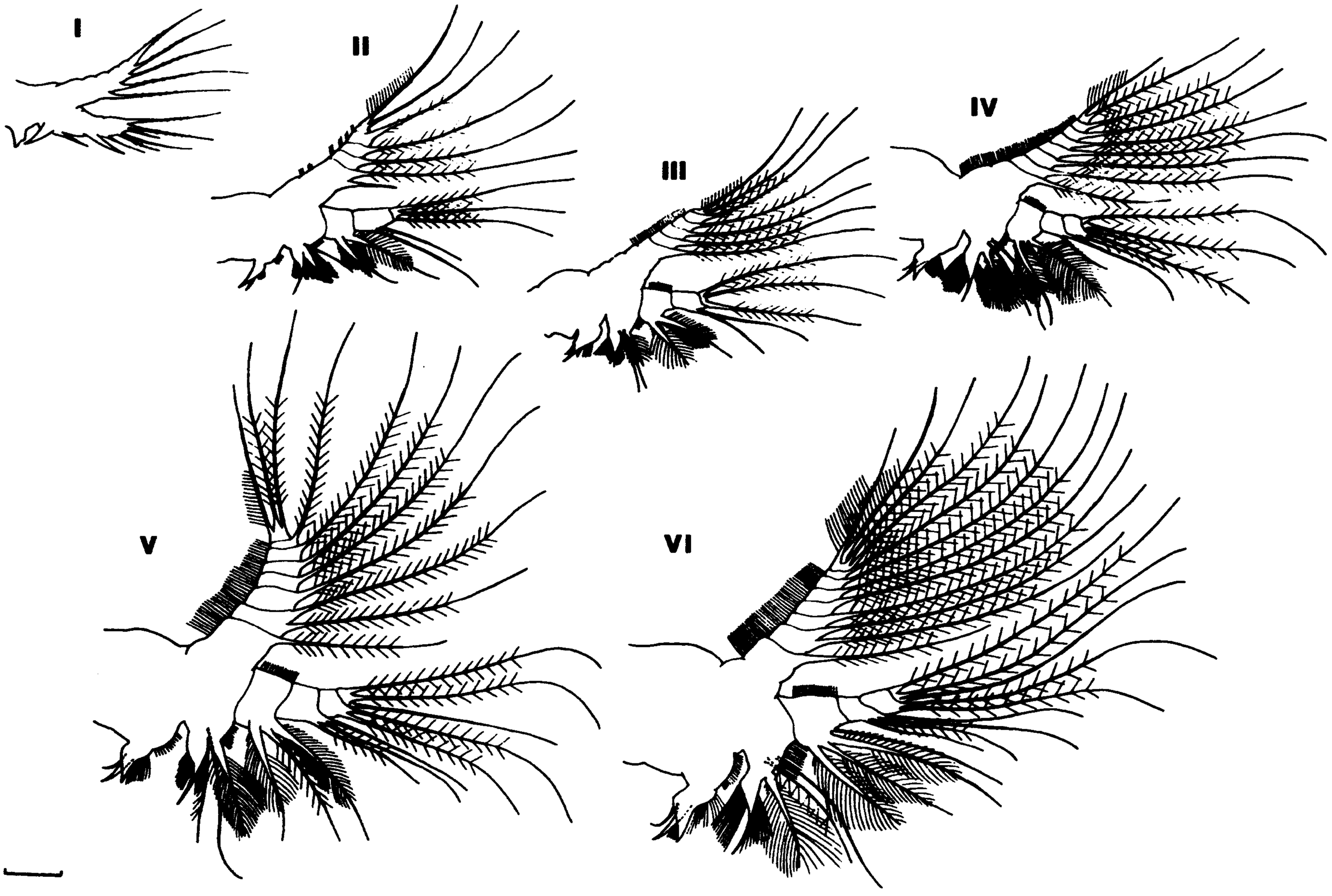


Figure 5: Dorsal view of the right antenna of *Pollicipes pollicipes* nauplii. Scale bar is 50 µm.

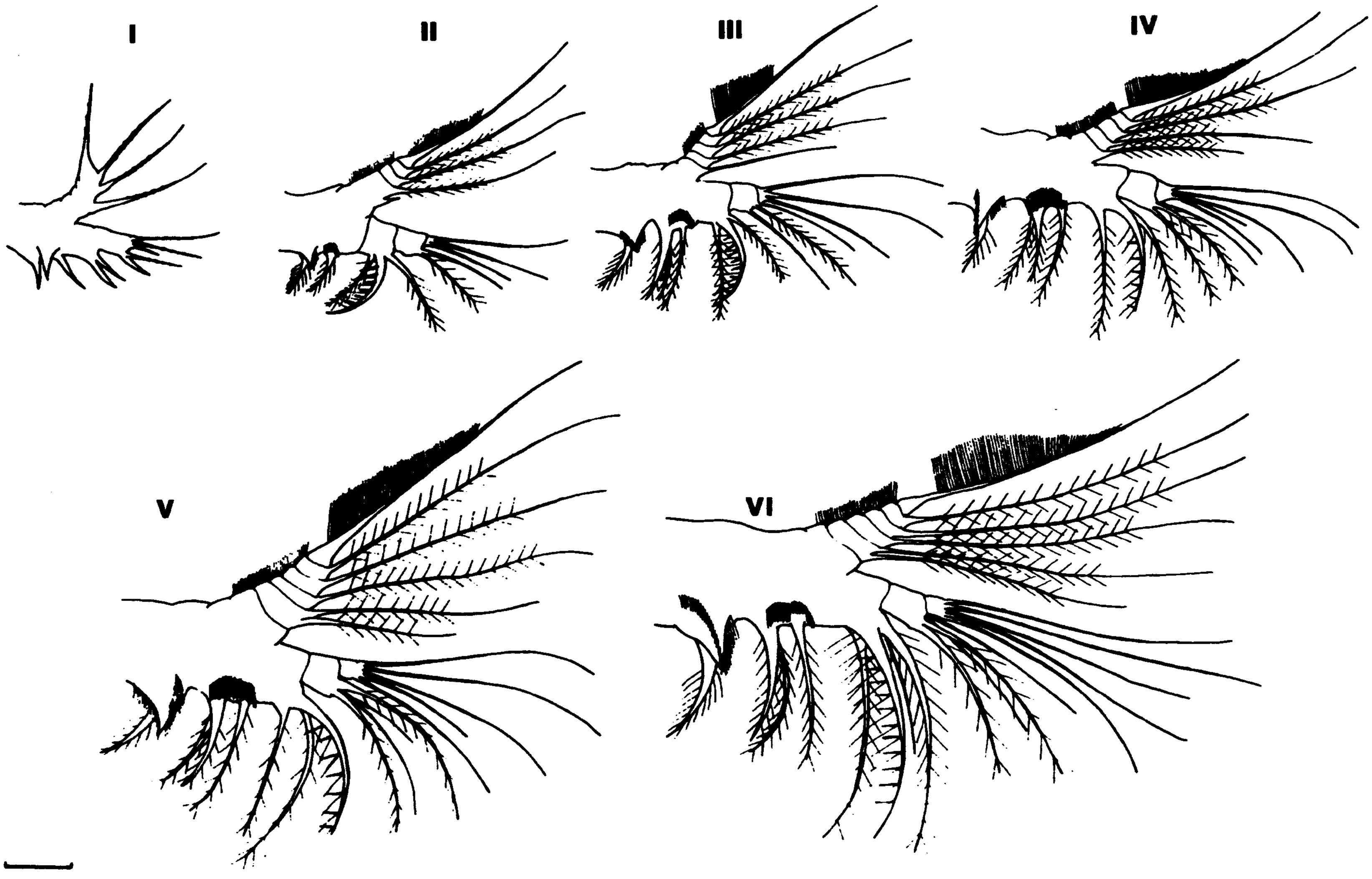


Figure 6: Dorsal view of the right mandible of *Pollicipes pollicipes* nauplii. Scale bar is 50 μm.

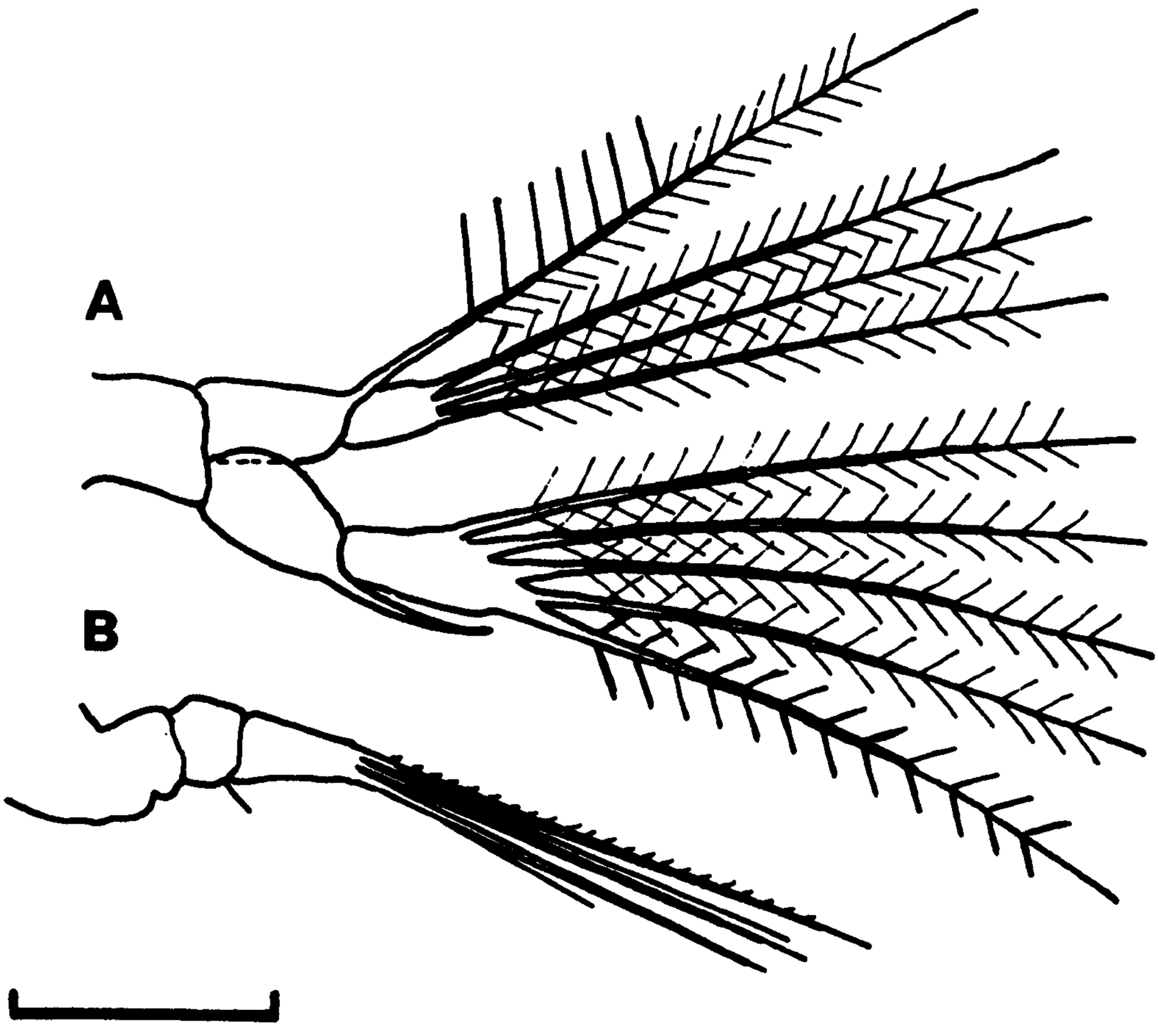


Figure 7: The thoracic appendage (A) and caudal appendage (B) of *Pollicipes pollicipes* cyprids. Scale bar is 50 μm.

Stage	Antennule	Antenna		Mandible	
		exopodite	endopodite	exopodite	endopodite
VI	S:P:P:PPSS:SP:P:PS:S	PPPP:8P	PPSPP:SSPS:SFFF:SPFH:G	P:5P	SSSSS:SSPP:SPCP:PPP:G
V	S:S:P:PPSS:SP:S:P :S	P PP:8P	PPSPP:SSP : FPF:SPFH:G	P:5P	SSSSS:SSPP: PCP:PPP:G
IV	S:P:PPSS:SP: :P :S	P P :7P	PPSPS: SS : FSF:SPFH:G	P:4P	SSSSS: SPP: PCP:PPP:G
III	S: :PPSS:SP: :P :S	P P :5P	PP P : SS : F F:SSFH:G	P:3PS	SS S : SPS: PCP:PPP:G
II	SPSS:SP: :P :S	P S :4PS	PP P : SS : F F: SFH:G	P:3PS	SS S : PS: PCP: P:G
I	SSSS:SS: :S :S	S :4S	SS S : SS : S S: SS:G	S:3S	SS S : SS: SS: S:G

P=plumose seta; S=simple seta; F=feathery seta; H=hispid seta; C=comb seta; G=gnathobase

Table 4: Setation formulae for all naupliar stages of *Pollicipes pollicipes*.

of the mandibular endopodite setae are thicker and slightly less abundant than those on the plumose setae of the exopodite.

Table 4 shows the limb setation formulae for antennule, antenna and mandible of each naupliar stage using the notation of Newman (1965) and Sandison (1967) but excludes references to variable setal type, presence and length.

No differences were observed between the six paired biramous thoracic limbs of the cyprid. One limb is shown in Figure 7A together with one of the pair of caudal appendages (Fig. 7B). The caudal appendage is simple with five terminal setae, the anterior one bearing setules to the front edge, and one short seta on the penultimate segment (Fig. 7B). The thoracic limbs show three types of plumose setae (Fig. 7A). The posterior seta of the external ramus has less abundant, thicker setules than the other plumose setae of the ramus and the anterior seta of the internal ramus bear 6 or 7 long stiff setules, thicker still than those on the other ramus.

Development of larvae

At room temperatures of 15-24 °C, 18 cultures of larvae, collected at stage I or stages I and II, showed >80 % survival of stage VI to the cypris stage in 11-24 (mean 15) days.

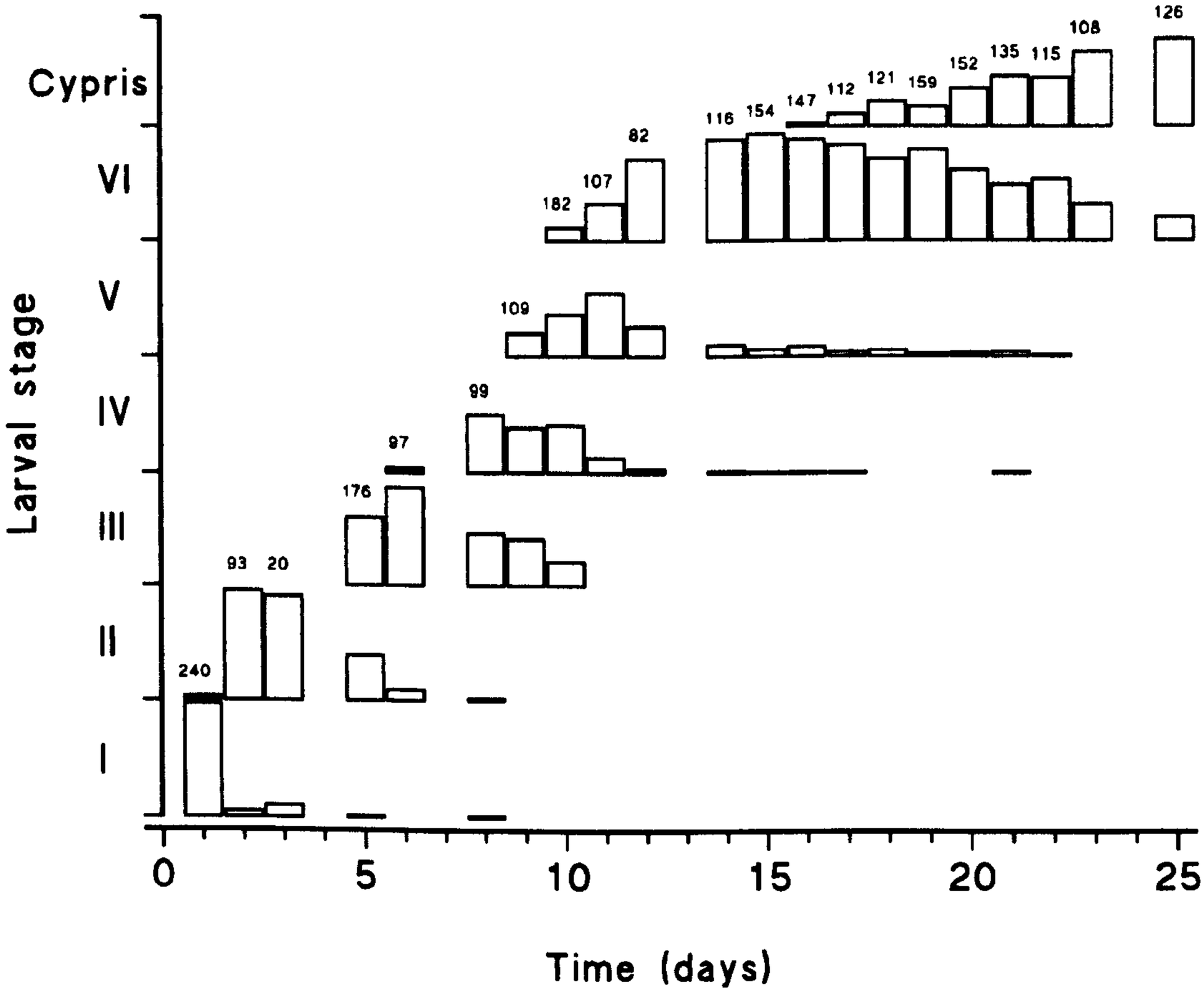


Figure 8: Development of a single laboratory culture of the larvae of *Pollicipes pollicipes*. Number above bar indicates number in sample for which the size of bar indicates frequency of stage.

Figure 8 shows the development of a single culture of larvae collected at stage I soon after release and maintained within the range 16-22 °C. It is apparent from the figure that stage I nauplii moult to stage II within hours of release. Examination of the nauplii indicated that in common with many other planktotrophic cirripedes, stage I do not feed. Of the two species of microalgae offered, stage II nauplii appeared to take only *R. reticulatum* whilst stage III nauplii and metanauplii consumed either or both species. Of 201 stage III-VI larvae examined, 41 % of animals had been feeding on *S. costatum*

alone, 25 % on *R. reticulata* alone, and 34 % on both species. No pattern was evident between stages and proportion of animals containing either or both micro algae.

Settlement of cyprids

Cyprids from 21 larval cultures were used in 27 settlement assays. Each assay contained between 400 and 3000 cyprids. The cyprids used in each assay had an age range of 3-4 days based on the fact that >80 % stage VI larvae had metamorphosed to cyprids 3-4 days after the first appearance of cyprids within most cultures (e.g. see Figure 8). Cyprids aged 0-17 days were used in assays. None of the substrata, whether clean or with adsorbed E_1 or E_2 extracts, elicited a great or consistent settlement response. Indeed 18 settlement assays produced no settlement.

Settled cyprids were distinguished from exploring cyprids by rinsing with fresh water and observing the presence of cypris permanent cement around the antennules. Nine assays resulted in settlement of a very small proportion (<1 %) of the cyprids within each assay. A total of 115 settled cyprids were recorded. One was on ground Perspex with adsorbed conspecific E_1 extract, 2 on *Pollicipes pollicipes* models with adsorbed conspecific E_2 extract, 5 in pits on light grey slate and 107 on live adults. Two assays, each containing four live adults, produced 90 settled cyprids. Sixty were found on the integument of the capitulum, between the plates and along the operculum and hinge, 22 on the basal integument and 8 between plates on the peduncle. Metamorphosis of 64 % of cyprids in one assay (28 settled) took 6 days from settlement, 18 % were still metamorphosing and 18 % failed to metamorphose. Surviving cyprids in the other assay (62 settled) were shedding the carapace at 4 days from settlement. Successfully metamorphosed cyprids from the other 7 settlement assays (25 settled) took 2-9 days to

shed the carapace. Only cyprids less than 10 days old settled. Most cyprids were aged 8 days or less at settlement; 90 were aged 4 days or less and only 16 cyprids in the age range 7-10 days settled. Cyprids older than 17 days had very little visible lipid left but a number were still alive after 21-24 days.

DISCUSSION

The morphological characteristics of *Pollicipes pollicipes* are similar to those of *P. polymerus* described by Lewis (1975a). Lewis (1975a) noted the similarity of *P. polymerus* larvae to those of *Chthamalus aestuarii* Stubbings, 1963 described by Sandison (1967). The larvae of the two *Pollicipes* species are also similar to those of *C. fragilis* Darwin, 1854 described by Lang (1979).

The dimensions of *P. pollicipes* larvae are similar to those reported by Lewis (1975a) for *P. polymerus* both in mean size and range. The coefficients of variation indicate similar relative magnitudes of variation for each stage of *P. pollicipes* in all dimensions, and are analogous to those of *P. polymerus* (see Lewis, 1975a). *P. pollicipes* larvae tend to be larger in all dimensions and stages but *P. polymerus* dimensions were never more than 11 % smaller.

The similarly sized larvae produced from tank maintained adults and from adults on the Algarve coast indicate that the LDF could be employed to assess the stages of large numbers of larvae using only two measurements. However, smaller stage III nauplii can only be confidently differentiated from stage II nauplii by (at least) shape. Since the difference in shape between these stages is obvious it would serve little purpose to reconstruct the LDF to more confidently discriminate small stage III from stage II larvae.

The size and shape of the larvae of *P. pollicipes* have also been described recently by Molares *et al.* (1994). Their sizes are in general agreement with those presented here but slightly larger and with a more rounded shape. The larval outlines of *P. pollicipes* here bear a closer resemblance to those figured for *P. polymerus* by Lewis (1975a).

Corresponding to *P. pollicipes*, Lewis (1975a) noted the lack of large spines or ornaments on the cephalic shield of *P. polymerus* larvae. Larger spines of the dorsal and ventral thoracic processes and the maxillae show the same pattern in development as that described for *P. polymerus* by Lewis (1975a). The labrum of each species presents only minor differences and the setation formulae also correspond closely (*cf.* Lewis, 1975a).

The antennal exopodite shows differences only in the setulation of the terminal two setae, the number of setae is the same at each stage. *P. pollicipes* bears setules only on the anterior edge of the first terminal seta in stages II-VI whereas Lewis (1975a) figures less abundant setules on the posterior edge in stages II-VI, a setal type which *P. pollicipes* appears to have as the second terminal seta in stage VI. The antennal endopodites of the two species differ only in the appearance of three setae at an earlier stage in *P. polymerus* (at stages III, IV, & V), a plumose seta in the first postaxial group in *P. pollicipes* which is simple in *P. polymerus*, and a possible extra, simple seta in the axial group of *P. polymerus* in stage VI. Increasing abundance of setules in the proximal region of the antennal exopodite is not uncommon among cirripede families having been shown for *Verruca ströemia* (Müller, 1776), *Elminius modestus* and *Balanus crenatus* by Stone (1989).

The mandibular exopodites of *P. pollicipes* and *P. polymerus* have the same setation formulae but differ again in the terminal seta. Lewis (1975a) figures setules on the posterior edge of the seta in stages III-VI which are lacking in *P. pollicipes*. The mandibular endopodites of both species have the same formulae at stage VI but *P. polymerus* again shows the appearance of three setae at stages III, IV and V which appear in *P. pollicipes* one stage later.

The antennules of both species have the same setation formulae, but the insertion of the first postaxial group of two seta in *P. pollicipes* is into the penultimate segment of the antennule in nauplii and into the terminal segment in metanauplii (see Fig. 4). In *P. polymerus* the setal insertion is into the terminal segment throughout development (Lewis 1975a). A change from the penultimate segment to the terminal segment at stage IV appears to be commonly figured where segments are clearly defined (see Grygier, 1994). In a recent review of antennular setulation, Grygier (1994) reported these setae to arise on the fourth (terminal) article in 13 species of cirripedes and to arise on the third (penultimate) in 11. He also noted that in most species these setae arose from the terminal article by stage IV.

Grygier (1994) also realized that of the three preaxial setae the middle one has been illustrated as inserted into the terminal segment to stage V and then inserted into the penultimate segment in stage VI for some species including *P. polymerus* but noted that reports on the same species generally disagree. The insertion articles for this seta in *P. pollicipes* and *P. polymerus* (Fig. 4 and Lewis, 1975a) in this respect also disagree, which may lend support to Grygier's (1994) hypothesis for changing boundary expressions of virtual articles.

The thoracic limbs of *P. pollicipes* cyprids are similar to those figured for the only other described member of the genus, *P. spinosus* (Quoy & Gaimard, 1827) (Batham, 1946). The setulation of the setae (Fig. 7A) appears to be more complex in *P. pollicipes*, but the number of setae on each ramus is the same for each species. The caudal appendages (Fig. 7B) are also similar to those of *P. spinosus*, but those of *P. pollicipes* carry more terminal setae. The cypris antennule (Figure 4) is similar to that illustrated and described for *Lithotrya dorsalis* (Ellis & Solander, 1786) (Dineen, 1987) and *P.*

spinosus (Batham, 1946) and balanomorphs generally (Walker *et al.*, 1987), therefore conforming to the view that cirripede antennules are generally conservative structures. A small seta at the terminus of the fourth segment, observed by Gibson and Nott (1971) in *Semibalanus balanoides* using SEM, was not seen in *P. pollicipes* although it cannot be precluded.

The development of *P. pollicipes* differs from that of *P. polymerus* only superficially. Lewis (1975b) indicates a development rate from stage II-VI of 0.14 stages/day using a diet of *Prorocentrum micans* Ehrenb and *Platymonas* sp. Her cultures showed low mortality (15 %) up to stage VI but only 3 % of the metanauplii metamorphosed to cyprids. The development rate of *P. pollicipes* here is twice that of *P. polymerus* at *ca.* 0.3 stages/day (Fig. 8) from stage II-VI. Although mortality was not estimated, large numbers of cyprids were obtained from each culture. Lewis (1975b) collected egg lamellae from field populations and maintained larval cultures at 12-16 °C. Adult *P. pollicipes* were maintained at 21 °C and larval cultures at 16-22 °C in the present study, hence an increase in development rate with temperature could have been expected. Lewis (1975a) reported little visible lipid in cyprids, whereas an abundance of lipid globules was observed in *P. pollicipes* cyprids here which indicates healthier cyprids (e.g. see Lucas *et al.*, 1979) resulting from healthier nauplii which could also account for the difference. Molares *et al.* (1994) report an extended development time for *P. pollicipes* fed only on *Isochrysis* sp. also noting that lipid globules were not observed in the cyprids. They did not report mortality but (Lewis 1975b) had earlier shown that *P. polymerus* fed solely on *Isochrysis galbana* Parke, 1949 only reached stage IV, and then at a slow rate.

Patel & Crisp (1960) and Crisp (1962) have shown that larval size in six balanomorph species correlates with latitude, such that larger larvae tend to be produced in higher

latitudes from larger eggs matured at lower temperatures. Stone (1989), using six balanomorph species, found that larvae reared on diatoms were often larger than larvae grown on flagellates. *Pollicipes polymerus* larvae fed solely on *Skeletonema costatum* died at stage II, hence Lewis (1975b) did not use diatoms in her later cultures. Similarly, *P. pollicipes* nauplii appear to be unable to ingest *S. costatum* until stage III. The limbs of *P. pollicipes* nauplii show the same features that chthamalids possess for feeding on small particles (see Moyse, 1987, Stone, 1989). Differences apparent in size and development rate between the two pollicipid species could thus be as much environmental (methodological) as genetic.

The differences in morphological characteristics between *P. polymerus* (Lewis 1975a) and *P. pollicipes* include the setulation of the antennal terminal seta(e), early stage labrum, insertion of antennular postaxial and one preaxial setae and appearance of setae at a later stage in *P. pollicipes* but these are difficult to interpret without more information on planktotrophic scalpellids. Of over 400 extant lepadomorphs (Zevina, 1981, 1982; Foster & Buckeridge, 1987) the larvae of only 14 lepadomorph species have so far been fully described. The larvae of *P. elegans* Lesson, 1831 have yet to be described, but Darwin (1851) regarded the adult of this species as showing a greater resemblance to *P. pollicipes* than to *P. polymerus*. All three species arose from Mesozoic radiation (Newman, 1987) with *P. elegans* retaining the most primitive characteristics (Foster & Buckeridge, 1987). The larvae of *P. elegans* might be expected to mirror the affinities of the adults.

The results for settlement experiments concur with those of other workers for *Pollicipes* (Lewis, 1975a, Molares *et al.*, 1994). Even adequate volumes of stored lipid in the present cyprids (hence assumed better condition *cf.* Lewis 1975a, Molares *et al.*

1994), did not result in the consistent settlement seen in balanomorphs under similar assay conditions (see Crisp & Meadows, 1963).

The influence of adults in pollicipid settlement is emerging as a recurrent theme in the present study and those of others (e.g. Lewis, 1975a, Hoffman, 1988, 1989). Although settlement of *P. pollicipes* was to a greater degree on conspecifics here, it was by only a small percentage of cyprids within each assay indicating the lack of strong stimulation. Indications are thus that other cues for settling have greater effect than the stimulus provided by arthropodin alone and these cues were not provided in this investigation.

The superficially indiscriminate settling of *P. polymerus* on solid substrata throughout the intertidal zone (Bernard, 1988) further highlights the need to investigate other less tractable cues than appear adequate to encourage many balanomorphs to settle. Relatively large numbers of *P. pollicipes* spat may also be found directly attached to rock, particularly under mussel aggregations, on the Algarve coast (Yule, pers. obs.). The conditions effecting such settlement were clearly lacking under the current experimental conditions.

Both *P. pollicipes* and *P. polymerus* inhabit areas of strong wave action. Recently, Pineda (1994) has noted how *P. polymerus* larvae may prefer to settle in sites with strong unidirectional flow. It was established that they settle in grooves cut into gutter shaped plates placed in the field at least 0.5 m from the nearest *P. polymerus* cluster, at abundances of up to 12 mm^{-2}. The provision of unidirectional (or at least bidirectional) flow and an appropriately orientated groove on the surface may be a greater stimulus to settlement, ensuring adequate safety and feeding conditions, than that of conspecific adults, ensuring potential procreation. *P. pollicipes* do exhibit a limited form of locomotion on surfaces (Chapter 3) and the proximity of a mate may be of lesser

consequence on the wave swept shores of the Algarve than the provision of safe adequate feeding conditions.

CHAPTER 3

Active relocation in lepadomorph barnacles

Initial observations made in the course of this investigation were published as:

Kugele, M. and Yule, A.B., 1993. Mobility in lepadomorph barnacles. *Journal of the marine biological Association U.K.*, **73**, 719-722.

ABSTRACT

Circumstantial evidence of mobility by the lepadomorph barnacle genus *Pollicipes* in the literature is supported by observations which indicate that the phenomenon is real. Comparative morphology of the cement delivery system of three lepadomorph barnacles indicates that the lepadid *Lepas anatifera* is unable to relocate voluntarily, whilst two scalpellids *Pollicipes pollicipes* and *Capitulum mitella* are able to do so. Speeds of up to 50 $\mu m\ d^{-1}$ were measured for both species but were considered underestimates of maximal rates through lack of directed stimulation. Neither gravity nor unidirectional flow proved effective stimuli for directed mobility although such was observed in the laboratory. The two scalpellid species use quite different mechanisms to effect relocation at the leading edge of the base, but both slough basal material at the trailing edge. It is hypothesised that growth alone can account for the mobility of *P. pollicipes* but *C. mitella* is likely to employ muscular activity.

INTRODUCTION

Except for certain parasitic copepods and isopods, barnacles have been considered the only crustaceans that are sedentary or non-motile when adult (Waterman and Chace, 1960). Indeed, evidence of post-settlement mobility in juvenile balanomorph barnacles, on smooth surfaces, was probably due to lateral pressure exerted by adjacent barnacles forcing flow in an essentially viscoelastic adhesive (Crisp, 1960). Lepadomorph barnacles however, with flexible muscular stalks, have the potential to exhibit true, voluntary, mobility.

Some evidence for active mobility in pollicipid lepadomorphs has been reported. Hoffman (1984) found that *Pollicipes polymerus* juveniles (<7 mm total contracted length) settled on adults were distributed such that their relative position on the adult peduncle was directly proportional to their size. The smallest juveniles were always the most abundant and clustered close to the capitulum. He noted that such a distribution could result from vertical adult growth near the capitulum, but it would not explain how large juveniles were found attached to the basal disc, a region where small juveniles were only rarely found. Chaffee and Lewis (1988) found that the two most mitotically active regions in the peduncle of *P. polymerus* were immediately below the capitulum/peduncle junction and at the stalk base. Lateral extensions of the base are common in *Pollicipes* and rapid somatic growth is indicated within these extensions (Chaffee and Lewis, 1988). Such extensions were most obvious in juveniles attached to adult peduncles, but also present in adults within clusters. Only one extension per animal was reported. Possession of a peduncular extension and degree of disc thickening seemed to be proportional to wave action, suggesting these basal modifications were adaptations

to increase adhesion through a greater adherand surface area. Hoffman (1984) had earlier suggested that these extensions may be forced downward by peduncular haemolymph pressure to seek a new or enlarged attachment site. Evidence from Fyhn *et al.* (1973), Crenshaw (1979) and Walker and Anderson (1990) indicates that lepadomorph barnacles generally exhibit higher haemolymph pressure than acorn barnacles. Crenshaw (1979) noted how the peduncle of *P. polymerus* seemed well designed to sustain high internal pressure. He found the integument to stretch equally well in longitudinal and circumferential directions under relatively low, sustained stresses. Chaffee and Lewis (1988) noted that basal extensions thickened and seemed to release cement, but could not determine the cement's origin. Such properties would be necessary in the development of true mobility utilising growth rather than muscular action as exhibited by, for example, gastropods (see Miller, 1974). The question arises as to whether the organism is indeed simply increasing its attachment area in response to environmental conditions, or whether there is directed mobility in a real sense. This chapter further addresses questions regarding the mechanism of, stimuli(us) for and ecological significance of the phenomenon for three lepadomorph species; the coastal scalpellids *Pollicipes pollicipes* and *Capitulum mitella* and the oceanic lepadid *Lepas anatifera*.

METHODS

Pollicipes pollicipes were collected intertidally from the Algarve (Portugal), *Capitulum mitella* from exposed shores on Hong Kong island, and *Lepas anatifera* from driftwood washed ashore on the south-west coast of Anglesey (North Wales). Animals were maintained in tanks under constant conditions of light, temperature and water flow. Unfiltered seawater from the Menai Strait was constantly flowing through the tanks. *L. anatifera* specimens were dead when collected but were also kept in a flowing seawater tank for a few days prior to use. Live barnacles were fed on *Artemia* sp. nauplii (freshly hatched), *Skeletonema costatum* and *Rhinomonas reticulata* (both cultured in the laboratory, see Yule, 1984) every other day.

The peduncle bases of a number of *P. pollicipes*, *C. mitella* and *L. anatifera* were dissected to compare the cement delivery (duct and pore) systems of the three species. A transverse cut across the peduncle near the base facilitated removal of peduncular musculature revealing the translucent integument of the base. Basal integument was carefully dissected away above the ducts and pores where the cement system was not clearly visible through the integument. Drawings of the duct and pore systems were made with the aid of a drawing tube or *camera lucida* attached to a binocular microscope.

To estimate rate of mobility and assess light, gravity and unidirectional flow as stimuli for the relocation phenomenon in *P. pollicipes*, a number of animals were attached to clear polyacrylamide plates with a minimum quantity of cyanoacrylate adhesive. Eight 75 mm x 50 mm plates were used, each roughened with aluminium oxide rubbing compound (Aloxite N°. 50). Four similar sized animals were attached to each plate along the centre line of the greatest dimension. Four plates were attached to the floor (vertical

aspect animals) and four plates to a wall (horizontal aspect animals) of a flume tank. The Perspex flume tank was constantly illuminated from above with fluorescent light. The water temperature fluctuated within the range 16-24 °C over the study period. The position occupied by animals under study was 80 mm wide in a water depth of 75 mm with turbulent flow. The mean water velocity 10 mm upstream of the barnacles at capitulum level was 21 cm s^{-1} (SD = 3 cm s^{-1}), estimated using a calibrated induction impeller (Novonic Streamflow).

The centre of the base, nominally assigned by eye when viewed through the plate, was used to locate an individual's position. The position of an animal on its plate was measured using a travelling microscope with incorporated vernier scale. The location of the animals was ascertained at 54, 106, 149 and 188 days following initial fixing. Others were measured at different times either if they fell off and were reattached or if they were replacements for those which had died. Before each barnacle was attached to a plate its rostro-carinal (RC) length (from the tip of the rostrum to the tip of the carina), width (from the tip of the sub-carina to the outermost part of the sub-rostrum) and thickness (at the capitulum/peduncle junction) was measured. These dimensions were remeasured for remaining, intact, barnacles at the time of the final position measurement.

To each of a further four, relatively large, *P. pollicipes* two smaller animals were attached opposite each other at the capitulum/peduncle junction. The four host barnacles were in turn attached in pairs to either side of a dark grey slate plate. The slate plate was then supported at the edges in a 25 l plastic tank which was subjected to a near natural daylight regime and variable water depth such that the animals were emersed for two three-hour periods in every 24 h. These barnacles were periodically photographed over the succeeding 251 days. The tank was serviced with continuously flowing seawater and

the temperature fluctuated within the range 13-23 °C. The temperature was prevented from exceeding 23 °C by use of a glass cooling coil and tap water.

In a similar manner to that employed for *P. pollicipes*, a number of *Capitulum mitella* were attached to plastic plates either singly, or in closely associated groups (the more natural configuration) where their bases were near but not actually touching. The perimeter of the base attached to the plate was marked on the underside of the plate with water resistant ink. These animals were maintained in the flume tank under the conditions described for *P. pollicipes*.

RESULTS

Mobility of *Lepas anatifera*

Figures 1 and 2 show typical duct/pore layouts in the peduncle base of *Lepas anatifera*. The duct (d) and pore (p) sizes increase towards the principal duct (pd). Duct termini (dt) were always present and indicate a conserved system from settlement simply increasing in size with growth. The ducts followed more or less straight lines, although usually diverging with increasing basal diameter (Figs. 1 and 2). Crossing of the ducts was only ever observed close to the duct terminus (e.g. Figs. 1 and 2). Figure 3A shows a dorsal view of the dissected base of an adult (10 mm basal diameter) in the region of the duct terminus. The typical pattern of cypris cement (cs) exuded around the antennules at fixation, is clear. The cement ducts (d) terminate in the antennules (an). A small cement pore (p), developed when juvenile, is a continuation of the same duct. The larger diameter cement duct (d) is representative of the larger adult size. Figure 3B is a ventral view of the base after dissecting out an antennule. The adhesive disc (ad) and fourth segment (fs) with terminal setae (s) are clear. All specimens examined conformed to this pattern, with a convergence of cement ducts towards the duct termini where cyprid antennules were still present. Since antennules are present, marking the site of settlement, and duct-pore layouts conform to a pattern of growth only, it is highly unlikely that *L. anatifera* is able to (voluntarily) relocate.

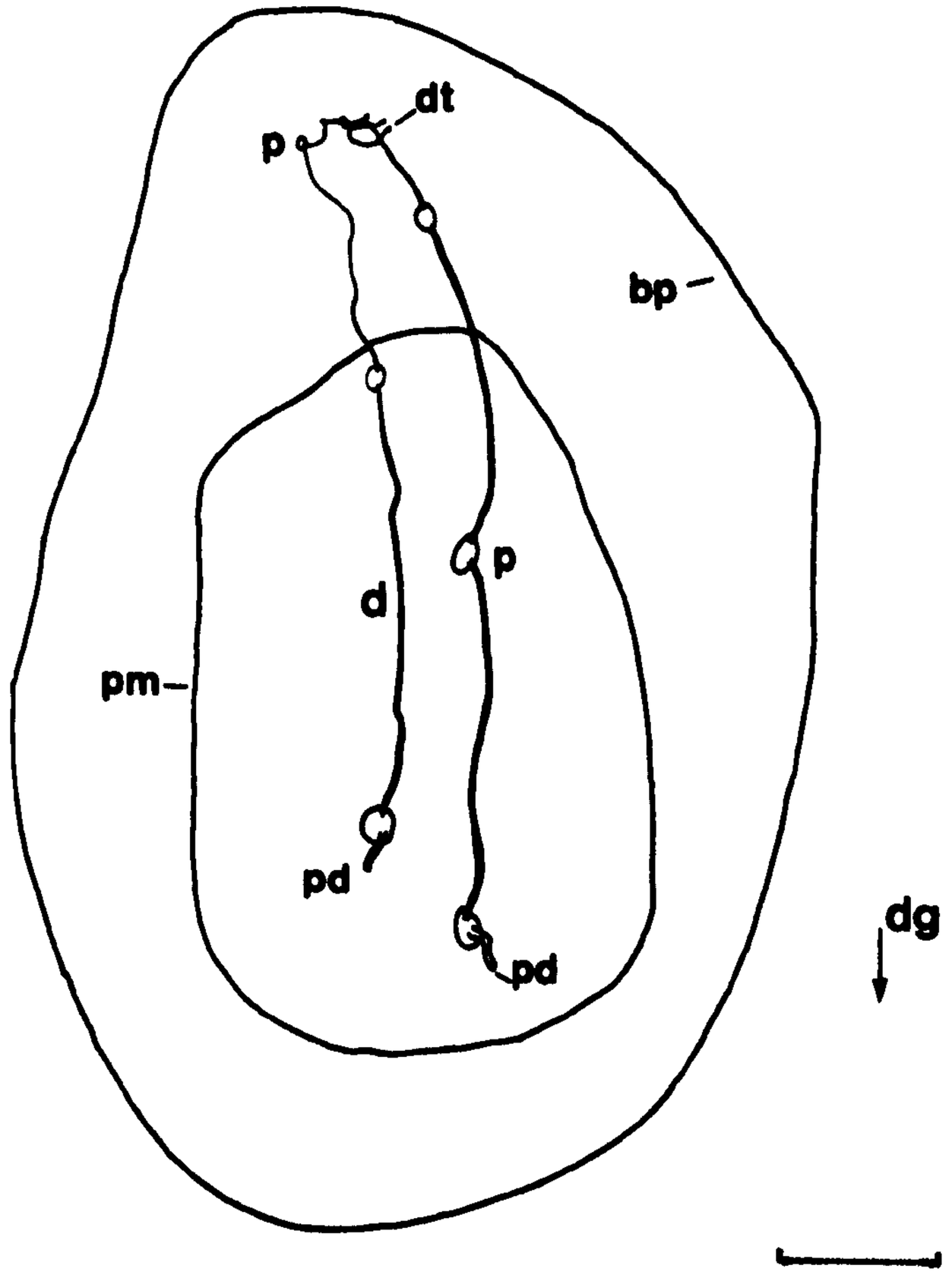


Figure 1: Duct and pore layout of *Lepas anatifera*. A simple linear pattern with ducts (d) and pores (p) increasing in size with distance from duct terminus (dt). Largest and newest pores are adjacent to principal ducts (pd). The basal perimeter (bp) extends in the direction of growth (dg). pm demarks the inner margin of the peduncle integument. Scale bar is 1mm.

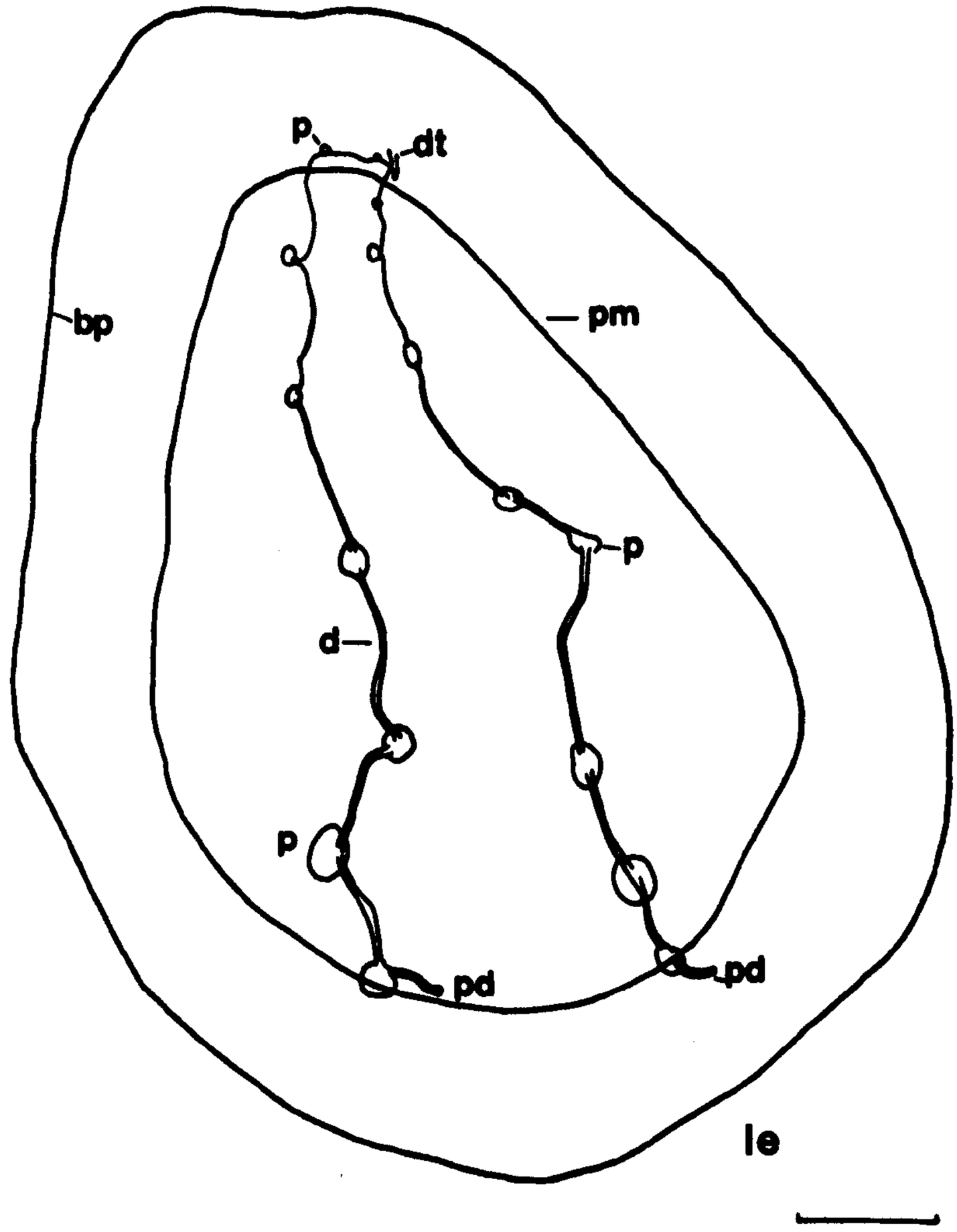
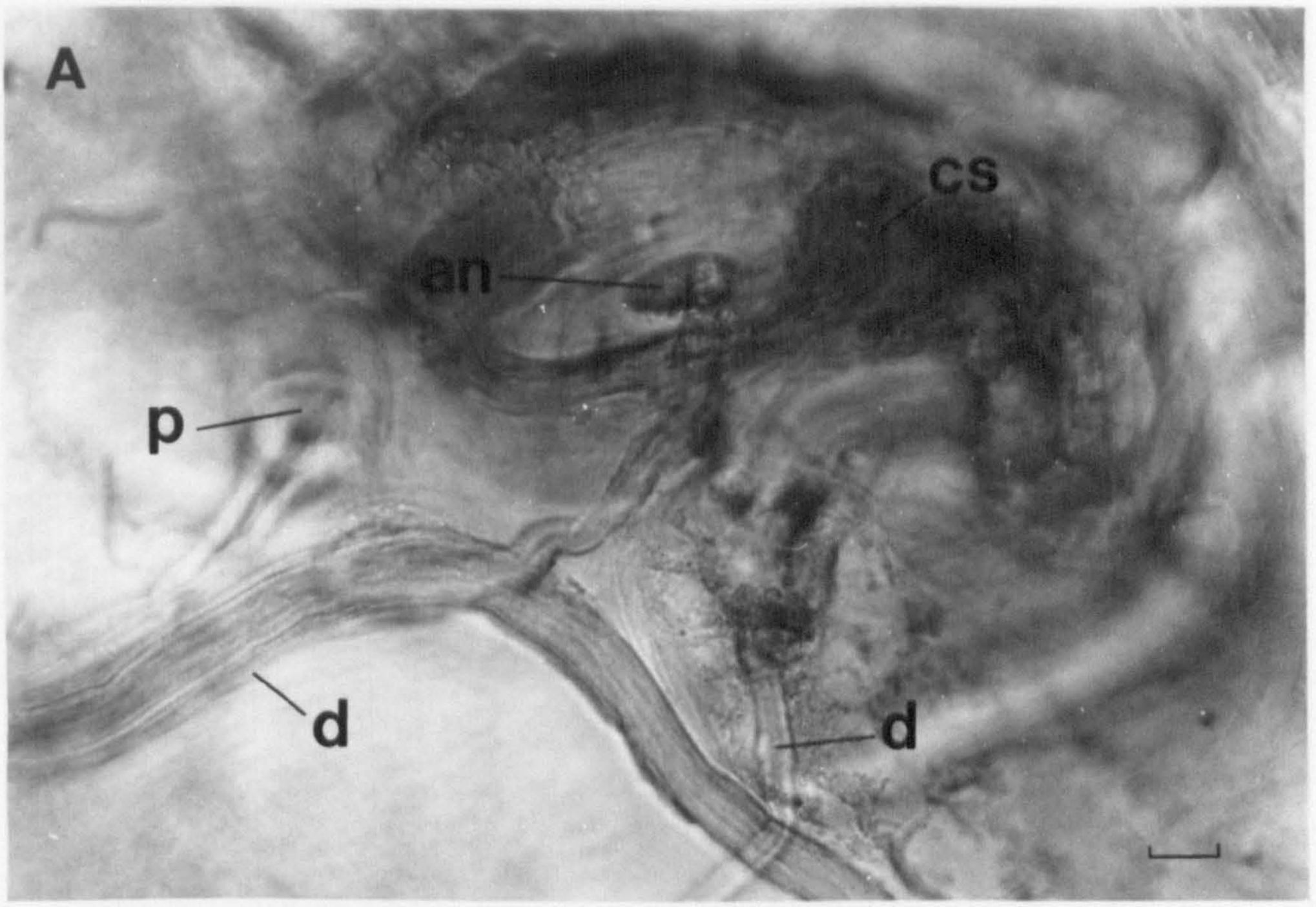


Figure 2: Typical duct and pore layout of *Lepas anatifera*. with a simple linear pattern of ducts (d) with cement delivery pores (p). Largest and newest pores are always adjacent to principal ducts (pd) at the leading edge of growth (le). Crossing of the ducts was only ever observed at the duct termini (dt). Scale bar is 1mm.



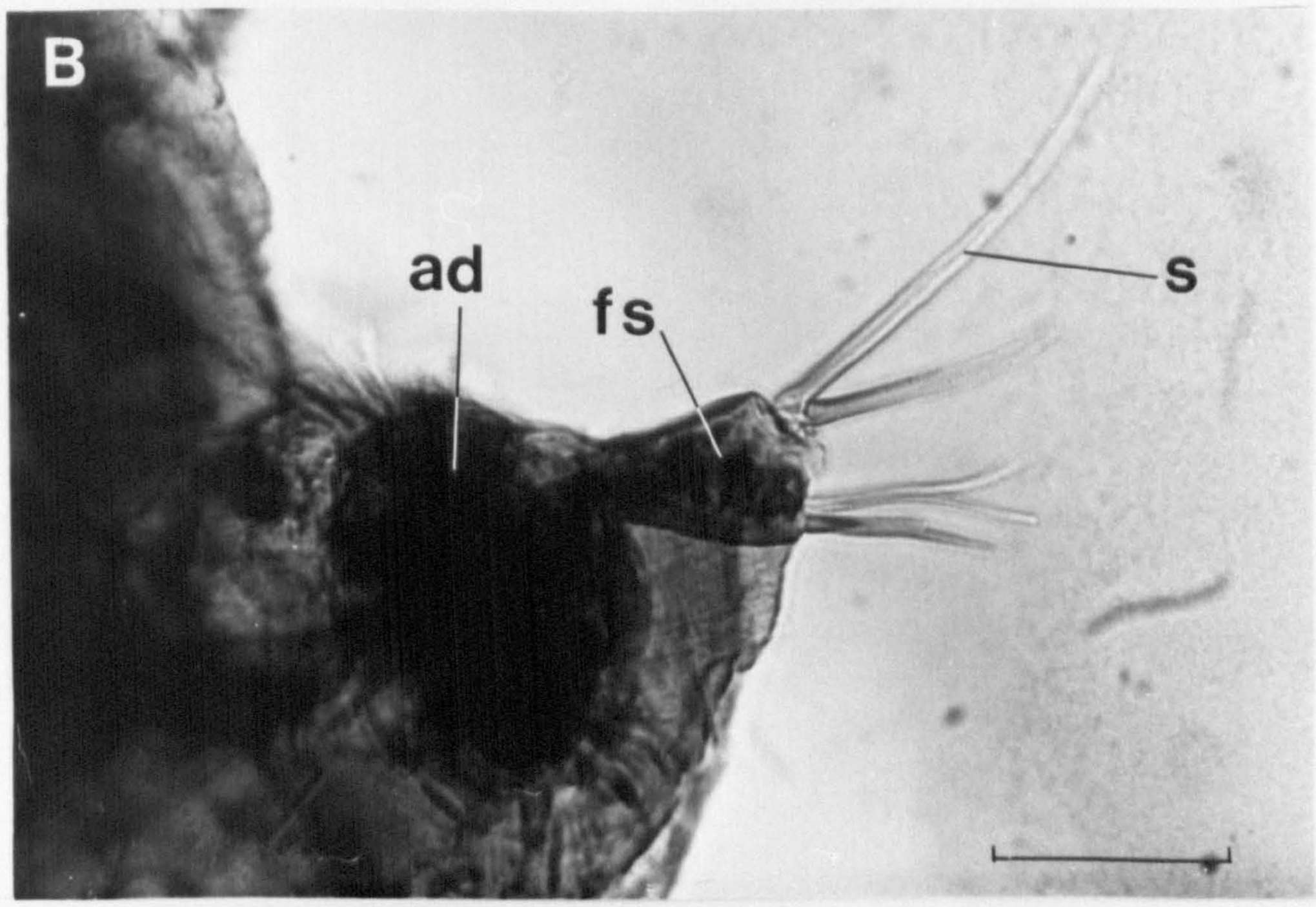


Figure 3: Dissected base of *Lepas anatifera*. A; dorsal view of duct terminus area with cypris antennules (an) embedded in cypris permanent cement (cs). Ducts (d) lead from antennules to small juvenile pores (p) and larger adult ducts. B; Ventral view of a dissected antennule from A, with adhesive disc (ad) of the third segment and the fourth segment (fs) with setae (s) still present. Scale bars are 20 µm.

Mobility of *Pollicipes pollicipes*

Table 1 shows the dimensions of the four size classes of scalpellid barnacles attached to the plastic plates. Each plate bore a single size class and each size class was represented by plates bearing vertical animals (attached to the flume tank floor) and horizontal animals (attached to the flume tank wall). Size classes were easily distinguished by rostro-carinal (RC) length alone, which had little overlap in range (Table 1).

Dim.	Size	Mean	CV	range	n
RC	1	15.23	7.7	13.36 - 17.26	11
	2	11.31	9.3	10.00 - 13.20	9
	3	8.18	12.2	7.00 - 10.50	10
	4	5.85	10.6	4.60 - 6.40	11
W	1	15.67	9.2	13.12 - 17.90	11
	2	11.50	10.7	10.40 - 13.34	9
	3	8.12	9.2	6.60 - 9.56	10
	4	5.62	14.2	4.56 - 6.88	11
T	1	9.01	8.4	8.04 - 10.28	11
	2	6.16	10.7	5.62 - 7.26	9
	3	3.93	12.7	2.88 - 4.56	10
	4	2.77	14.4	2.00 - 3.28	11

Dim, dimension: RC = rostro carinal length, W = width, T = thickness, see text for definition. CV = coefficient of variation. n = number in sample.

Table 1: Various measurements (mm) of the 4 size classes of *Pollicipes pollicipes* attached to plastic plates.

To discriminate moving animals (with 95 % confidence), known precision of base centre estimate was essential for reliable estimates of movement rates. To evaluate the relative precision of position location on the plate (in two dimensions), the absolute differences between all replicate X and Y measurements for each individual were

Size	n	median	mean rank	h	p
1	48	0.16	101.7	10.96	0.01
2	48	0.21	115.1		
3	48	0.13	89.6		
4	48	0.13	79.7		
Total	192		76.5		

Size class	Mean abs. diff. (mm)	SD (mm)
1	0.36	0.47
2	0.35	0.45
3	0.21	0.21
4	0.16	0.12

h = test statistic, p = probability of obtaining magnitude of test statistic by chance.

Table 2: Kruskal-Wallis test of absolute differences (mm) of repeated measurement of nominally assigned base centre location for 4 size classes of *Pollicipes pollicipes* attached to plastic plates.

calculated. Table 2 shows the result of a Kruskal-Wallis test of the ranked absolute deviations for each size class, which indicated a significant difference between at least two size classes. Dunn's method for all pairwise comparisons of mean ranks revealed a significant difference between size classes 2 and 4 (Sd_{diff} = 11.34, $Z_{0.0042}$ = 2.64) suggesting a size dependent precision. Table 2 also shows, for each size class of animal, the mean absolute deviation and standard deviation (SD). Not surprisingly, the largest animals base centres were determined with the least precision and those of the smallest with the greatest. Any animal with a measured basal location less than two standard deviations (in both axes, and for their size; see Tables 1 and 2), from their last measured position, were excluded from rate estimation. This procedure ensured that *ca.* 95 % of measured differences which could be due to simple measurement variability were not included as moving animals. Of 41 animals used, 20 moved beyond the measurement

Source	DF	SS seq.	SS adj.	MS adj.	F	p
Time (d)	1	10.28	7.05	7.05	7.24	0.01
Attitude	1	3.93	0.28	0.28	0.29	0.59
Attitude x time	1	0.21	0.21	0.21	0.22	0.64
Error	39	37.97	37.97	0.97		
Total	42	52.39				

Term	Coeff.	SE	t	p
Constant	0.34	0.28	1.25	0.22
Time	0.01	0.004	2.69	0.01

Table 3: Analysis of variance of cumulative distance (weighted, mm) with time (covariate, days) for vertically and horizontally attached *Pollicipes pollicipes* (seq. = sequential, adj. = adjusted for entry order).

precision. Of 121 subsequently measured locations 43 were attributed to (20) moving animals. The cumulative vector lengths (total distance travelled) of moving animals showed a significant correlation with time (Pearson's $r = 0.467$, $df = 41$, $p < 0.001$). The majority of animals deemed to have moved were the smaller animals (60 % and 91 % of size classes 3 and 4 respectively). No size class 2 animals moved in excess of measurement variability and only 36 % of size class 1 animals moved.

Table 3 shows the result of an analysis of variance of distance moved with time (covariate) for vertical and horizontal affixed animals. A weighted model was used to account for increasing error variance with time. A significant relationship between distance and time was confirmed ($F_{[39,1]} = 7.24$, $p = 0.01$) but the relative rates of travel for vertical and horizontal animals were not significantly different ($F_{[39,1]} = 0.22$, $p = 0.642$). For the term of the available data (25-188 days) the average speed was

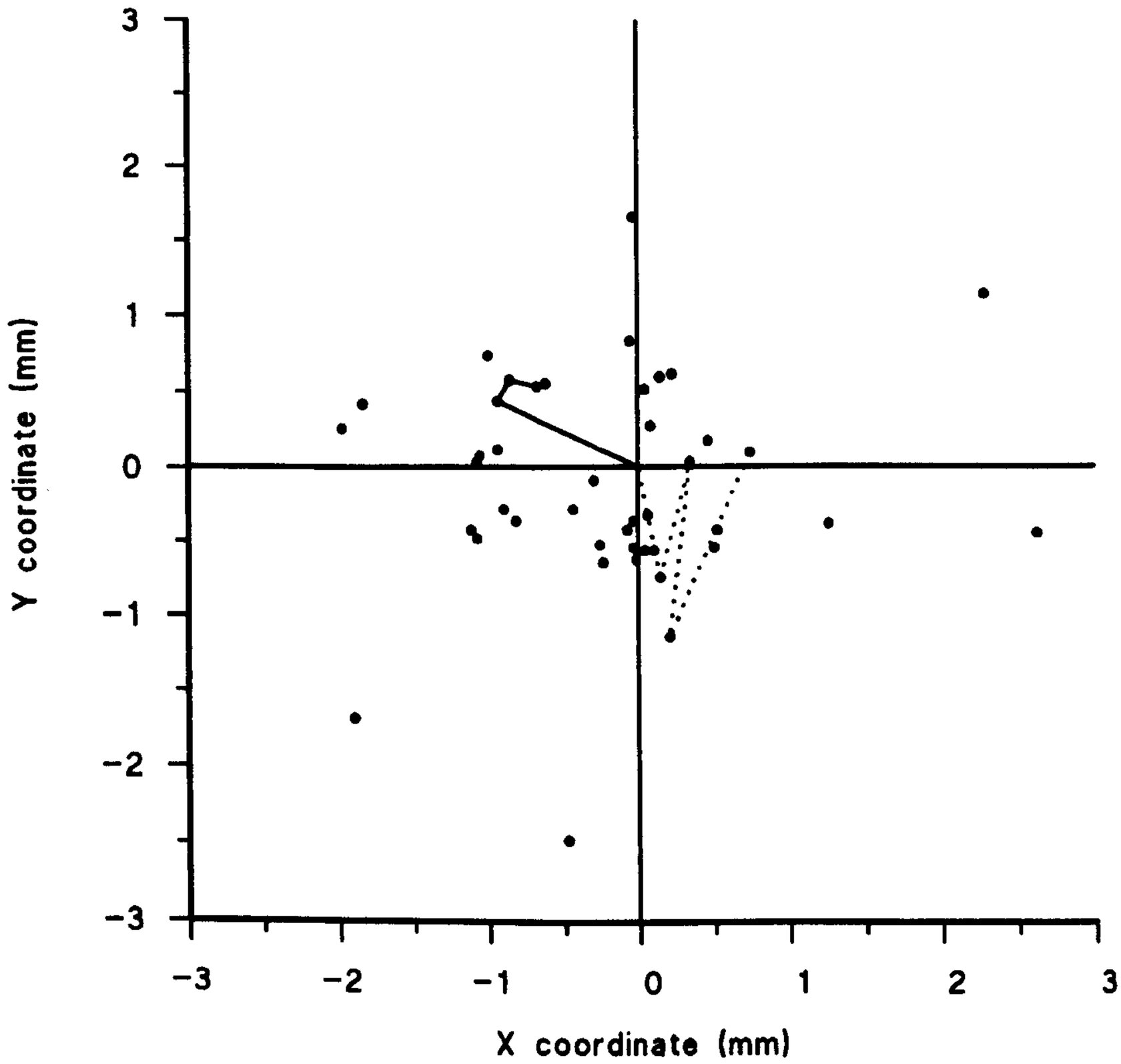


Figure 4: Measured coordinates from their initial locations (0,0) of *Pollicipes pollicipes* moving on plastic plates over a 188 day period. A uniform distribution of bearings, with most animals moving less than 1mm before changing direction, evidences lack of stimulation. Movement of 20 individuals was recorded. Connected points represent the path of 2 individuals between 5 measured positions.

ca. 10 μm d^{-1}. However, there were six measurements from three animals indicating speeds in excess of 30 μm d^{-1}, the fastest being 50 μm d^{-1}.

Rayleigh's test for the random distribution of bearings based on a unit circle indicated no directed travel for vertical (mean vector length $r = 0.27$, $n = 26$, $p = 0.15$) or horizontal animals ($r = 0.19$, $n = 17$, $p = 0.55$). Figure 4 shows the measured positions of all animals that had moved throughout the study, which confirms a uniform distribution of bearings and shows that few animals moved any great distance from their

origin. Indeed the correlation of distance (as opposed to cumulative distance) from original location position and time for moved animals indicated no relationship (Pearson's $r = 0.147$, $df = 41$, $p = 0.35$), which suggests that the majority of animals were moving about their initial location. The connected points in Figure 4 represent the measured positions of 2 animals throughout the study, which illustrates the typical back and forth movement about the point of initial fixation.

Of the twenty animals that moved, 11 were originally fixed facing downstream, the rest facing upstream. At their last measured positions only 5 were facing downstream and 11 upstream or normal to the flow; four died and the capitulum was lost. Assuming no directed change in the initial orientation of the barnacles and the probability of facing downstream initially as 0.55 the binomial probability of finding 5 or fewer barnacles facing downstream at the end would be 0.049, suggesting a significant change in the orientation of the population. Three of the four barnacles that died originally faced upstream and it is therefore unlikely that they would have changed their orientation.

	moving				stationary			
Dim.	r	df	t	p	r	df	t	p
RC	-0.11	18	-0.47	0.64	0.24	20	1.11	0.28
W	-0.43	18	-2.02	0.06	0.17	20	0.77	0.45
T	-0.43	18	-2.02	0.06	0.05	20	0.22	0.82

Table 4; Pearson correlation coefficients (r) for capitulum dimension (RC; Rostro-carinal, W; width, T; thickness) with time for (surviving) moving and stationary *Pollicipes pollicipes* attached to plastic plates.

Table 4 shows the correlation coefficients for capitulum dimensions with time for moving and stationary barnacles. The lack of any significant correlation between time and each dimension indicated no measurable growth in either group of animals. Likewise, there was no significant correlation for any dimension with time for moving animals initially facing upstream or downstream (maximum absolute Pearson's $r = 0.473$, $df = 8$, $p = 0.17$).

Of the 8 animals attached to larger animals at the capitulum/peduncle junction, only 2 moved appreciably throughout the duration of the study. The other six either fell off, died or did not move measurably. Figure 5 shows the positions of the two mobile animals (ma) and two non-mobile animals (na). The mobile animal in Fig. 5A had moved down the peduncle to the slate and was in the process of moving off the larger animal onto the slate. The mid-line of the peduncle had moved some 8 mm in 203 days, an average rate of 40 $\mu m\ d^{-1}$. Analysis of similar photographs taken throughout the study indicated a maximum rate of travel for this animal of 50 $\mu m\ d^{-1}$ over a period of 90 days. The mobile animal shown in Fig. 5B (ma) had also moved down the peduncle but against gravity and at a slower rate. The maximum rate of travel estimated for this animal was 20 $\mu m\ d^{-1}$ measured over 251 days. Estimated rates of travel for these animals are comparable to the faster moving animals attached to plastic plates. Large peduncular extensions of the base were not seen in these animals although some degree of encroaching integument was evident at the leading edge (e.g. le in Fig. 5B). Likewise, the majority of animals attached to plastic plates that had moved lacked obvious basal extensions.

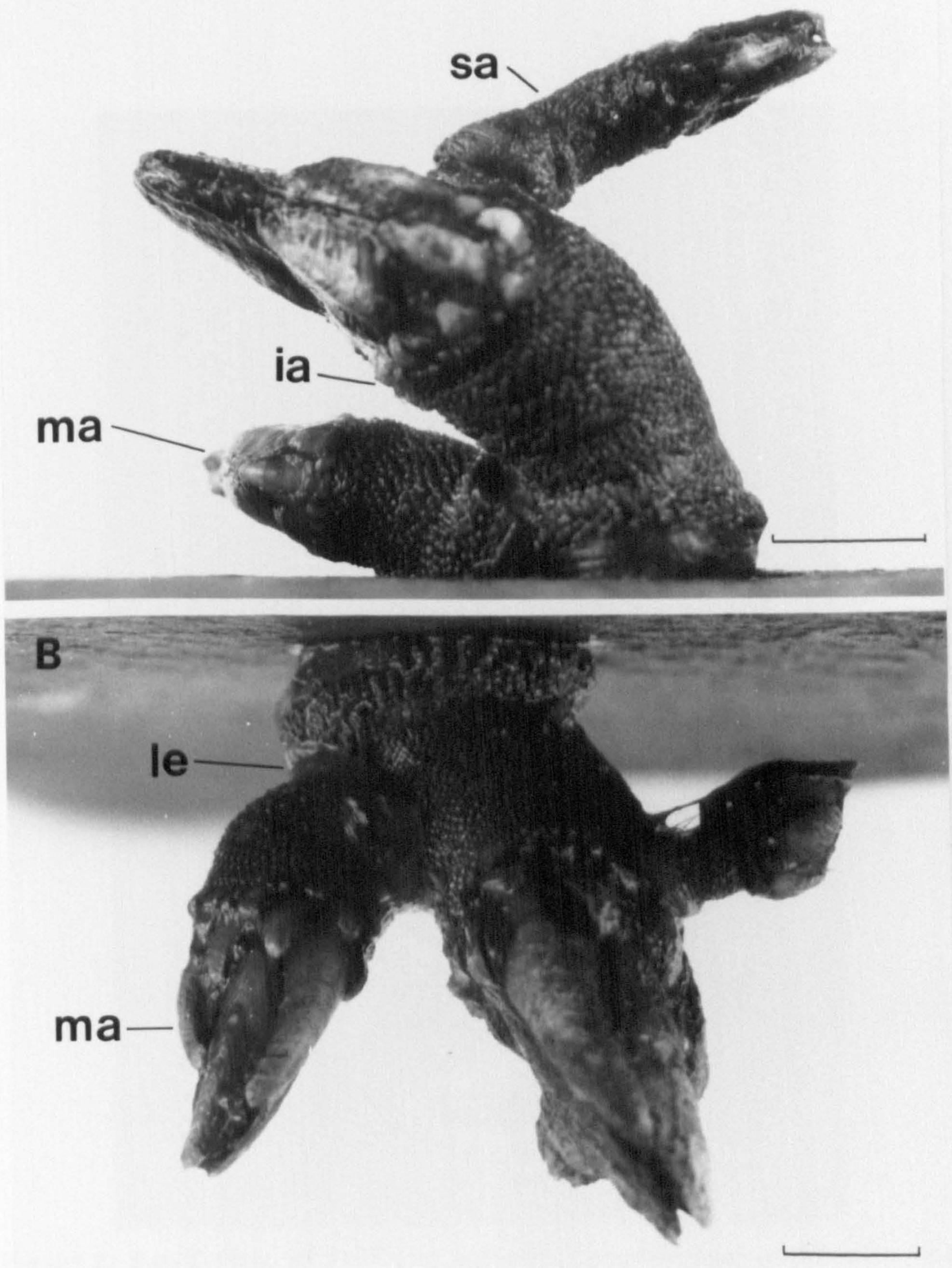


Figure 5: Large *Pollicipes pollicipes* bearing smaller conspecifics, initially attached at the capitulum peduncle border (ia). A; A large animal attached to the topside of a slate plate with a smaller, mobile, animal (ma) having moved toward the slate and a stationary animal (sa) still at the site of initial fixation. B; A large animal attached to the underside of a slate plate with a smaller, mobile, animal (ma) again having moved towards the slate. The leading edge (le), in the direction of travel, lacks an obvious peduncle extension. Scale bars are 5 mm.

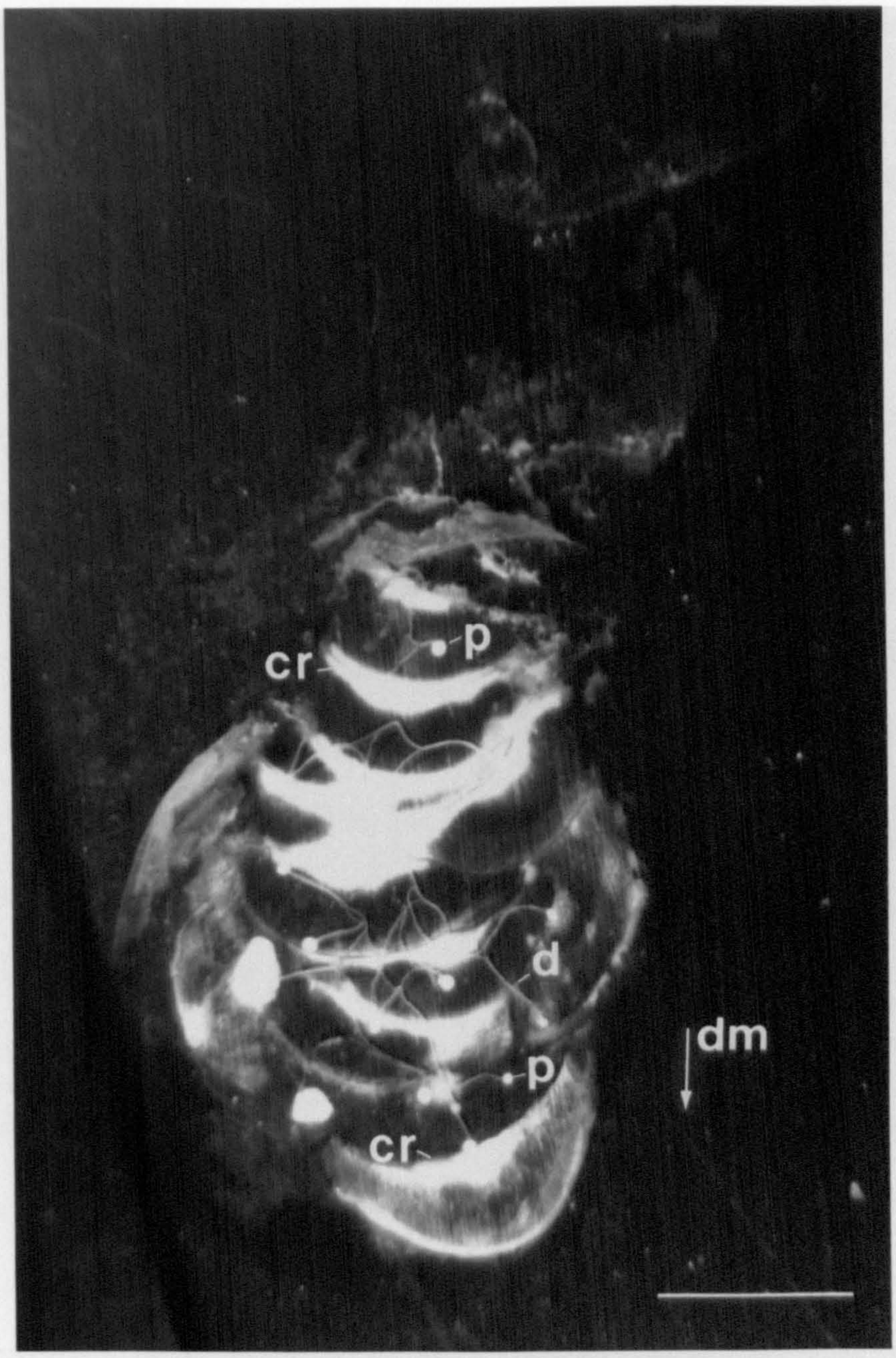


Figure 6. Dorsal view of *Pollicipes pollicipes* peduncle base attached to black plastic (Darvic) with all tissue removed except basal cuticle with ducts (d) and pores (p). The animal had moved in the direction indicated (dm) and cement ridges (cr) of bright white cured cement indicate incremental lateral peduncle base extensions. Scale bar is 2 mm.

Figure 6 shows the dissected base of an animal attached to black plastic that had moved in the direction indicated (dm). Incremental adhesion of basal integument is evident from the cement ridges (cr) caused by flow of pre-cured cement. The network of ducts (d) and pores (p) has no obvious pattern except to demark the line of travel. Figures 7 and 8 show the typical cement delivery system in the basal integument of adult *P. pollicipes*. The ducts are much convoluted in comparison to those of *Lepas anatifera* (Figs. 1 and 2). The pores are more or less the same size throughout the system. Remains of antennules were only ever found in the smallest (previously undisturbed) juveniles. Figure 8 shows an animal that had previously moved (dm, Fig 8). The principal ducts (pd, Fig. 8) lead to pores (p, Fig. 8) that are nearer the leading edge (le, Fig. 8) of movement than the trailing edge (te, Fig. 8). In this respect the pattern is similar to that found for *L. anatifera*. The trailing edge has pores and ducts that are likely to be lost through sloughing as the animal moves (*cf.* Fig 6).

Figure 9 shows a developing *P. pollicipes* peduncle extension viewed from below. In Figure 9A the extension is about 4.6 mm long with 3 discrete areas of recently exuded cement (cm). In Figure 9B the same extension, of smooth thin integument, had increased in length by about 1 mm in 25 days with an associated increase of discrete cement masses from 3 to 11. Such extensions were commonly seen in animals attached to the edge of plastic materials and maintained in tanks. Each discrete cement mass must have been delivered from a newly formed pore.

Figure 10 shows a selection of adult *P. pollicipes* with attached juveniles. In all cases travel is indicated by cement ridges (cr) similar to those in Figure 6. Movement of the juveniles is towards the peduncle base of the host adult. Figure 10 is typical of all animals examined at various sites on the south-west coast of Portugal. Where movement

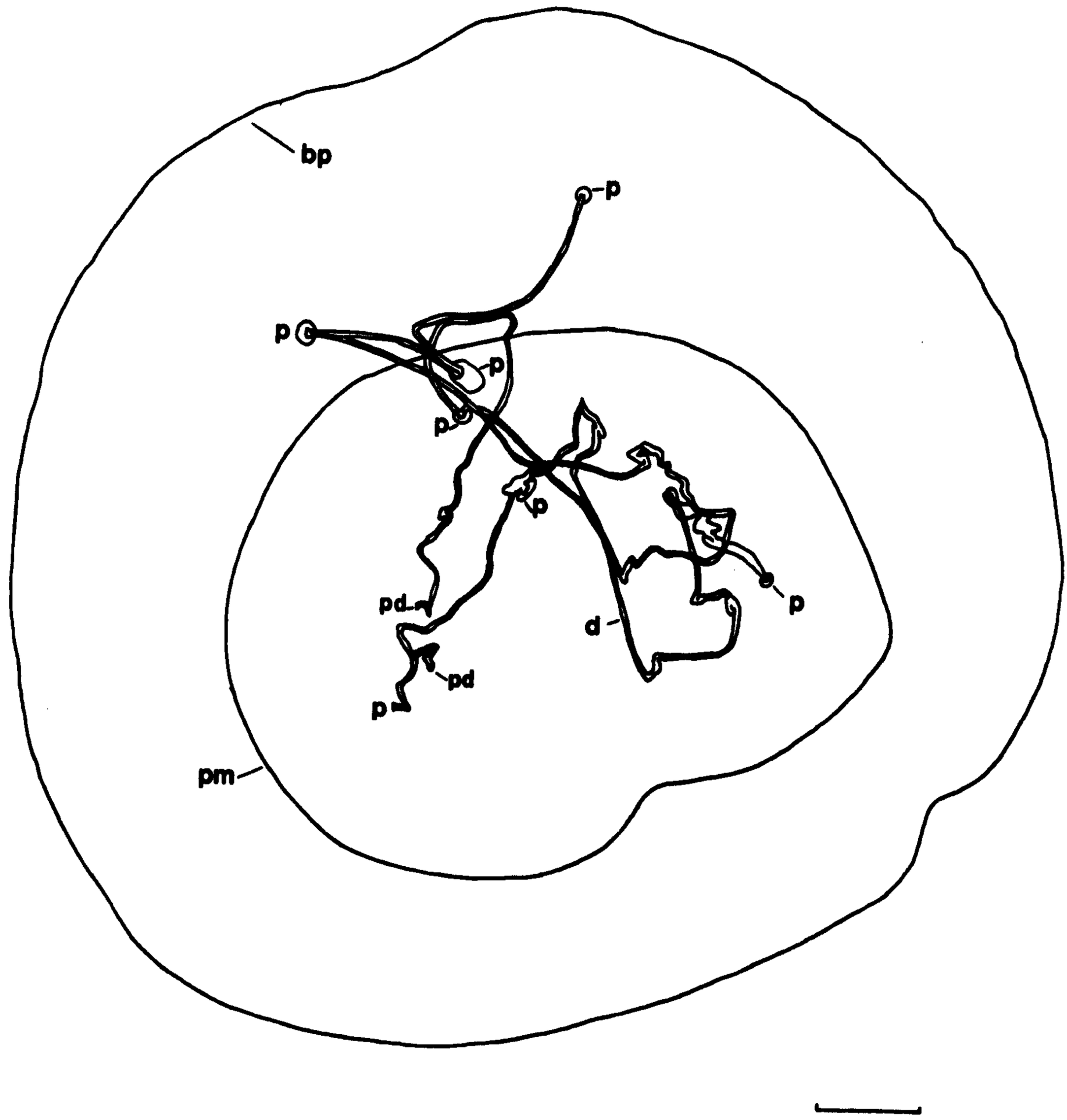


Figure 7: A *camera lucida* drawing of the cement delivery system in the peduncle base of *Pollicipes pollicipes*. Convoluted ducts (portrayed as branched for simplicity) run closely associated but are separate. Ducts (d) and pores (p) may appear randomly scattered and are of more or less constant size. bp marks the base perimeter and pm the inner margin of the peduncle integument. Scale bar is 1 mm.

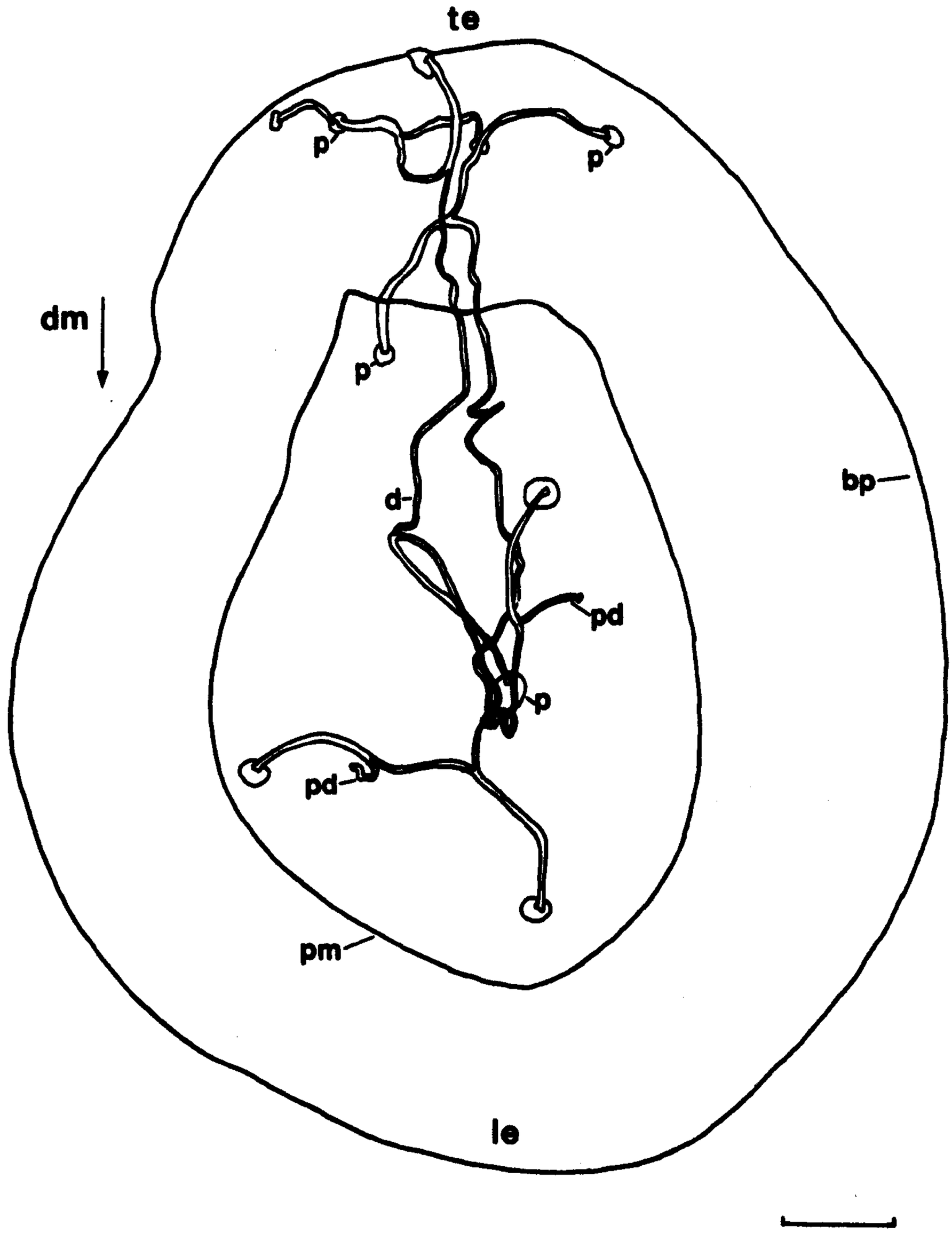


Figure 8: Typical cement delivery system in *Pollicipes pollicipes*. A *camera lucida* drawing of an animal that had been moving in the direction indicated (dm). Pores (p) adjacent to the principle ducts (pd) are toward the leading edge of travel (le). Ducts (d) and pores between the base perimeter (bp) and peduncle integument margin (pm) at the trailing edge (te) are about to be lost through sloughing. Scale bar is 1 mm.

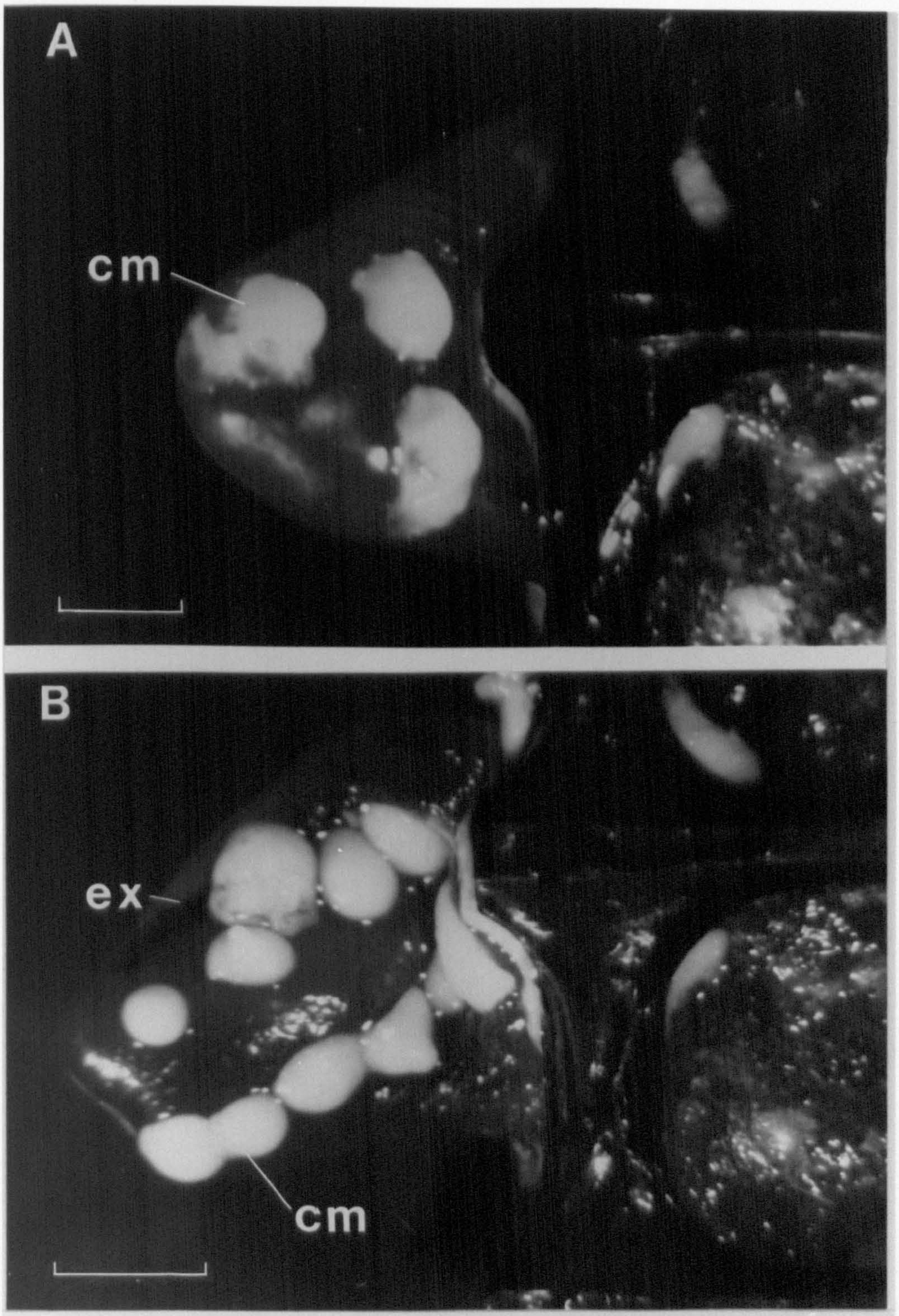


Figure 9: A developing peduncle extension in *Pollicipes pollicipes* attached to black plastic mesh. A; The developing extension of thin, smooth, turgid, integument is supplied with 3 cement pores, evident from 3 discrete cement masses (cm). B; 25 days later the extension (ex) has increased in size with the addition of 8 pores delivering cement. Thickening of the integument is perceptible adjacent to the plastic mesh mount. Scale bars are 2 mm.

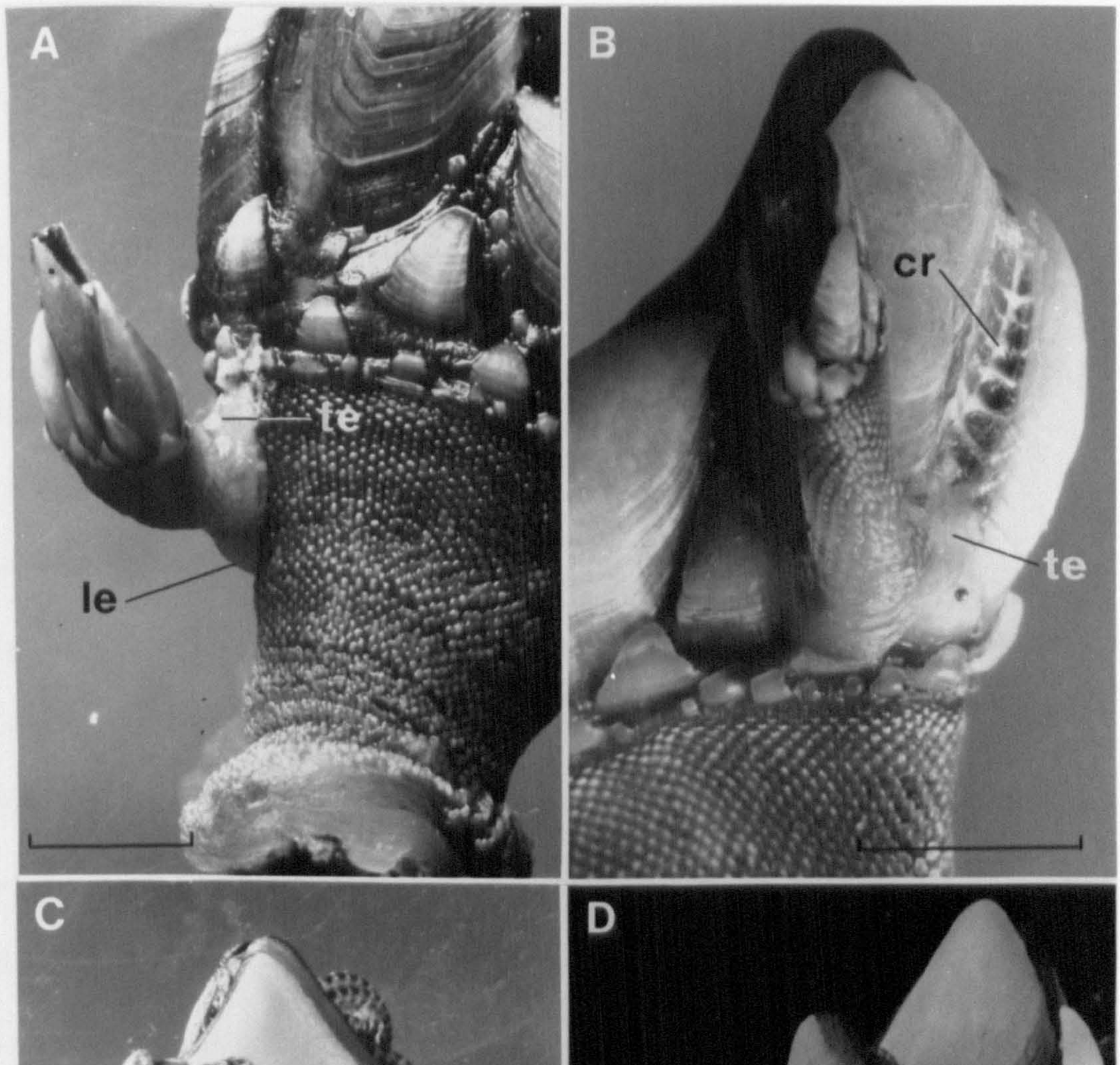


Figure 10: A selection of mobile *Pollicipes pollicipes* juveniles settled on adults, collected from the Algarve (Portugal). Cement ridges (cr) indicate direction of travel down the host peduncle. Peduncle extensions (pe) are common at the leading edge (le) of directed travel. Extensions at the trailing edge (te) and cement ridges are indicative of sloughing. All juveniles are moving towards the adult base regardless of adult attitude on collection. Scale bars are 5 mm.

of juveniles was evident, it was always towards the base of the adult, regardless of the attitude of the adult. Peduncle extensions (pe) are obvious at the leading edge (le) of juveniles shown in Figure 10 (excepting 10D). Extensions at the trailing edge (te) of these juveniles are indicative of material about to be sloughed off in the relocated individual.

Mobility of *Capitulum mitella*

Figure 11 shows clearly the method employed by *Capitulum mitella* for relocation mobility. Figure 11A shows a group of animals originally fixed close together but not touching. The foremost animal (ai) had an apparent extension to the peduncle base (pe). Reference to Figure 11B, where the dotted outlines mark the perimeter of the base initially in contact with the substratum of each individual, shows that movement was in the opposite direction to the apparent extension. The peduncle was laid to the substratum in the direction of travel and cemented, unlike *Pollicipes pollicipes* which employs obvious, large, true extensions. The cement ridges (cr) in Figure 11B must be due to additional cement pores delivering cement. The ridges therefore represent increments of new integument being cemented to the substratum as for *P. pollicipes* (e.g. Fig. 6). Figure 11B also shows that movement of closely separated animals is generally towards other animals resulting in a cluster with peduncle bases touching.

Figure 12 shows a solitary animal that was moving in the direction indicated (dm) with an apparent peduncle extension (pe) opposite to the leading edge (le) of travel. Viewed from below (Fig. 12B), peduncular plates are evident on the ventral surface (base). The three discrete cement masses (cm), beyond the area of initial fixation marked by the dotted line, are indicative of at least three new cement pores secreting adhesive.

The final stage of relocation is illustrated in Figure 13 which shows rear views of an individual *C. mitella* which had moved to its right (dm). In Figure 13A the method of laying the peduncle to the substratum results in an apparent basal extension (pe) on the left side of the animal which is later sloughed off to result in a repositioned animal with a shorter peduncle shown in Figure 13B.

Although animal positions on the plates were not measured as for *Pollicipes pollicipes*, the greatest rate of travel determined from sequential photographs was estimated at 33 $\mu m\ d^{-1}$ (7.7 mm in 236 days) which is comparable to that for *P. pollicipes*.

Large extensions of the peduncle like those of *P. pollicipes* (e.g. Fig. 9) were never seen in *C. mitella*. However, a degree of separation of peduncular plates at the leading edge of moving animals, together with an area of newly attached integument devoid of peduncular plates (e.g. see Figs. 11B and 12B), indicate growth and some degree of stretching in this area.

Figures 14 and 15 show typical cement delivery system layouts in the peduncle base of adult *C. mitella*. Duct/pore systems were very similar in appearance to those of *P. pollicipes* (Figs. 7 and 8) with convoluted ducts and a variable number of similarly sized pores. Again there was no obvious simple pattern but the position of the newest pores (nearest the principal ducts and towards the leading edge of travel for moving animals), lack of residual antennules and older pores and ducts about to be sloughed off at the trailing edge of travel (for animals exhibiting active relocation) conform to the pattern observed here for *P. pollicipes*.

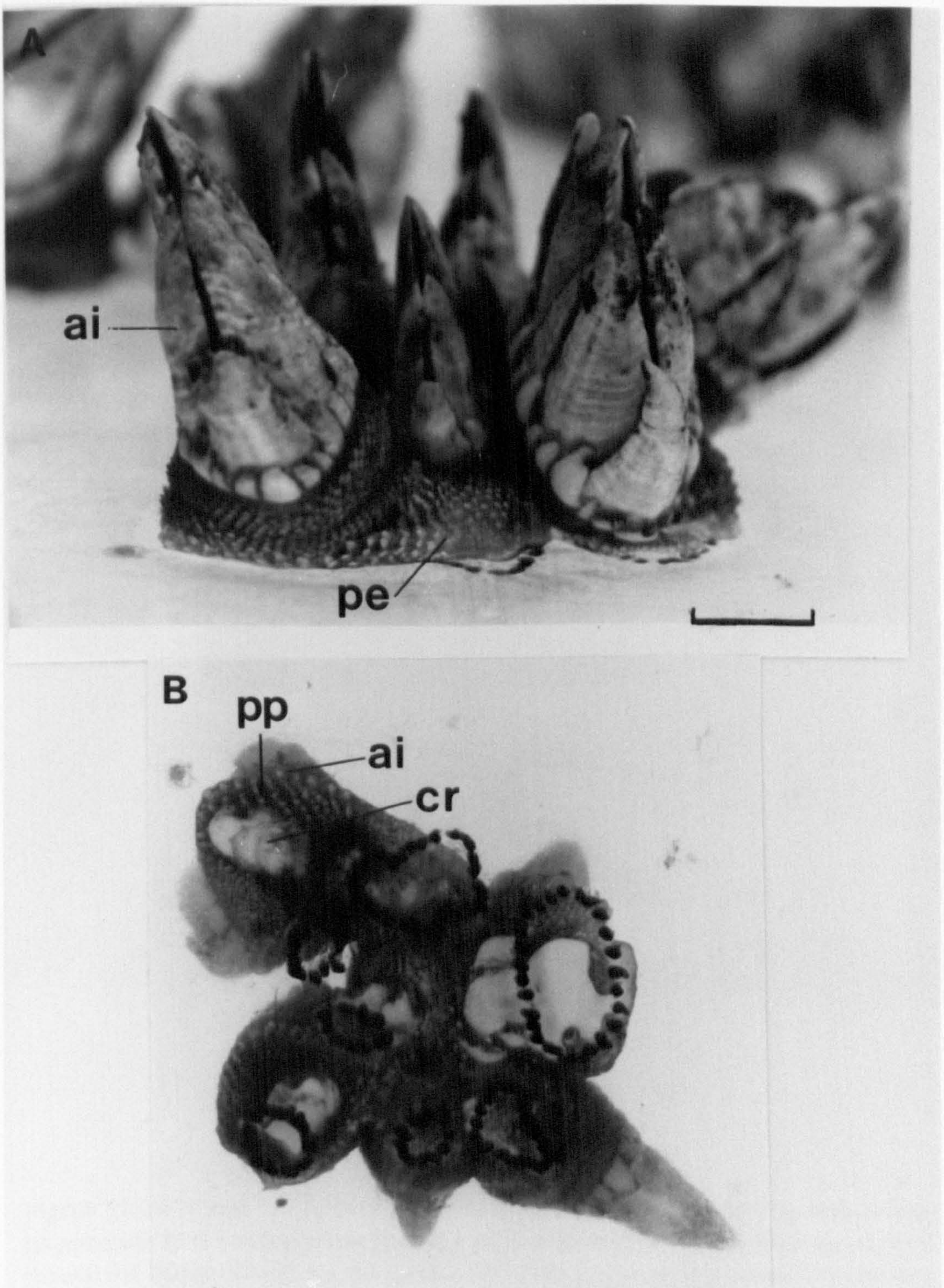


Figure 11: Relocation of *Capitulum mitella*. A; A cluster of animals with one (ai) possessing a large extension to the base (pe) which is at the trailing edge of travel, evident from B; a ventral view through the transparent plate mount with the original fixed position outlined. Ventral peduncular plates (pp) indicate incremental laying of the peduncle to the substratum demarked by cement ridges (cr). Other animals have moved together from their original separated positions forming a cluster with closely associated bases. Scale bar is 5 mm.

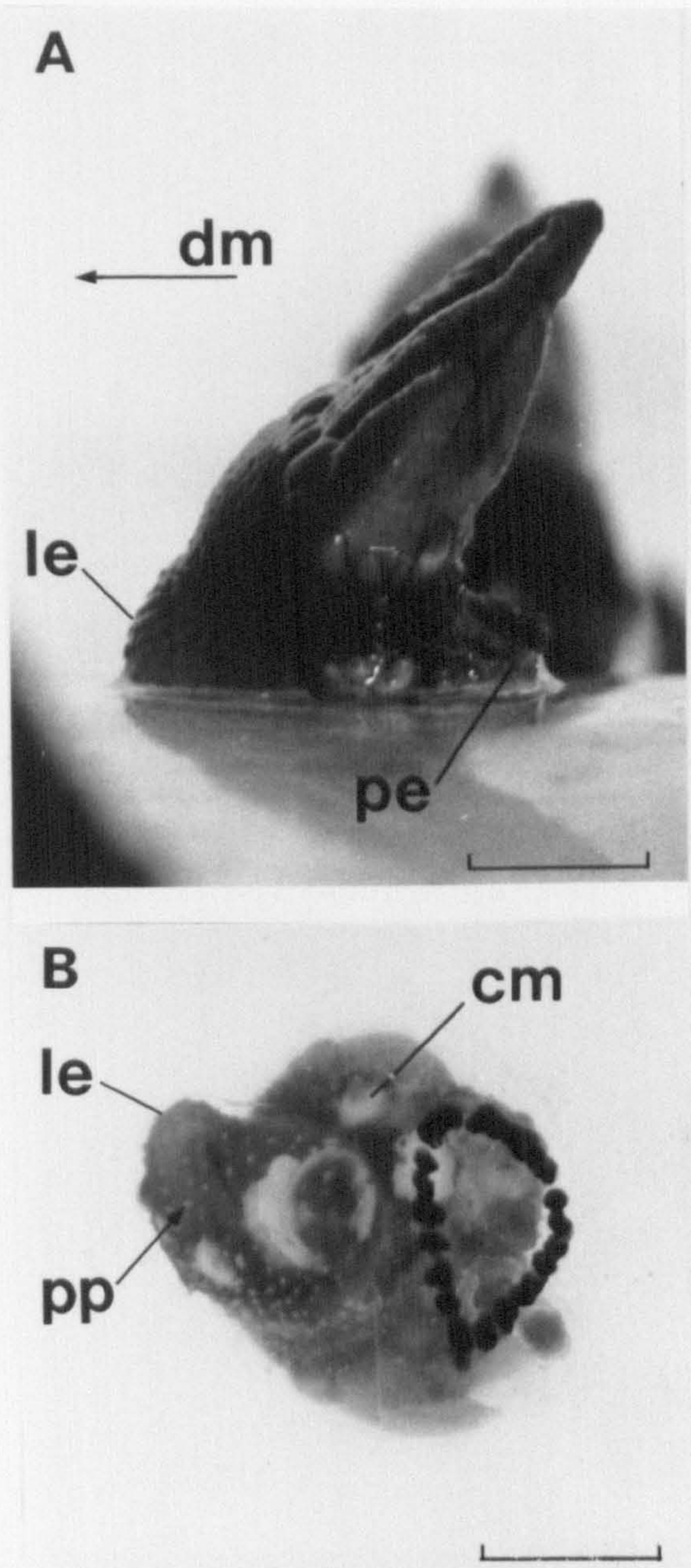


Figure 12: A; A mobile *Capitulum mitella* moved to its right (dm) by cementing its peduncle to the substratum forming an extension (pe) at the trailing edge of movement. Some growth and/or stretching of the peduncle integument is evident at the leading edge (le) of travel resulting in B; separated peduncular plates (pp) in the now basal integument. New pores delivering cement to the new basal integument are evident from discrete cement masses (cm). Scale bars are 5 mm.

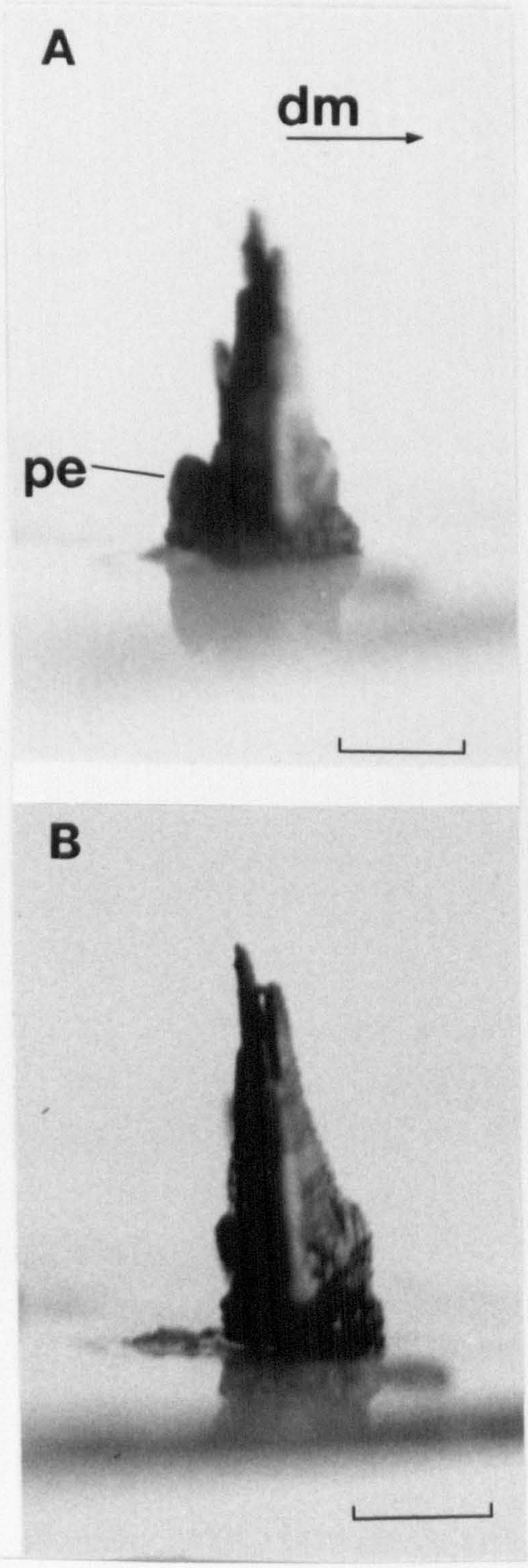


Figure 13: A; A mobile *Capitulum mitella* having moved to its right (dm) by laying and cementing its peduncle to the substratum, thereby reducing its total height from *ca.* 19mm and forming an apparent basal extension (pe), which in B; later sloughs off to result in an animal with a total height of *ca.* 14 mm and thus a peduncle shorter by *ca.* 5 mm. Scale bar is 5 mm.

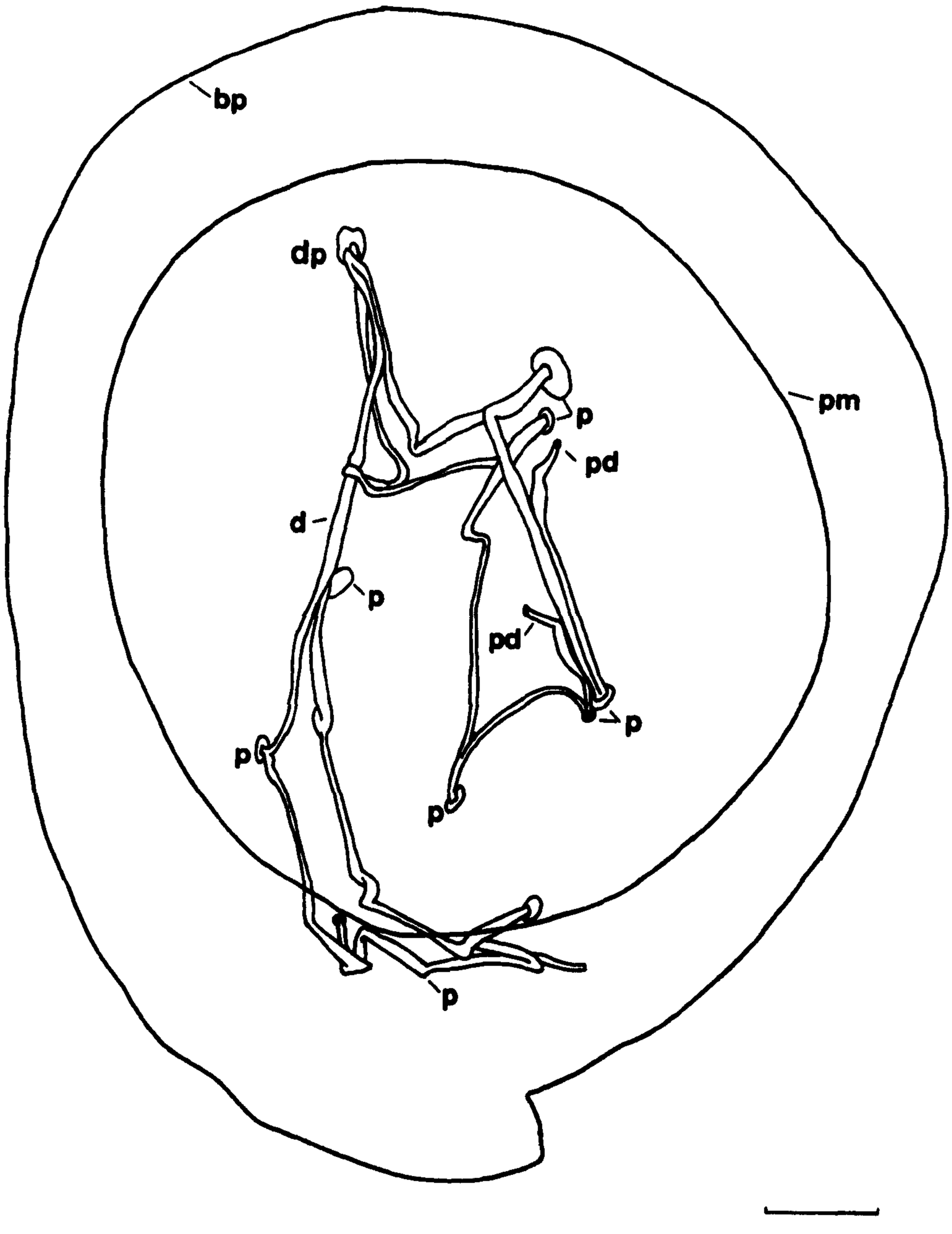


Figure 14: Duct and pore layout in *Capitulum mitella* showing convoluted ducts (d) which deliver cement to pores (p). The apparent connection of ducts from separate principal ducts (pd) is due to closely associated ducts and a double pore (dp). Scale bar is 1 mm.

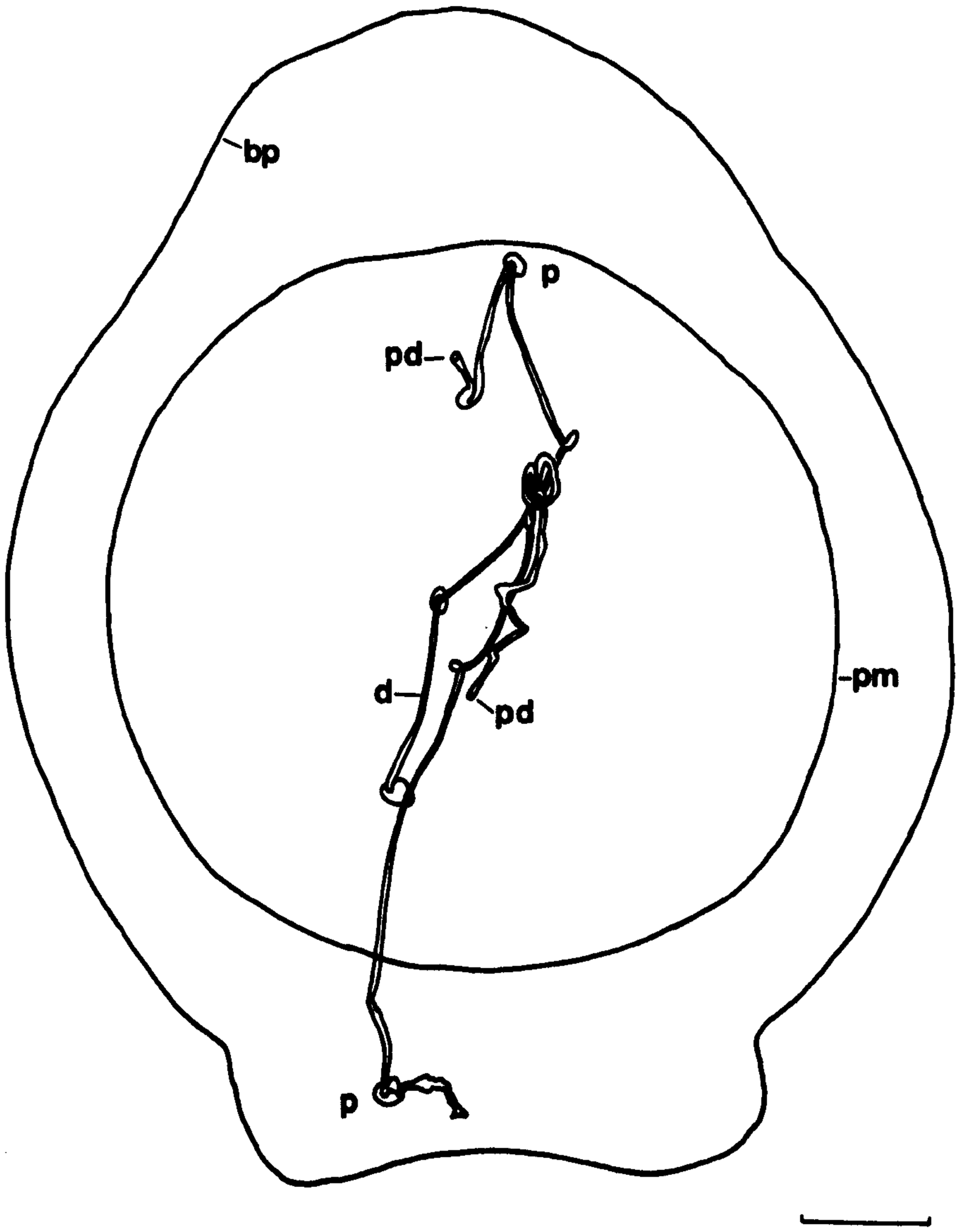


Figure 15: Duct and pore layout in *Capitulum mitella*. An almost linearly arranged system with few pores (p) and lacking in convoluted ducts (d) resulting most likely from a linear travel path. The base perimeter (bp) indicates travel toward the top of the figure. A pore and open-ended duct between the base perimeter and peduncle integument inner margin (pm) were being sloughed off. Scale bar is 1 mm.

DISCUSSION

Investigation of the cement delivery system (ducts and pores within the base integument) of *Lepas anatifera* shows that this barnacle can not actively relocate. The presence of antennules at the duct termini agree with Pagenstecher's (1863, cited in Saroyan *et al.*, 1968) interpretation that the cement ducts of adult *L. pectinata* Spengler, 1793 lead from the remains of the antennules. The presence of cypris antennules marking the settlement site, together with increasing duct and pore size along a linear (often divergent) path are strong evidence for immobility. Indeed, cypris antennules embedded in cypris cement are commonly present in balanomorph barnacles (Yule and Walker, 1984), where any observed mobility (Crisp, 1960) is the result of growth pressure from adjacent barnacles on very smooth surfaces. Crossing of the separate ducts only ever occurs near the duct termini and therefore probably represents orientation of the fixed cypris larva or early juvenile. Animals that had settled on conspecifics were attached close to the base, subsequent growth resulting in direct adhesion to the inanimate substratum. There is therefore no apparent necessity for this species to exhibit voluntary, active relocation.

The layout of the ducts and pores, increasing in size towards the principal ducts, indicates that pores are added adjacent to the principal duct with growth. No evidence of relatively small pores between established pores was found which corresponds to the more intricate basal duct and pore network of balanomorph barnacles where radial and circular (≡ secondary) ducts leading to pores (≡ terminal chamber) at the growing margin of the base are also formed adjacent to the principal ducts (e.g. Lacombe, 1967, Saroyan *et al.*, 1968, Bocquet-Védrine, 1970, Lacombe, 1970, Walker, 1973).

The basal cement delivery systems of *Pollicipes pollicipes* and *Capitulum mitella* are similar to that of *L. anatifera* in that duct and pores are added towards the leading edge of base growth. One major difference between these scalpellid systems and that of balanomorph barnacles and *L. anatifera* is the general absence of cypris antennules under adult bases. The obvious presence of antennules in juvenile, undisturbed scalpellids and absence in larger animals is in itself indicative of relocation. Absence of antennules is strong evidence for lost basal material as a result of relocation. The other major difference between the scalpellids and other non-mobile barnacles is the complex nature of convolutions of the ducts within the basal integument, which is evidence of growth in different directions.

The evidence points to growth and sloughing as the major factors in the relocation mechanism rather than muscular activity. For *P. pollicipes* there is no evidence to implicate muscular activity in relocation. The cementing of a growing peduncle extension supplied with newly formed pores in the direction of travel, coupled with the sloughing of basal material at the trailing edge is sufficient to cause movement of the base centre without the need to postulate peeling of the trailing edge from the surface and subsequent reattachment. Formation and enlargement of an extension through relatively high haemolymph pressure (Fyhn *et al.*, 1979, Crenshaw, 1979 and Walker and Anderson, 1990) stretching thin integument (Crenshaw, 1979) at a specific area of rapid somatic growth (Hoffman, 1984, Chaffee and Lewis, 1988) would not necessarily require muscle activity.

Results have demonstrated the active relocation of *C. mitella* at a similar rate to that of *P. pollicipes*. The method employed by *C. mitella* is similar to that of *P. pollicipes* with the sloughing of basal material at the trailing edge and growth and stretching at the

leading edge. However, unlike *P. pollicipes*, *C.mitella* does not utilise large peduncle base extensions but lays its peduncle to the substratum, resulting in a shorter peduncle. Such a method is much more likely to involve muscular action but the nature of the mechanism remains conjectural.

Observations and experimental measurements on *P. pollicipes* and *C. mitella* have verified circumstantial evidence (Hoffman, 1984, Chaffee and Lewis, 1988,) for relocation. Results demonstrate that the phenomenon is not restricted to smaller animals but was, perhaps, less measurable or is less common in larger animals.

P. pollicipes attached to plastic plates generally moved about the point of initial fixation with low rates of movement (average 10 $\mu m\ d^{-1}$). The stimuli offered in the flume were unidirectional water flow, gravity (horizontally or vertically fixed animals) and general, almost diffuse, fluorescent light from above. No directed response pattern was evident from the barnacles. At the end of the experiment, however, significant numbers of animals reorientated, so that their cirral nets were directed into the oncoming water instead of away from it. No obvious peduncular extensions were seen in animals which reorientated, turning presumably requiring little directed growth.

2 of 8 candidate *P. pollicipes* attached to larger individuals in the laboratory did show directed travel. Both moved toward the slate substratum in accordance with field observations where animals attached to larger animals had always moved down the peduncle regardless of the attitude of the host. These laboratory animals moved 2-4 times faster than animals showing random movement over small distances. Neither gravity nor directed seawater flow were causal to any observed directed movement so other stimuli must be sought.

The occurrence of relocation in field *P. pollicipes* shows that the phenomenon has survival value. Gregarious settlement in barnacles is a well recognised phenomenon with spat settling in close association with adults increasing the probability of survival and reproductive fulfilment (see Crisp, 1974). Hoffman (1989) reported settlement of larval scalpellids high on adult conspecifics in a position which may afford protection from predation and certainly provides access to moving seawater and an adequate food supply. The ability of *P. pollicipes* juveniles to relocate ensures the survival of the juvenile beyond that of the host adult, maintains the adult cluster pattern and frees settlement space high on the host peduncle. It is clear that not all larvae adopt the same settlement/relocation strategy since metamorphosed juveniles can be found at the base of adult *P. pollicipes* and on bare rock substratum under juvenile mussels in the field. The mix of settlement strategies is clearly successful and responsible for the characteristic rosette shaped clumps of *P. pollicipes* found on the coasts of the Algarve.

C. mitella mounted in closely associated groups and maintained in constant conditions in an upright attitude generally moved toward each other forming tighter clusters with touching bases (e.g. Fig. 11). One benefit of such a strategy for individuals is that close association with other members of the cluster would produce mechanical interlocking reducing shear stresses on the cement. The stimulus for the movement is currently speculative but must ultimately involve some appreciation of the proximity of others even if the process begins as random reorientations, as seen for *P. pollicipes* on plastic plates, resulting in contact with conspecifics which is then maintained. A few juveniles were found high on adult barnacles but in the limited sample there was no evidence for the relocation of juveniles to the substratum. The major difference seen in the methods

of relocation employed by the two scalpellids is indicative of convergent evolution of the behaviour of organisms living in similar habitats.

The results have shown that mobility is perhaps the wrong term for scalpellid movement. Because measured positional change is always due to the loss of basal material at the trailing edge of movement (*cf L. anatifera* as immobile) active relocation seems more descriptive of the phenomenon.

CHAPTER 4

The dissolution of lepadomorph cement in sterile and asterile seawater

ABSTRACT

The cements of two intertidal scalpellid lepadomorphs, *Pollicipes pollicipes* and *Capitulum mitella*, are shown to dissolve in sterile seawater, but at very low rates of *ca.* 0.14 % d^{-1} for masses with initial weights of *ca.* 560 µg. Significantly higher rates of dissolution for cement in flowing seawater and in the presence of strains of bacteria isolated from the cements could be explained solely by the relative surface area:volume ratios and hence size, of the samples employed indicating resistance to degradation by marine bacteria. Dissolution of lepadomorph cement in seawater supports hypotheses for solidification of the compositionally similar balanomorph cement through the formation of hydrophobic complexes, hydrogen and electrostatic rather than covalent bonding.

INTRODUCTION

The proteinaceous cement of adult barnacles is well known to be highly resistant to chemical breakdown creating difficulty in analysing the pre-cured (pre-polymerised) moieties and hence determining the 'bond' type(s) causing solidification. Indeed, since major research into the chemical nature of barnacle cement began in the late 1960s direct characterisation has been, until recently (see Naldrett, 1993), limited to amino acid profiles and microcombustion analyses (e.g. Cook, 1970, Saroyan et al., 1970a, Walker, 1972, Lindner and Dooley, 1973, Barnes and Blackstock, 1974, Walker and Youngson, 1975), revealing the cement to be predominantly protein. Attempts to solubilise cement to characterise the protein constituents have largely relied on dissolution using SDS-2Me to facilitate electrophoresis of the solubilised proteins (e.g. Barnes and Blackstock, 1976, Yan and Pan, 1981, Naldrett, 1993).

Lindner and Dooley (1973) reported that the cement of *Balanus crenatus* was highly resistant to solvents such as dilute acid and alkali, salt solutions and thioglycollate solution. Such an apparently unreactive material has obvious benefits to barnacles utilising its adhesive properties in a reactive solvent (seawater) under conditions where the ubiquitous action of microbial life encourages rapid decay and recycling of materials. Resistance to breakdown, and hence loss of adhesion/cohesion, could be achieved from an innate stability and/or by the exclusion of erosive and decaying agents from the material.

Naldrett (1993) noted that bacteria and algae are rarely found in the cement of balanomorph barnacles, attributing this to exclusion at a non-porous, dense outer zone in cured cement. However, apparently still healthy algae can sometimes be found under

the cement of some balanomorphs (Yule, pers. obs.), probably as a result of the barnacles growing over the plants, so bacteria may be similarly trapped. Moreover, lepadomorphs often have large cement masses around the peduncle base fully exposed to the rigours of the environment. As an extreme example, *Dosima fascicularis* (Ellis and Solander, 1786) (≡ *Lepas fascicularis*) utilises its cement as a float after initial settlement of the larvae on a small floating object.

Whilst much attention has been paid to cement reactivity with various solvents *in vitro*, no direct information exists on the longer term stability of cement under more natural conditions. The current work investigates the stability of cements from two intertidal scalpellid lepadomorphs, *Pollicipes pollicipes* and *Capitulum mitella*, in seawater and subjected to cultured bacterial isolates collected from the cement.

METHODS

Cement collection

Pieces of cement were collected from the bases of adult *Pollicipes pollicipes* and *Capitulum mitella* which were suspended upright, in flowing seawater tanks, by fishing line attached to the shell plates with epoxy resin. Initially, old cement was gently eased off the base and discarded. Removal of cured cement often resulted in the production of new cement from uncovered cement duct pores. The new, solidified, cement was collected by prising small beads of the material from the base. The time interval between clearing pores and collecting newly deposited cement varied from a few hours to 3 days. Cement beads were routinely cleaned of visible debris, washed in UV irradiated filtered (to 0.2 μm) seawater and if not immediately used were washed in distilled water and stored in a desiccator, in plastic tubes with nylon mesh covering the ends, until required.

Long term stability of cement in seawater

Sets of 6-20 *Pollicipes pollicipes* cement beads, covering a range of volumes, were placed in 30 mm diameter polystyrene tubes closed at both ends with nylon mesh (80 μm square). The tubes were suspended in a tank of flowing seawater housing adult *P. pollicipes*. The total wet weight of cement within each container was periodically determined (to the nearest 1 μg) with a Cahn-c31 microbalance. Pieces of cement were rinsed in distilled water and blotted dry before weighing.

In a similar manner, the weight loss of cement beads from *P. pollicipes* and *Capitulum mitella* was assessed in sterile seawater. A number of beads were kept in 20 ml universal jars with 10 ml UV irradiated, filtered (to 0.2 μm) seawater. After weighing the cement,

the seawater was changed and the jars were autoclaved (+1 bar for 15 minutes) and stored at room temperature without agitation.

Bacteria collection and isolation

Bacteria were collected from cement samples from *C. mitella* and *P. pollicipes* which had been kept in seawater, as above, for twenty days. OZR agar, as a best compromise medium (Sieburth, 1967), with ferric sulphate replacing ferric chloride, was used to isolate bacterial strains. Plates were incubated at 28 °C for 48 h before replating. Attempts to characterise bacterial isolates, using API, were unsuccessful even when isolates were gradually changed from a medium of 75 % seawater (OZR) to 7 % seawater (OZR). Bacterial isolates were, therefore, distinguished only by appearance and growth rate.

Bacterial effect on cement

Cement beads were placed in 20 ml universal jars with 7 ml UV irradiated, filtered (to 0.2 μm) seawater and 7 ml OZR (as above) broth. The jars were autoclaved (+1 bar 15 mins.) then each was inoculated, at random, with 100 μl of an OZR broth cultured bacterial isolate. Most jars contained one piece of cement. One jar contained two, and another three, smaller beads. Half the jars contained *P. pollicipes* cement and half *C. mitella* cement. All the jars were kept on a rotating wheel (8 rpm) at room temperature (18-25 °C). The lid of each jar was removed, over a flame, at least every other day to prevent anoxia.

Weights were periodically determined as before but, to reduce contamination, cement beads were blotted dry on autoclaved paper without rinsing and weighed on autoclaved

aluminium foil. OZR agar plates were periodically inoculated from the cement and medium in the jars to monitor potential contamination.

Bacterial protease

The presence of agar diffusible protease was assessed for each bacterial isolate using milk agar plates. The initial milk agar formula resulted in total clearance of many plates within 24 hours making comparisons between isolates difficult. Double substrate concentration plates were made by dissolving 8 g skimmed milk (Cow and Gate) in 60 ml of distilled water. Agar was prepared from 3 g agar powder dissolved in 130 ml seawater (filtered and irradiated) with 10 ml of 50 mM tris-HCl (pH 7.8). The milk and agar solutions were mixed aseptically after autoclaving (+1 bar, 15 mins), allowed to set in Petri dishes, then topped with a layer of OZR agar.

Confirmation of protease presence and a determination of whether it was membrane bound or released to the medium was assessed, spectrophotometrically, for 3 bacterial isolates. The method described by Sarath *et al.* (1989) using azocasein was employed, but enzyme presence was determined relative to a reagent blank only, hence no quantitative estimates were possible. Substrate and enzyme were incubated for 2 hours at 25 °C.

Protease released to the medium was concentrated from 1 l OZR broth cultures of 2 bacterial isolates cultured at 32 °C for 48 h. The bacterial cultures were centrifuged at 12000 g for 30 minutes and non-specific proteins were precipitated from the broth, over ice, with an equal volume of saturated (room temperature) ammonium sulphate. Precipitated protein was centrifuged (12000 g, 15 minutes) then resuspended in 10 ml Tris-HCl (50 mM, pH 7.8) and dialysed overnight in 4.5 l tris-HCl (50 mM, pH 7.8) at

10 °C. Concentrate was stored at -70 °C until used. The presence and nominal activity of protease within the concentrate was determined by the method of Sarath *et al.* (1989). Cement beads were incubated with 0.5 ml of concentrated protein at room temperature for 35 days, without constant agitation, and weight change measured as above.

RESULTS

Figure 1 shows the relative weight (% of original weight) plotted against time for *Pollicipes pollicipes* cement beads kept in a flowing seawater tank (asterile cement). 3 samples (containing 14, 15 and 20 cement beads, with initial weights of 5, 3 and 15 µg respectively) showed an apparent initial weight gain of up to 40 % (squares, Fig. 1) before a consistent weight loss which began after *ca.* 18 days (Fig. 1). Another 3 samples (containing 6, 9 and 16 cement beads, with initial weights of 10, 7 and 13 µg respectively) appeared to undergo either virtually no change in weight or a very low weight loss (triangles, Fig. 1) for about 18 days, followed by a much increased weight loss. The first set of cement beads (squares, Fig. 1) were placed in the flowing seawater tank some 94 days after the tank was cleaned whilst the second set (triangles, Fig. 1) were placed in the tank between 13 and 31 days after it had been thoroughly cleaned. Nematodes, ciliates and harpacticoid copepods were found within all the sample containers placed in the tank, hence a fouling community developed within the containers during immersion. It seems reasonable to attribute the initial measured increase in weight of the first group of beads to the establishment of a surface associated community. The cleaner nature of the tank for the second group of beads meant that the fouling community build up was very much slower and masked by other processes affecting the measured weight of the cement.

For both sets of samples, an increase in relative weight loss occurred at about 18 days, and must have represented an abrupt change in the predominant process determining the measured weights (Fig. 1). Using least squares linear regression, 3 samples lost weight at a rate of 2.4 ±0.5 (SE) % d^{-1} ($r = -0.84$, $df = 10$, $p < 0.001$,) and 3 at a rate of

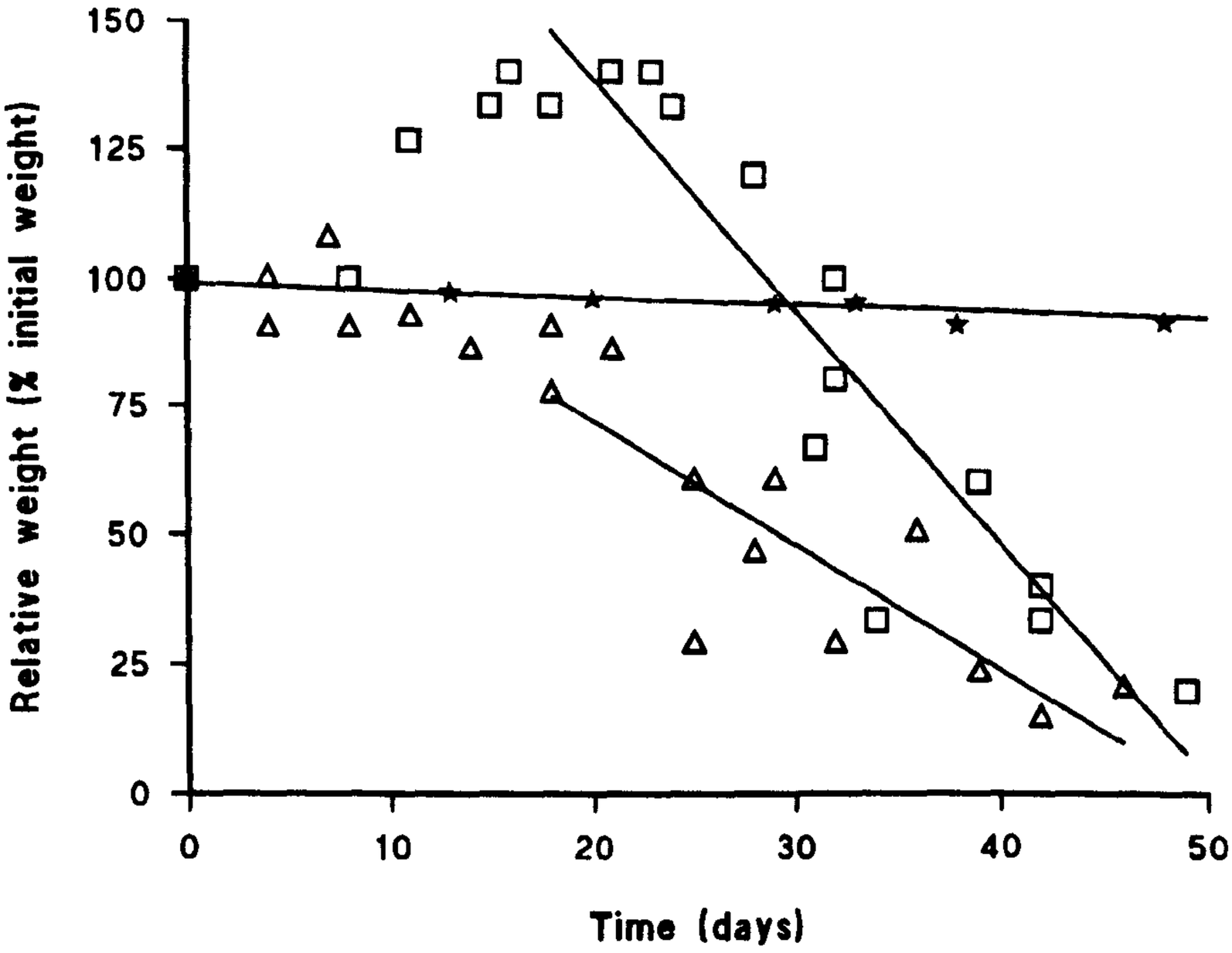


Figure 1: Relative weight (%) of *Pollicipes pollicipes* cement beads immersed in unagitated sterile seawater (stars), a flowing seawater tank (squares) and a flowing seawater tank comparatively recently cleaned (triangles). Lines fitted by least squares linear regression (see text).

4.5 ±0.6 (SE) % d^{-1} ($r = -0.92$, $df = 11$, $p < 0.001$). The linear relationships between relative weight and time, from day 18, for both sets of samples are shown in Figure 1.

A change in appearance of the cement beads was observed for cement samples shown in Figure 1 over the period in which weight was measured. Fresh cement beads were always bright white (e.g. see Fig. 9, Chapter 3) but changed colour through cream, light brown and brown to dark brown, at which time they fell apart and could not be weighed.

Sterile samples of *P. pollicipes* and *Capitulum mitella* cement also reduced in weight with time. 14 beads of *P. pollicipes* cement were used with a total initial weight of 5.76 mg and 12 beads of *C. mitella* cement with a total initial weight of 9.01 mg. Over

a period of 57 days weight reduced significantly in cement samples from *P. pollicipes* ($r = -0.878$, $df = 6$, $p = 0.004$) and from *C. mitella* ($r = -0.842$, $df = 6$, $p = 0.009$). An analysis of variance of relative weight (% of initial weight) with time (covariate), shown in Table 1, showed no significant difference between the rate of weight loss for cement from either species ($F_{[1,12]} = 0.92$, $p = 0.36$). Subsequently, the cement samples from both species were combined and weighed over a further 94 days. Least squares linear regression of relative weight (%) with time for *P. pollicipes* and *C. mitella* cement over 151 days ($r = -0.985$, $df = 12$, $p < 0.001$) indicated a relative weight loss of 0.14 ± 0.01 (SE) % d^{-1} with an intercept of 99.19 %, not significantly different to 100 (SE = 0.56, $p = 0.17$). The relationship and relative weights with time (to 50 days) for sterile *P. pollicipes* and *C. mitella* cement samples are also shown in Figure 1 (stars).

Source	DF	SS (seq.)	SS (adj.)	MS (adj.)	F	p
Time	1	115.69	115.69	115.69	34.81	<0.001
Species	1	7.42	0.02	0.02	0.01	0.942
Time x Spp.	1	3.05	3.05	3.05	0.92	0.357
Error	12	39.88	39.88	3.32		
Total	15	166.04				

Table 1: Analysis of variance of relative weight (%) and time (days; covariate) for *Pollicipes pollicipes* and *Capitulum mitella* cement (seq = sequential, adj. = adjusted for entry order).

Sterile cement beads underwent a similar change in appearance with time to asterile cement. From bright white at day 0, beads were variously cream coloured or light brown when last weighed at 151 days and at a predicted relative weight of 79 %.

An estimated reduction of 21 % in weight over 151 days for sterile scalpellid cement in seawater must have been due to dissolution, albeit slow, whether or not enhanced due to repeated autoclaving.

P. pollicipes cement samples in flowing seawater appeared to undergo much greater rates of weight reduction (2.4-4.5 % d^{-1}), falling to *ca.* 17 % of their initial weight in less than 50 days (Fig. 1). However, the difference in rates of weight reduction between sterile and asterile cement samples could be attributed to the relative surface area of the cement beads. Assuming the surface area (SA) of a cement bead to be roughly the 0.67 power of weight, then the average relative SA:volume ratios (SA:V) for the three treatments are thus: sterile = 0.12, clean tank = 1.01 and dirty tank = 1.28. The ratios of weight loss to the SA:V, using the 95 % confidence intervals of weight loss, for the three treatments are then; sterile 1.0-1.3, clean tank 1.3-3.5 and dirty tank 2.4-4.6, showing little difference in rate which could not be accounted for by differences in surface area and, perhaps, agitation. The build up of biofilm on the asterile treated beads also appears directly related to surface area and tank conditions. The differences between asterile treatment rates is clearly due to the dynamics of the biofilm formation rather than effects on the cement dissolution since both sets of results predict zero weight for the cement beads at around 50 days (50 days for clean tank and 51 days for dirty tank).

Bacterial degradation of the cement of *Pollicipes pollicipes* and *Capitulum mitella*

Six bacterial strains were isolated from the cement of *Pollicipes pollicipes* and six from *Capitulum mitella* cement on the basis of growth and appearance on the nutrient agar. The strains were labelled with a number (1-6) and letter distinguishing which species cement they had been isolated from (P, *P. pollicipes* and M, *C. mitella*).

	C. mitella cement			*P. pollicipes* cement		
BI	r	df	p	r	df	p
1P	0.360	12	0.206	0.022	11	0.949
2P	-0.577	12	0.031	-0.449	12	0.107
3P	-0.375	11	0.207	-0.831	11	<0.001
4P	-0.764	12	0.001	-0.951	11	<0.001
5P	-0.786	12	0.001	-0.687	11	0.009
1M	-0.700	11	0.008	-0.119	12	0.685
2M	-0.832	12	<0.001	-0.939	12	<0.001
3M	-0.633	12	0.015	-0.717	12	0.004
4M	-0.383	11	0.196	-0.590	11	0.034
5M	-0.900	11	<0.001	-0.728	11	0.004

Table 2: Correlations (Pearson's product moment, r) of lepadomorph cement weight (μg) with time (days) in the presence of various bacterial isolates (BI).

All but two isolates (1P and 3P) showed the presence of agar diffusible protease when cultured on milk agar plates. 3 isolates (5P, 5M and 6M) cleared the whole petri dish within 72 h but also grew over the same area. The other 7 isolates grew as discrete colonies of various sizes and cleared a zone ranging from 7-45 mm in diameter within 72 h.

Ten of the twelve isolates, excluding two (6M and 6P) were each used to inoculate a jar containing *P. pollicipes* cement and one containing *C. mitella* cement. 6M and 6P were virtually identical to 5P and 2M, respectively, in appearance and growth rate hence were not used in attempts to degrade the cement. Table 2 shows Pearson's correlation coefficients (r) for cement weight with time (to 131 days) in the presence of one of 10 bacterial isolates. In 30 % of cases no significant reduction in weight with time was indicated. Half of the isolates were consistent in that significant reduction in weight occurred for both species cement (Table 2). A bead of *P. pollicipes* cement, in the

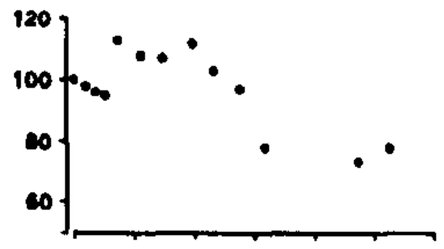
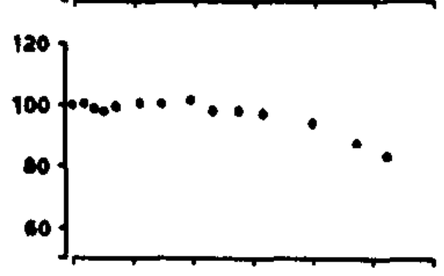
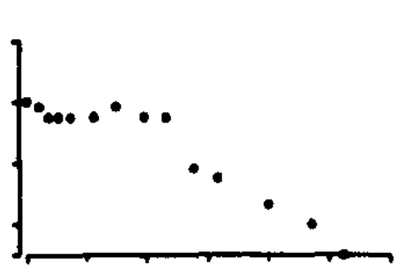
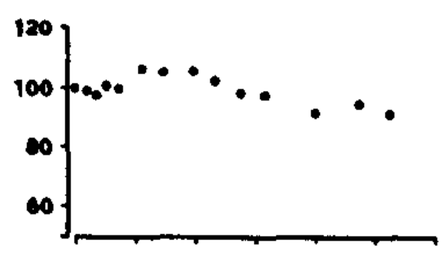
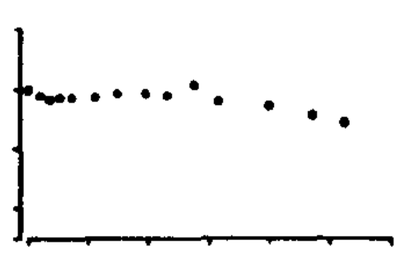
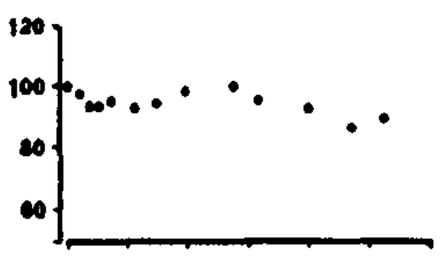
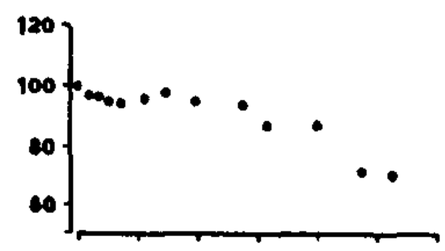
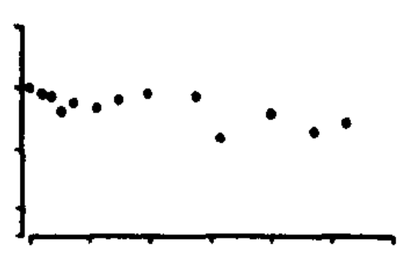
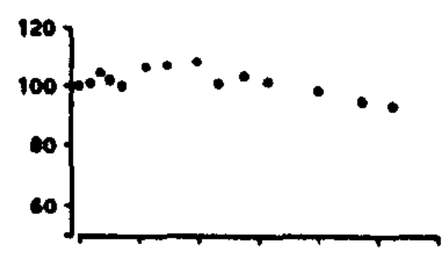
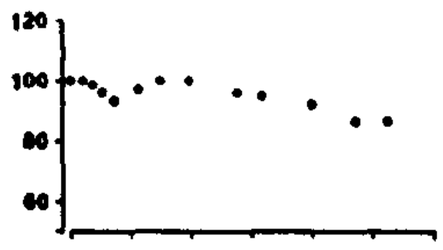
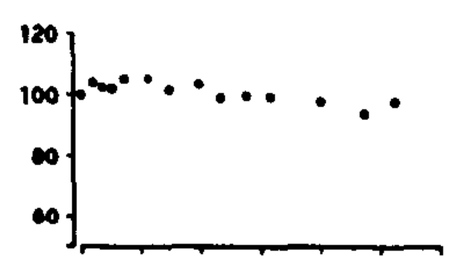
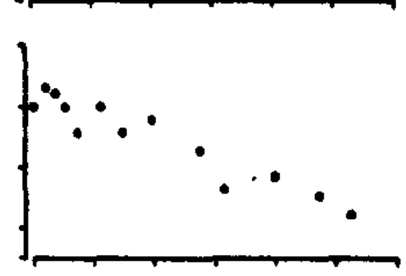
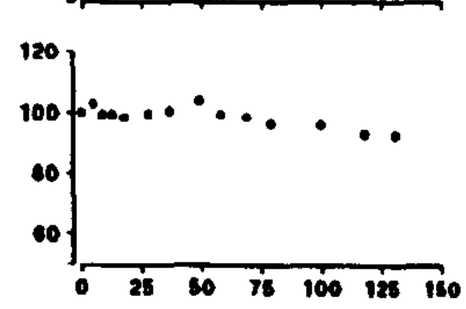
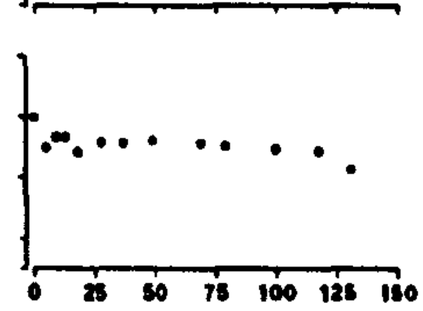
Bacterial Isolate	*Capitulum mitella* cement	*Pollicipes pollicipes* cement
1M		
2M		
3M		
4M		
5M		
2P		
3P		
4P		
5P		

Figure 2: Measured relative weights (%, abscissa) of *Pollicipes pollicipes* and *Capitulum mitella* cement beads showing significant weight loss with time (days, ordinate) in the presence of bacterial isolates.

presence of one of the two bacterial isolates that expressed no evidence of agar diffusible protease (1P and 3P, Table 2) exhibited a significant weight reduction with time (3P, Table 2).

Figure 2 shows the relative weight (%) plotted against time for each cement sample showing significant weight loss (Table 2) in the presence of a bacterial isolate. A pattern comparable to that found for asterile *P. pollicipes* cement (Fig. 1) was evident in all cases, with highly variable weight change until around 28 days into the experiment. For this reason relative cement weights (%) from day 28 were used in an analysis of variance of relative cement weights since the reduction appeared to be consistently linear in all cases after this period (Fig. 2).

Table 3 shows the result of an analysis of variance of relative cement weight (%) with time (covariate) for each bacterial isolate, including sterile cement for comparison. Significant differences in the rate of weight loss between isolate treatments was indicated ($F_{[14,103]} = 14.27$, $p < 0.001$, Table 3). Only 4 of 14 cement samples in the presence of a bacterial isolate reduced in relative weight at a significantly higher than the average rate of 0.18 % d^{-1} (Table 3) whilst 6 isolate treatments showed a significantly lower than average rate of weight reduction (Table 3). Four isolate treatments, together with sterile cement, were not significantly different to the average rate (Table 3). No consistent species to species pattern in weight reduction was evident, hence differences appear random.

Should the significant differences between the rates of relative weight loss (Table 3) have been attributable to initial weight alone (and hence relative surface area) one might expect cement samples having a significantly higher rate than the mean rate (Table 3) to be those with a greater initial weight and so on. Figure 3 shows the actual weight loss

Source	DF	SS (seq.)	SS (adj.)	MS (adj.)	F	p
Time	1	5112.09	5059.17	5059.17	453.28	<0.001
Isolate	14	5020.98	1131.67	80.83	7.24	<0.001
Time x Isolate	14	2229.12	2229.12	159.22	14.27	<0.001
Error	103	1149.61	1149.61	11.16		
Total	132	13511.80				

Average rate (% d^{-1}) = -0.179 ±0.01 (±SE)

Differences, with 95% confidence interval (dotted line), between estimated rates and the average rate for all 15 samples.

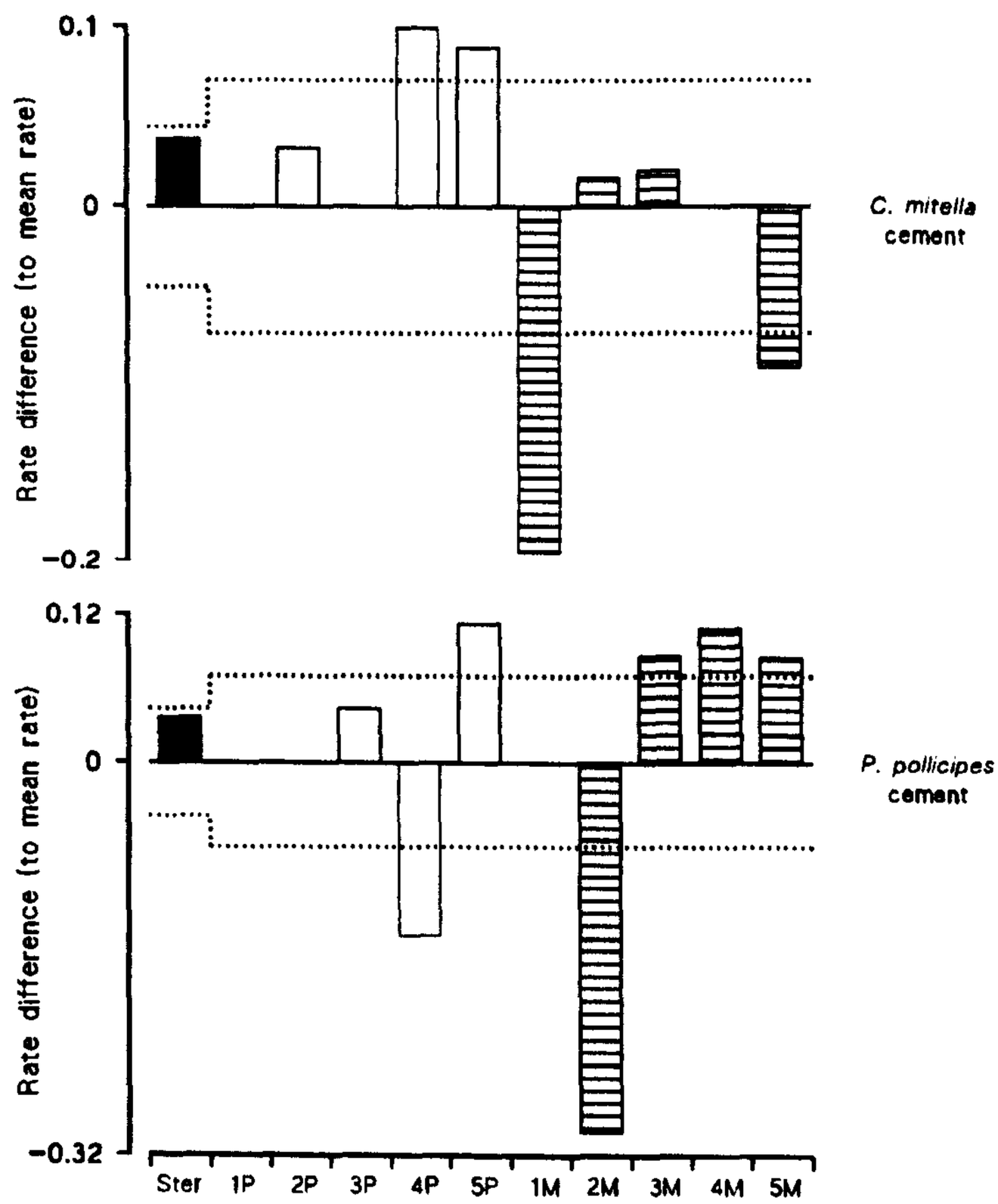


Table 3: Analysis of variance of relative weight (% initial weight) and time (days; covariate) for sterile lepadomorph cement and in the presence of bacterial isolates (seq = sequential, adj = adjusted for entry order).

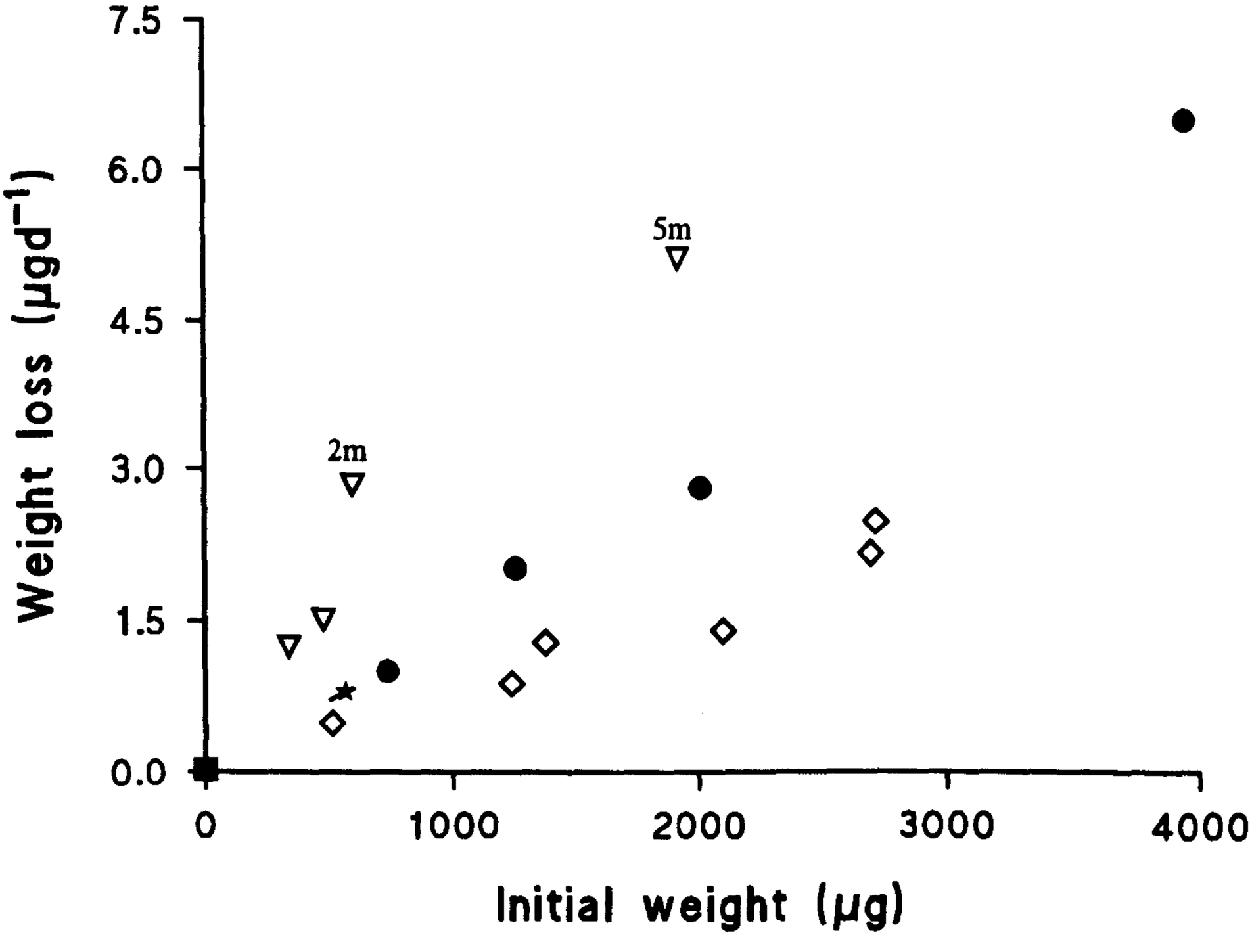


Figure 3: Estimated weight reduction (µg d^{-1}) with initial average bead weight within sample for *Pollicipes pollicipes* and *Capitulum mitella* sterile cement (star), asterile cement in flowing seawater (squares), cement with bacterial isolates showing significantly lower weight reduction rate than average (diamonds), cement with bacterial isolates showing significantly higher rate than average (triangles) and cement with bacterial isolates showing average rate of weight loss (circles).

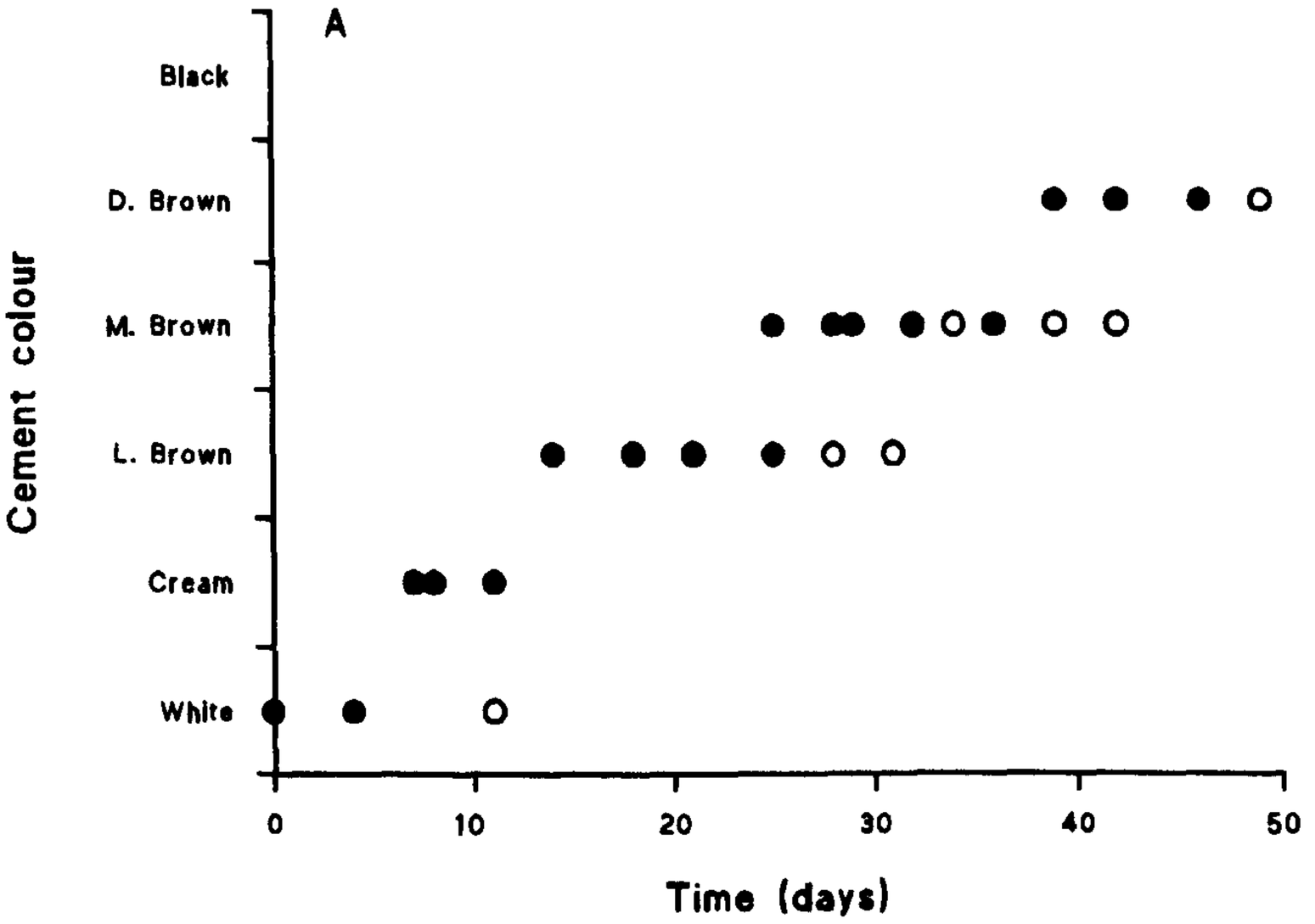
per day (in µg, estimated from the relative weight loss and initial weight) plotted against initial weight for all the cement samples employed. There was indeed a clear increase in weight loss with increasing initial weight (r 0.763, df 15, p <0.001, Fig. 3). However, Figure 3 shows 3 distinct groupings for the results over roughly the same range of initial sizes (excepting one large bead of 4 mg). Samples with significantly higher than average weight loss (triangles, Fig. 3) are elevated on the abscissa, those with average weight

loss(solid symbols, Fig 3) in the middle and those with significantly lower than average weight loss (diamonds, Fig. 3) lowered. Thus, superimposed on the expected SA:V association, there is a clear trend which can only be attributed to the action of the bacteria and perhaps cement bead shape.

Protein was concentrated from bacterial isolates 2M and 5M, chosen because cement beads in the presence of these isolates reduced in weight at a significantly higher rate than average (Table 3 and Fig. 3). Proteolytic enzyme activity was evident in the protein concentrate from both bacterial isolates at 0.03 ±0.01 (±SE, n = 3) activity units (as defined by Sarath *et al.* 1989) for that concentrated from bacterial isolate 5M and 0.06 ±0.01 (±SE, n = 3) for that concentrated from bacterial isolate 2M. Concentrated protein from both bacterial isolates was incubated with a bead of cement from *P. pollicipes* and one from *C. mitella* for 35 days. Although all cement beads were similarly sized (initial weights 228-327 µg), a weight reduction was measured for *P. pollicipes* cement only in the presence of protein concentrated from bacterial isolate 5M. Moreover, with an initial weight of 281 µg and weight reduction of 9 µg after 35 days, the estimated rate of weight reduction (0.26 µg d^{-1}) was less than that predicted for an equivalent weight of cement in sterile seawater at 0.4 µg d^{-1}.

Even though no consistent effect of protease could thus be quantified, the cement samples did change in appearance, from an initial bright white colour to black after the 35 days.

A change in appearance of cement samples with time was common to all cement samples. Figure 4A shows the observed colour of asterile cement samples plotted on an arbitrary scale against time. A similar pattern of darkening with time was evident for both



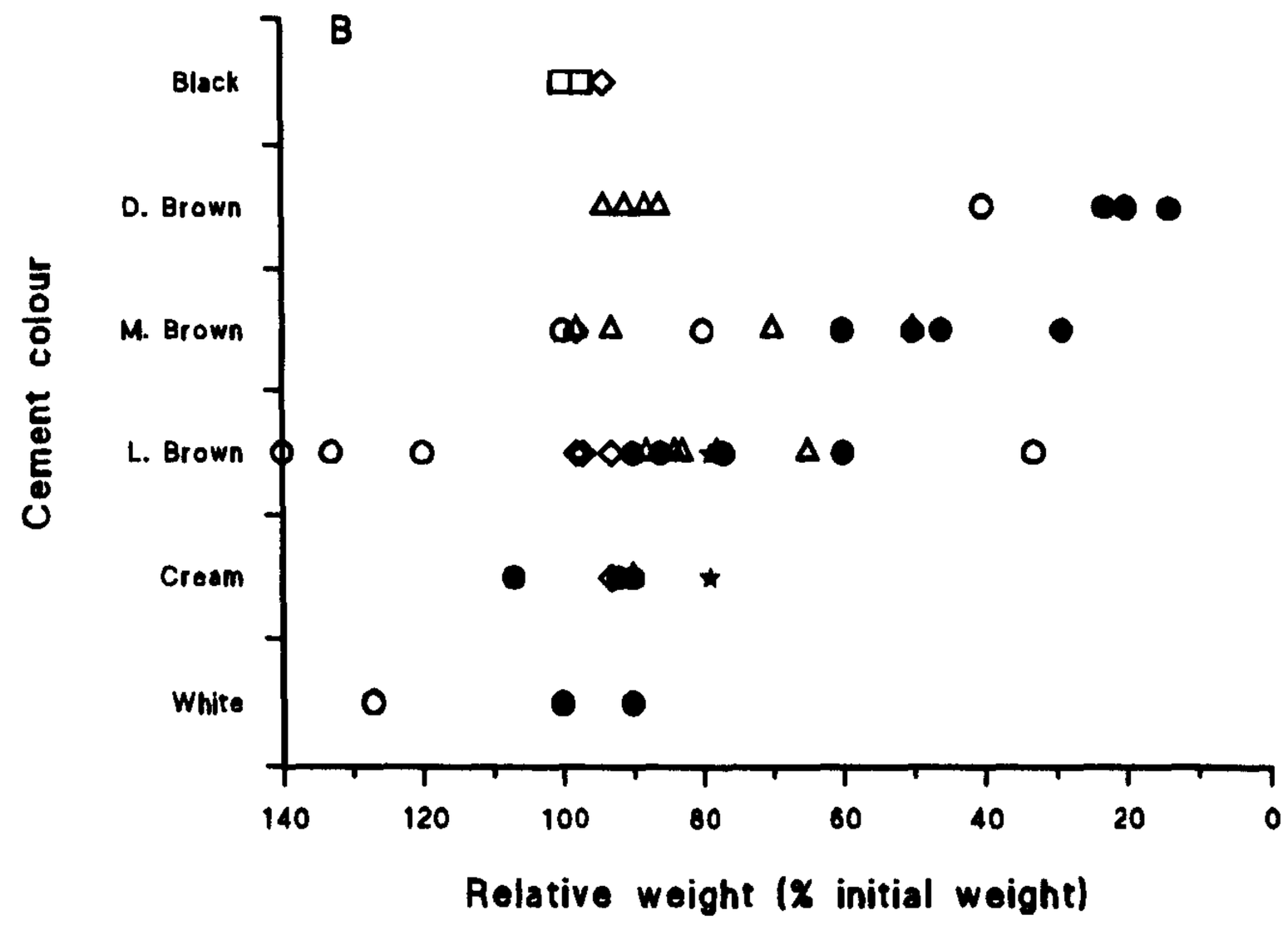


Figure 4: A, a trend of progressive darkening with time of immersion (days) in flowing seawater for *Pollicipes pollicipes* cement samples (filled circles = samples in recently (up to 1 month) cleaned tank, open circles = samples in tank cleaned 3 months previously). B, colour of cement samples with relative weight. A trend is maintained for cement in relatively clean tank (solid circles) and for sterile cement (stars), but not for asterile cement in dirty tank (open circles) or for *Capitulum mitella* and *Pollicipes pollicipes* cement in the presence of bacterial isolates whether with significant weight reduction (triangles) or not (diamonds), or for cement subjected to concentrated bacterial protease (squares).

asterile cement that consistently lost weight (solid circles, Fig. 4A) and for samples that apparently increased in weight initially (open circles, Fig. 4A).

Figure 4B shows the progressive colour of all cement samples in relation to their associated relative weights (%). A positive trend of darkening colour with decreasing weight was evident for all asterile samples showing consistent weight reduction with time (solid circles, Fig 4B). The colour of sterile cement samples (solid stars, Fig 4B) at 79 % of their initial weight also complied with the trend but asterile samples exhibiting a weight increase initially (open circles, Fig. 4B) did not. Departure from the trend of darkening with reducing relative weight therefore indicates changes in weight measurement resulted from causes other than loss of cement.

The colour of cement in the presence of a bacterial isolate was noted only when they were last weighed. Like asterile cement samples with obvious initial (but almost certainly decreasing, see Fig. 1) overestimates of weight, cement in the presence of bacterial isolates did not generally fit the trend of darkening with reducing relative weight, whether they exhibited significant weight reduction (open triangles, Fig 4B) or not (open diamonds, Fig 4B). It is likely then that weight measurements for cement with bacterial isolates were also overestimates of their true weight, most probably due to the formation of a surface biofilm contamination.

All cement samples incubated with concentrated bacterial protease (open squares, Fig 4B) and a cement bead in the presence of one bacterial isolate (*P. pollicipes* cement with 2P), turned black. Such a colour change, at apparent weights in excess of 94 % (Fig. 4B) of initial weight, could not be explained beyond speculation that would serve little

purpose. However, none of these samples lost weight at a significantly greater rate than that for sterile cement.

DISCUSSION

The cements of *Pollicipes pollicipes* and *Capitulum mitella* are clearly very stable materials. Sterile cement from both species had similar, low, solubility in seawater which concurs with all other reports for cirripede cement's resistance to dissolution in various solvents (e.g. Lindner and Dooley, 1973, Barnes and Blackstock, 1976 and Yan and Pan, 1981).

Asterile cement samples from *P. pollicipes* in flowing seawater lost weight at a significantly higher rate than sterile cement to the extent that they disintegrated after 50 days. Such a difference, however, could be explained solely by the relative surface area to volume ratios (sizes) of the beads employed. Furthermore, evidence of decreasing overestimates of cement weight with time due to biofilm contamination would have resulted in high estimates of relative weight reduction as a dynamic balance was achieved between cement dissolution, biofilm accumulation and biofilm loss. Differences in rates were, thus, not great enough to suggest measurable cement degradation by microorganisms.

Reduction in weight for *P. pollicipes* and *C. mitella* cement beads in the presence of a bacterial isolate generally followed the same pattern as that for cement beads in flowing seawater, with consistent weight reduction after a similar period of time, again indicative of a dynamic biofilm formation (see Cooksey and Wigglesworth-Cooksey, 1995). Only 14 of the 20 cement samples showed significant weight loss with time. It is most likely that the failure to measure a weight reduction in the other six samples was due to biofilm contamination on the surface of the beads, either equalling any weight reduction of the cement or even reducing or preventing dissolution. Continuous rotation of the relatively

small volume sample containers may not have had the same effect on the biofilm as flowing seawater (i.e. dislodgement), and organisms capable of grazing the biofilm were excluded from the experiment.

Groups of cement samples with bacterial isolates exhibited trends which were independent of initial sample mass (Fig. 3). However the action of the isolates across species and within isolates was so inconsistent in magnitude and direction of effect (Tables 2 and 3) that the relatively high loss rate of one group of samples (triangles, Fig. 3) could not be confidently attributed to the action of the associated bacterial isolate. Furthermore, the lack of any significant effect of isolated protease from two of the bacterial isolates in question point to variability in microtopography and shape as a major factor causing increasing variability in weight loss with increasing initial weight.

In at least one case, the presumed combination of biofilm development followed by a reduction in biofilm mass appears to have caused overestimation of cement dissolution rates (squares, Fig. 1.). The colour change of the cement beads lends support to this interpretation. Cement masses from *P. pollicipes* and *C. mitella* are porous containing almost discrete, small, volumes of water which impart an opaque white appearance to the cement under incident light through reflection/dispersion, whilst the cement is translucent brown when viewed with transmitted light (see Chapter 5). As cement dissolves increasing space within the cement, a change in light reflection/dispersion and hence observed colour would thereby give an indication of the relative weight. A positive relationship between darkening colour and time of immersion and relative weight was evident for asterile cement samples showing no initial weight gain (solid circles, Fig. 4A,B). Asterile cement samples showing obvious initial weight gain, although following the trend with time (open circles, Fig. 4A), did not follow the trend with relative weight

until they showed evidence of weight loss (open circles, Fig. 4B). Cement beads in the presence of bacterial isolates were generally darker than would have been expected from their relative weight when last measured (diamonds and triangles, Fig. 4B) indicating that cement had probably been lost but the mass of the accumulated biofilm obscured its measurement. Consequently, there was no tangible evidence that *P. pollicipes* cement in flowing seawater or *P. pollicipes* and *C. mitella* cement in the presence of bacteria isolated from the cements was subject to any action that caused a weight reduction significantly greater than that measured for sterile cement from both species.

Considering the very low rates of lepadomorph cement dissolution in seawater (0.14 ±0.02 % d^{-1} (±95 % CI)), and the great compositional similarity between balanomorph and lepadomorph cement (e.g. see Chapter 5 and references therein) it is not surprising that Lindner and Dooley (1973) reported the adult cement of *Balanus crenatus* to be resistant to dissolution in salt solution and could note only an 'extremely slight swelling' of cypris permanent cement after one week in 0.2 N NaOH. Both Lindner and Dooley (1973) and Naldrett (1993) suggest that hydrogen or salt bonds (electrostatic) are too weak to be involved in the polymerisation or solidification of balanomorph cement because of its resistance to dissolution in salt solutions. Evidence here indicates that dissolution of lepadomorph cement does occur in seawater so the total rejection of hydrogen and/or electrostatic bonding by Lindner and Dooley (1973) and Naldrett (1993) may be invalid. Barnes and Blackstock (1976), Yan and Pan (1981) and Naldrett (1993) have indicated that the hydrophobicity of protein moieties may be an important factor in the solidification of balanomorph cement. Naldrett (1993) noted that such interactions between proteins are enough to make a matrix resistant to marine bacteria (and dissolution) by preventing water from entering. The present results lend support to

the hypothesis by confirming resistance to marine bacteria collected from the cement and showing that dissolution is very slow.

The dissolution of lepadomorph cement in seawater has obvious implications for its effectiveness as an underwater adhesive. The cement masses used here were in contact with the (often moving) medium over their whole surface area, yet dissolution rates were still very low. Although lepadomorph barnacles may be found with large cement masses around the peduncle base (e.g. *Lepas*, Darwin, 1851) adhesion of *P. pollicipes* and *C. mitella*, like that of balanomorphs, is attained by a thin layer of adhesive between the base and substratum. As has been noted by Walker (1971) and Naldrett (1993), cement is zoned and the interface with water is less porous (see Chapter 5). The area across which dissolution could take place is thus very small relative to the volume of cement present. Barnacles also moult quite regularly, laying down new adhesive material as they grow (Fyhn and Costlow, 1976, Yule and Walker, 1987) hence any small loss through dissolution will readily be made up during the next moult cycle.

CHAPTER 5

The solidification of scalpellid lepadomorph cement

ABSTRACT

Measurement of the carbon and nitrogen content of *Pollicipes pollicipes* and *Capitulum mitella* cements indicated that both species cements, like those of other cirripedes, are predominantly protein. The cement of *P. Pollicipes* was shown to be delivered as a clear liquid in nl quantities, curing in around 2 hours, as has been previously demonstrated for balanomorph adult and cypris permanent cements. The zonation of lepadomorph cement here has been shown to be due to variable space within the cement, a result of differential degrees of curing. Such partially cured cement was porous, able to absorb its own weight in water. The porosity of cured cement allows for further deliveries of cement from the same pore. Further curing of the partially cured zone of cement masses could be induced by heating to dryness at temperatures in excess of 70 °C from a minimal volume of water. Such treatment resulted in an homogeneous appearance (no zonation) of the cement and reduced the space within the cement by 39-45 %, but the cement remained porous. It was determined that the presence of water alone was essential for curing but has an inhibitory effect resulting in the characteristic zonation. Cement delivered and cured in air or nitrogen had no such zonation. Histochemical treatment of cement prior to heat treatment indicated that disulphide bonds and tyrosine-OH groups play no part in the solidification process after delivery supporting growing evidence that hydrophobic complexing is central to the solidification process.

INTRODUCTION

Research into barnacle cement has had two main industrially based goals. One is to understand the properties of the adhesive such that protective systems for plant in the marine environment may be developed to reduce or even prevent fouling (Saroyan *et al.*, 1970a). Harris (1946) considered barnacles the most important fouling organisms since they are resistant (once settled) to toxins used in antifouling coating systems by virtue of their cirri being some distance from the substratum, avoiding lethal concentrations of toxin, and with a shell that persists after death increasing drag and providing a non-toxic substratum for more fouling. The cost of barnacle fouling has been estimated at around 200 million pounds a year (Christie and Dalley, 1987). Of lepadomorph barnacles, only *Conchoderma* and *Lepas* species are considered fouling organisms. The other major reason for interest in barnacle cement is to elucidate the chemical properties that bestow the cement with the ability to be applied and to set underwater, with sufficient strength and stability to resist failure in a harsh environment. No synthetic adhesive has yet been developed that can be used in such a manner (Naldrett, 1993), although many epoxy resin glues have much greater durability (see chapter 4) and strength than barnacle cement (Yule and Walker, 1984b). The marine environment has similarities with the biomedical environment; wet, salty and biochemically active (Baier, 1982). The similarity has led to suggestions for barnacle glue as a potential dental restorative adhesive (Carderelli, 1968) and the development of homologues designed for use in dentistry and surgery (Crisp, 1973).

Research into the biochemical composition of barnacle cement has been chiefly restricted to the cement of adult balanomorphs. However, Barnes and Blackstock (1974)

found the biochemical composition of the cement of the lepadomorph *Dosima fascicularis* adult to be similar to that of balanomorph barnacles. Analysis of the cement of *Lepas anatifera* by Walker and Youngson (1975) confirmed the similarity of lepadomorph and balanomorph adult cements. Indeed, Yule and Walker (1987) further concluded that balanomorphs and lepadomorphs employed a common adhesion mechanism since balanomorph and lepadomorph cements are biochemically similar.

Although much research into barnacle cement has been restricted to that of the adults, Saroyan *et al.* (1970a) reported no significant differences, based on staining with various dyes, between cypris permanent cement (that used for fixation) and what they termed primary (exuded under normal conditions) and secondary (used for repair and reattachment) adult cement. Furthermore, Yan *et al.* (1983) used electrophoretic techniques to demonstrate little difference in the composition of so called primary and secondary cements of *Balanus reticulatus* Utinomi, 1967. Lacombe's (1967), histochemical study of the cement apparatus of *B. tintinnabulum* concluded that the unicellular gland cells secreted an acid mucopolysaccharide adhesive but Walker (1970) found that the conclusion was erroneously based on a histochemical reaction with the ribose of ribonucleic acid.

It is widely accepted that cirripede cement is delivered as a liquid which cures through protein polymerisation or cross-linking to a rubbery mass. However, after research spanning almost 30 years, the nature of polymerisation remains elusive. Various curing or solidification methods have been suggested based on biochemical and histochemical analyses of cured cement and histochemical studies of the gland cells that secrete the cement. Determination of amino acid profiles, lipid, carbohydrate and ash content of barnacle cement has been inconclusive in providing evidence for the curing method.

Cook (1970) reported a similarity between his analysis of *B. crenatus* cement and that of lobster fibrinogen, presumably suggesting a similar protein structure and polymerisation process. Harris (1946) had suggested that settling cyprids secrete '... a protein cement which hardens by a tanning process...'. Shimony and Nigrelli (1972), finding arylsulphatases (ASases) in mantle homogenate of *B. eburneus*, speculated that the enzyme functioned in the phenolic tanning of cement by hydrolysing phenolic sulphuric esters, providing phenolic groups capable of cross-linking proteins. No evidence of ASases in tissue associated with the cement apparatus (see Walker, 1981) was, however, presented. Walker (1981) speculated that chemical dehydration, involving quinone selectively occupying strongly hydrated groups (see Vincent and Hillerton, 1979), could be a mechanism of cross-linking the adhesive proteins which would work underwater, a clear necessity for all marine bioadhesives. Since the general unreactivity of the cured cement made traditional biochemical analysis difficult, some workers employed histochemical techniques on both the cured cement and the glands producing the pre-polymerised adhesive. Combining histochemistry with solubility and composition analyses, Lindner and Dooley (1973) concluded that the cement of adult *B. crenatus* cured largely through a quinone type cross-link. When no phenolic precursors could be found, Lindner and Dooley (1973) speculated that a phenolase could oxidise phenolic groups of itself resulting in, so called, autotanning.

Walker (1970, 1971) demonstrated that the cement gland cells of *Elminius modestus*, *Semibalanus balanoides* and *Chirona hameri* adults secrete protein and contain a greater concentration of -SS groups over -SH groups, finding a greater degree of staining for -SH, using the DDD method, after reduction of -SS groups with thioglycollic acid. Cheung and Nigrelli (1972) also found evidence of -SH groups and -SS linkages in the

cement gland cells of *B. eburneus* cyprids and juveniles. From the results of histochemical reactions they concluded that both the cyprid and juvenile cements were collagenous, but the juvenile cement contained tyrosine whereas the cypris cement did not. Lindner and Dooley (1973) ruled out disulphide bonding polymerisation for the cement of *B. crenatus* because they could not dissolve cement in thioglycollate. Like Walker (1970), Fyhn and Costlow (1976) also found evidence of protein bound -SH and -SS groups but noted that they were concentrated at the secretory region of *B. amphitrite* cement gland cells, again with a more positive reaction for -SS groups. Results were negative for both -SH and -SS groups at the synthesis region of gland cells. Although cytological differences in gland cells have been reported (Lacombe and Ligouri, 1969, Walker, 1970) between cirripede species, there is no evidence that such extends to the exuded cements.

More recently advances in techniques for protein analysis have enabled Naldrett (1993) to conclude that the cement of *B. crenatus* is cured by hydrophobic interactions between protein moieties. He further indicated that disulphide bonds were made prior to the delivery of the liquid cement and played little or no part in the exogenous solidification. Naldrett (1993) found no evidence for quinones using NMR and could dissolve the cement in SDS buffers hence he concurred with Barnes and Blackstock (1976) and Yan and Pan (1981) in ruling out phenolic tanning from involvement in the curing process.

Although lepadomorph and balanomorph barnacle cements appear to be similar little use has been made of the fact that lepadomorph cement, which may be found encircling the base of the peduncle, can be easily collected in large quantities (Barnes and Blackstock, 1974, 1976, Walker and Youngson, 1975). The current work presents observations of the cement of the intertidal scalpellids *Pollicipes pollicipes* and

Capitulum mitella leading to a novel method employed to investigate the solidification process, made possible by virtue of the larger quantities of cement that can be collected from such barnacles in comparison to the much smaller amounts collected from balanomorph barnacles.

METHODS

Pollicipes pollicipes and *Capitulum mitella* adults were maintained in flowing seawater tanks as described in chapter 3. Recently cured cement beads were collected from the adults, cleaned and stored in a desiccator at room temperature, also as described in chapter 4.

Delivery and curing of cement

To estimate the exudation rate and curing time of *P. pollicipes* cement a binocular microscope, colour video camera (JVC TK1085E) and time lapse recorder (Panasonic AG-6010) were employed, enabling reviewing at 16-24 times real time. To facilitate recording cement delivery into seawater, air, nitrogen (oxygen free) and distilled water, animals were maintained base upward with their capitulum and most of their peduncle immersed in seawater. Initially, animals were fixed in position over a hole in the base of a petri dish with elastic plastic padding, following the methods for balanomorph barnacles (Walker, 1972 and Cheung *et al.*, 1977). However, muscular activity of the peduncle often caused failure of the seal around the peduncle allowing fluid leakage and/or ingress. Therefore, silicon discs (Silform N°. 1; Stag Polymers and Sealants Ltd.), *ca.* 6 mm thick, with a hole in the centre were moulded in Petri dish lids. Candidate animals were chosen with peduncle diameters slightly larger than the hole in the disc. Figure 1 shows the apparatus developed. The smooth, elastic, silicon rubber disc formed a watertight seal around the peduncle and was supported by the petri dish base which formed a vessel facilitating immersion of the peduncle base in fluid whilst the capitulum remained immersed in seawater in the glass jar below. To assess curing of cement in an

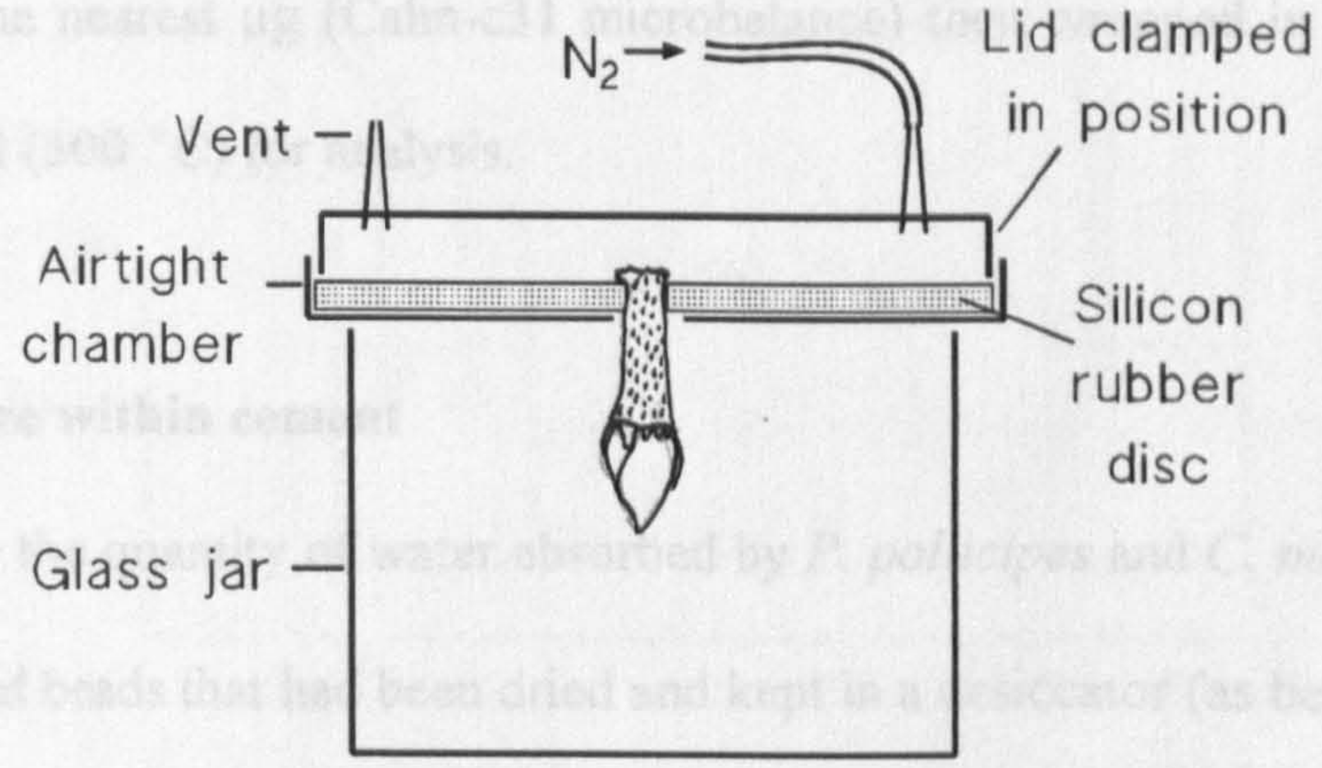


Figure 1: Apparatus developed to observe exudation and curing of *Pollicipes pollicipes* cement. Cement delivery and solidification, without contamination, could be observed in various fluids whilst the capitulum and peduncle were maintained in seawater.

oxygen free environment, the silicon disc was placed in a holed Petri dish lid. A chamber was formed by the addition of a clamped Petri dish base with a vent and gas delivery tube (see Figure 1). The combination of the silicon rubber seal and a N_2 supply at slightly greater than atmospheric pressure ensured no ingress of fluid into the chamber.

Carbon and nitrogen content of cement

A gas chromatograph, mass spectrophotometer auto-analyzer (Roboprep CN, Europa Scientific) was used to estimate the carbon and nitrogen content of cured *Pollicipes pollicipes* and *Capitulum mitella* cements. The analyzer was calibrated for carbon and nitrogen, using acetanilide ($CH_3CONHC_6H_5$), over a range of element content based on the expected CN content for the weight range of cement to be analyzed assuming a similar content to reported results for other cirripede species' cement. A number of dry stored cement beads (see above), over the size range available from each species, were

weighed to the nearest µg (Cahn-c31 microbalance) then wrapped in precombusted aluminium foil (500 °C) for analysis.

Variable space within cement

To estimate the quantity of water absorbed by *P. pollicipes* and *C. mitella* cements, uncontaminated beads that had been dried and kept in a desiccator (as before) for up to twelve months were used. Cement beads were again selected over the available size range and individually weighed on a Cahn c-31 microbalance to 1 µg then immediately rehydrated in distilled water for two hours. Following rehydration, wet weights for each cement bead were determined after blotting dry to remove surface water.

To determine the effect of heat on the water retention of the cement, stored samples were again selected, weighed, rehydrated and placed in *ca.* 50 µl of distilled water. The distilled water was evaporated and the cement fully dried out at 90 °C in a water bath before reweighing. Distilled water was used since initial results using seawater were inconsistent, which was interpreted as due to crystallisation of salts on evaporation.

A similar procedure using freshly collected cement beads, already hydrated and washed thoroughly in distilled water, was employed with a variety of solvents and a range of evaporating temperatures. Weights for these cement beads were not determined. Figure 2 shows the apparatus employed for evaporating off small volumes of fluid from cement samples. With most solvents an open system was employed with a simultaneously run control using distilled water. The partially sealed form of the equipment (Fig. 2) was only used for the deoxygenated water solvent. Nitrogen was continuously passed through the deoxygenated water chamber (at +0.2 bar) whilst air was likewise passed through the simultaneously run control chamber.

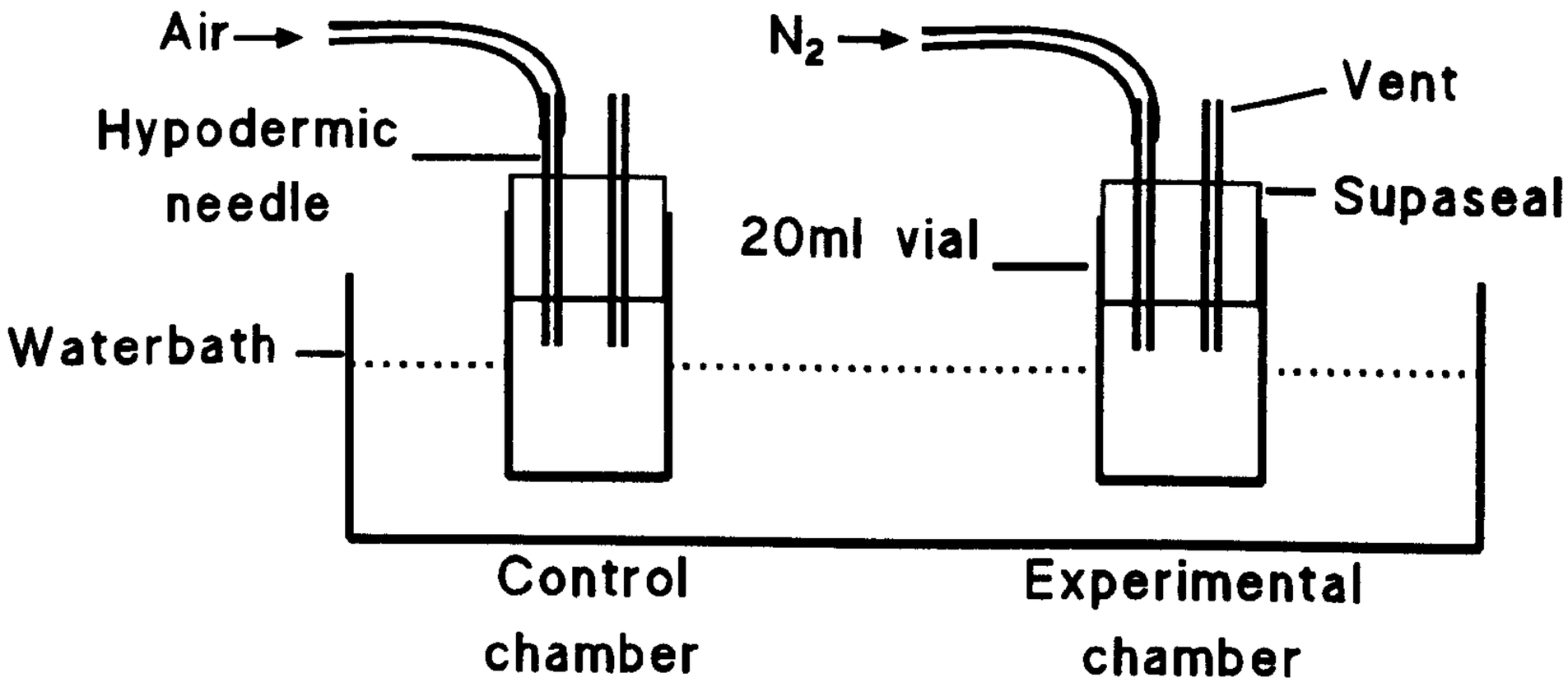


Figure 2: Apparatus employed to evaporate liquids from lepadomorph cement at various temperatures. The semi sealed control chamber was only required when deoxygenated water was used as the solvent.

Initially the strong oxidising agent sodium dithionite was used to deoxygenate water but crystallisation of salt on evaporation made it impossible to determine any effect of using such treated water on the cement. Deoxygenation of water was later accomplished using a similar method to that described by Carpenter (1965). Cement samples were placed in a semi sealed vial together with *ca.* 10 ml distilled water (see Fig. 2). The water was boiled under (oxygen free) nitrogen (+0.2 bar) for 20 minutes after which nitrogen was passed through the water for twelve minutes by pushing the hypodermic needle below the water level. Alternatively, nitrogen was passed through the water overnight with no previous boiling. Excess water was ejected, immediately before the drying process, by inverting the chamber whilst nitrogen flow was maintained.

Histochemical treatment of cement

Histochemical treatments of *P. pollicipes* and *C. mitella* cement were used either as stains to identify components or as blocking agents to prevent specific bonding reactions. Hand cut razor cut sections (300-500 μm thick) of cement beads were most often used. Where simultaneous control sections of material known to give a positive result were required (to validate the stain), cryostat sections (5-20 μm thick) were sometimes employed to improve the repeatability of the treatment. The following techniques were employed:

Bromphenol blue stain for protein. Hand cut and cryostat sections of cement were dehydrated through a series of ethanol solutions then stained for 15 minutes in an ethanolic (95 %) solution of bromphenol blue (1 mg ml^{-1}). Sections were then washed in 0.5 % acetic acid for 20 minutes, rinsed in tap water and chemically dehydrated through a series of ethanol solutions. Sections were finally mounted in DPX. Mouse skin sections were used to validate the stain.

Modified alkaline tetrazolium reaction to stain -SS and -SH groups. The method followed Pearse (1985). Cryostat sections (7-20 μm thick) of mouse skin and cement were serially dehydrated to absolute ethanol and covered with 2 % Colloidin in amyl acetate. When dry, sections were treated with 0.5 M thioglycollic acid (adjusted to pH 8 with 0.1 N NaOH), to reduce -SS groups to -SH, for 3 hours at 50 °C in a covered coplin jar. After rinsing in tap water, 1 % aqueous acetic acid and tap water again, sections were treated with freshly prepared nitro-BT reagent (Pearse, 1985) for 60 minutes at 37 °C. Subsequent washing of sections in 1 % aqueous acetic acid and rinsing in tap water preceded dehydration through alcohols, clearing in Histoclear and mounting in DPX.

Nitroprusside reaction for staining -SH groups. The method followed Hammet and Chapman (1938) and Pearse (1985). Hand cut sections of cement and mouse skin were placed in a cavity block with a minimal quantity of distilled water to which 50 µl of 27-29 % NH_3OH and 50 µl of a 1 % sodium nitroprusside solution were added. A small quantity (*ca.* 0.2 g) of crystalline ammonium sulphate was then placed close to the sections. Several sections were reduced in fresh thioglycollic acid (pH 8, as above) for 4-5 hours at 37 °C prior to nitroprusside treatment. The thioglycollic acid was removed, after reduction, by thoroughly washing in tap water, 1 % aqueous acetic acid, and tap water again. Prolonged washing of sections in tap water for 45 minutes to remove thioglycollic acid was required to ensure no colour development before the addition of crystalline ammonium sulphate.

Iodine oxidation blocking of -SH groups. The method again followed that of Pearse (1985). Hand cut sections of cement were washed in distilled water and treated for 4 hours at room temperature in a solution of 9.5 mg iodine + 8.25 mg potassium iodide in 25 ml of distilled water, adjusted to pH 3.2 with 0.1 N HCL. The sections were then washed overnight in 5ml of distilled water. Treated sections, together with untreated sections, were placed in a minimal quantity of distilled water and heated to dryness at 86.4 °C .

Iodoacetic acid blocking of -SH groups. Hand cut sections of cement were incubated for 20 hours at 37 °C in 0.1 M iodoacetic acid adjusted to pH 8 with 0.1 N NaOH following Pearse (1985). The sections were then washed in 5 changes of distilled water. Treated sections together with untreated sections were again dried from a minimal quantity of distilled water as above.

Nitrosonaphthol stain for tyrosine. The method adopted was that of Sundler *et al.* (1976) and Pearse (1985). Several hand cut sections of cement were pretreated, by drying from a minimal quantity of water. Nitrosonaphthol reagent was prepared from equal volumes of 0.1 % aqueous ethanolic nitrosonaphthol and 10 % HNO_3 with a trace of $NaNO_2$. Sections were incubated in the reagent for 45 minutes at 60 °C, washed in 1-2-dichloroethane for 5 minutes then rinsed in 98 % ethanol. Sections were examined dry using an epifluorescent microscope with an excitatory maximum around 450 nm (UG1 filter) and an emission filter to observe fluorescence maxima around 570 nm. Fluorescence validation was determined using sections of mouse skin and pancreas treated as above and a small quantity of tyrosine salt (*ca.* 5 µl) on filter paper to which the reagent was added.

Tyrosyl-OH blocking. Hand cut cement sections were treated with 0.5 g *p*-nitrobenzoyl chloride in 10 ml 98 % pyridine for 4 hours following Pearse (1985). After washing in distilled water for 30 minutes treated and untreated sections were dried from a minimal quantity of distilled water, as above, at 86.6 °C.

Iodination blocking of tyrosine. The method again followed Pearse (1985). Hand sectioned cement was air dried then incubated for 72 hours at 25 °C in 0.78 N I_2 in absolute ethanol. After thorough washing in absolute ethanol sections were rehydrated in distilled water and then dried, at 86 °C, from a minimal quantity of distilled water.

RESULTS

Delivery and curing of cement

Time lapse observations of *Pollicipes pollicipes* cement pores from which cured cement had been recently removed proved difficult to interpret because the exudation of cement was not visible under seawater. The only evidence of delivered cement was the gradual appearance of the increasingly opaque white cement mass over the pore. There was no evidence of the opacity starting in distinct zones or regions of the cement mass, merely a steady increase in opacity throughout the cement mass generally. The gradual change from a presumed transparent fluid (Cheung and Nigrelli, 1972, Cheung *et al.*, 1977) with the same (inferred) refractive index as seawater to an opaque, white cement mass was interpreted as representing the curing (or solidification) of the cement. The quantity of cement exuded under seawater could only be estimated from the size of the fully cured cement mass, equating the cement volume to that of a hemisphere of the same diameter as the cement mass. Cement volumes observed from the video recordings were rather small, ranging from 23-64 nl (mean 48 ±6 (SE) nl, n = 7), although much larger, irregularly shaped, cement masses over a single pore have often been observed on other occasions. It took, on average, 117 ±15 (SD) minutes (range 102-137 minutes) from the first sign of cement opacity until the cement mass attained its characteristic, fully opaque white colouration with no further visible change.

Observations of peduncle bases in air or under flowing nitrogen gave no visible signs of solidification (i.e. no opacity), although the cement was readily seen to be exuded from the pores as a clear, apparently low viscosity, liquid in a matter of (5-20) minutes compared to the 2 hours required for developing full opacity under water. Figure 3

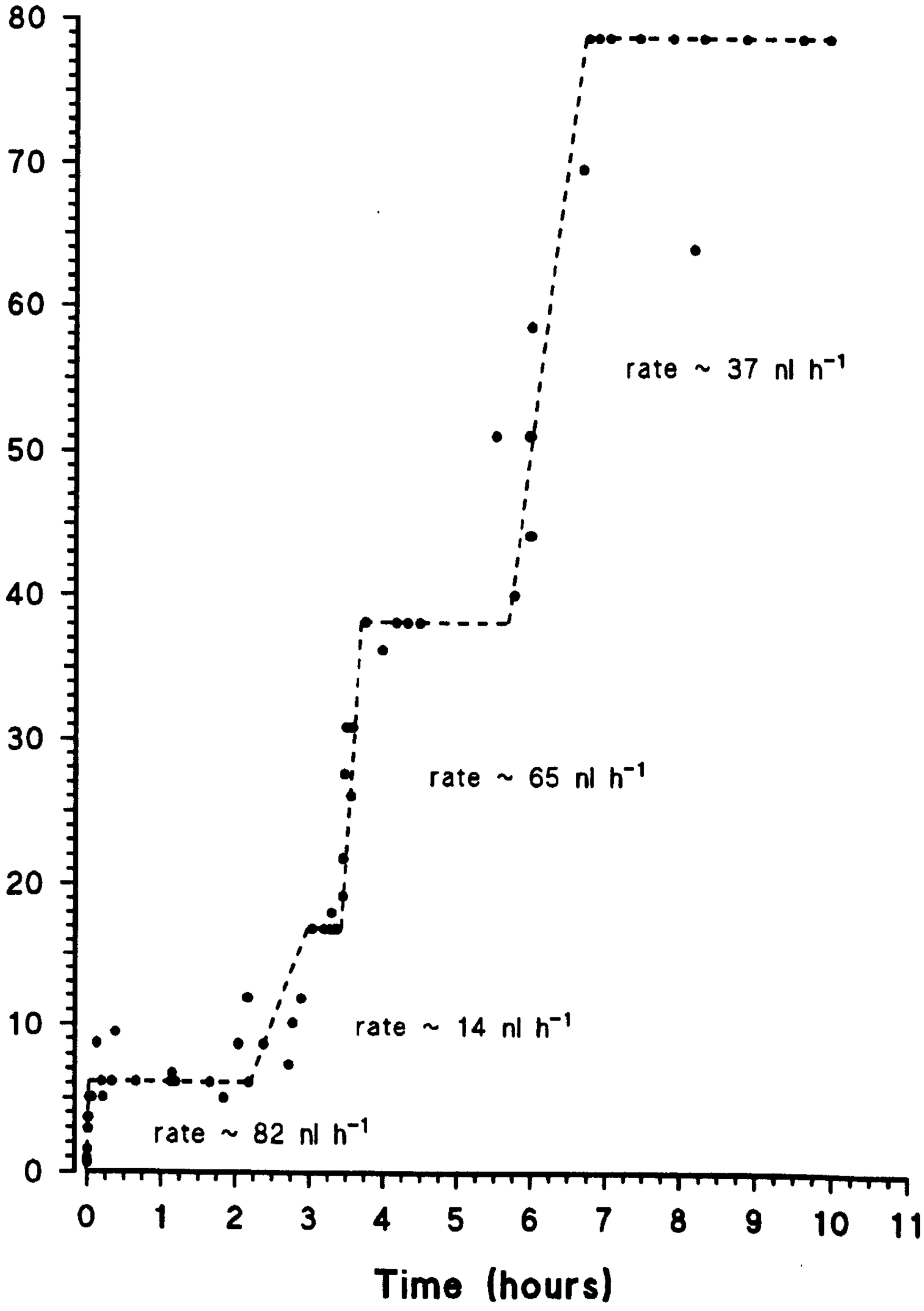


Figure 3: Estimated volume of liquid cement delivered, in air, from a single pore on the base of *Pollicipes pollicipes*. Successive deliveries from the same pore are evident from the step like pattern. The times between deliveries (*ca.* 2 h) are similar to the complete curing time of the cement (see text).

details the estimated volume of cement, exuded from a pore in air with time from the first appearance of liquid cement. Cement volume was again estimated from the diameter of the cement bead and the volume of an equivalent hemisphere. There is an obvious periodic addition of fluid forming a step pattern over the observation period (dotted line Fig. 3). The apparent increasing volume of successive cement deliveries was attributed to a greater spread (wetting of the moist basal integument) as the cement volume increased. Increased droplet spreading would result in overestimated cement volume as a result of the increased departure from the hemispherical shape used to approximate the volume of cement. The rate of exudation of the first delivery of cement was *ca.* 82 nl h^{-1}. Evaluation times for successive deliveries appear slower than the initial delivery, probably also due to continued (variable) spreading of the droplet after delivery time.

Figure 4 shows, in more detail, the change in estimated volume with time of a cement bead exuded under flowing nitrogen. The exudation rate (*ca.* 5 nl h^{-1}) is much smaller than that observed in air (Fig. 3), but the delivery time (*ca.* 20 mins., Fig. 3) is similar. The variability in volume, particularly around 10 and 20 minutes, was attributed to muscular action of the peduncle base moving the cement mass closer and away from the focal point of the camera. Movement of the base, indicating muscular activity in the peduncle, often occurred while the cement was exuded. The bases were, of course, not fixed firmly in position.

Although no evidence of curing (i.e. increasing opacity), of the cement was visible in air or N_2, the cement masses did become rubbery (i.e. non liquid) even though they remained fully translucent.

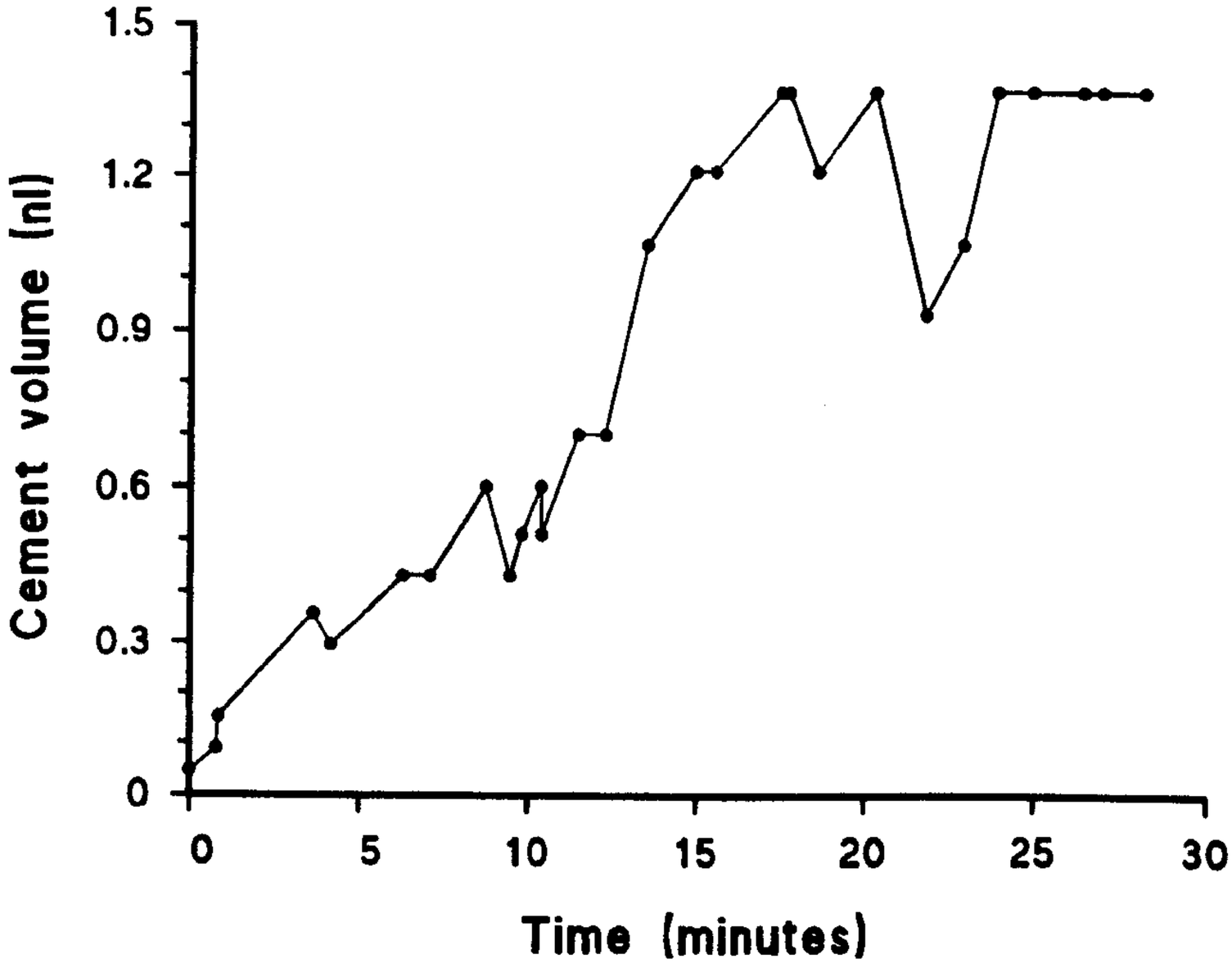


Figure 4: Estimated volume of liquid cement delivered, in flowing nitrogen, to the base of a *Pollicipes pollicipes*. The delivery time is short (20 mins.) compared to the curing time in seawater (*ca.* 2 h).

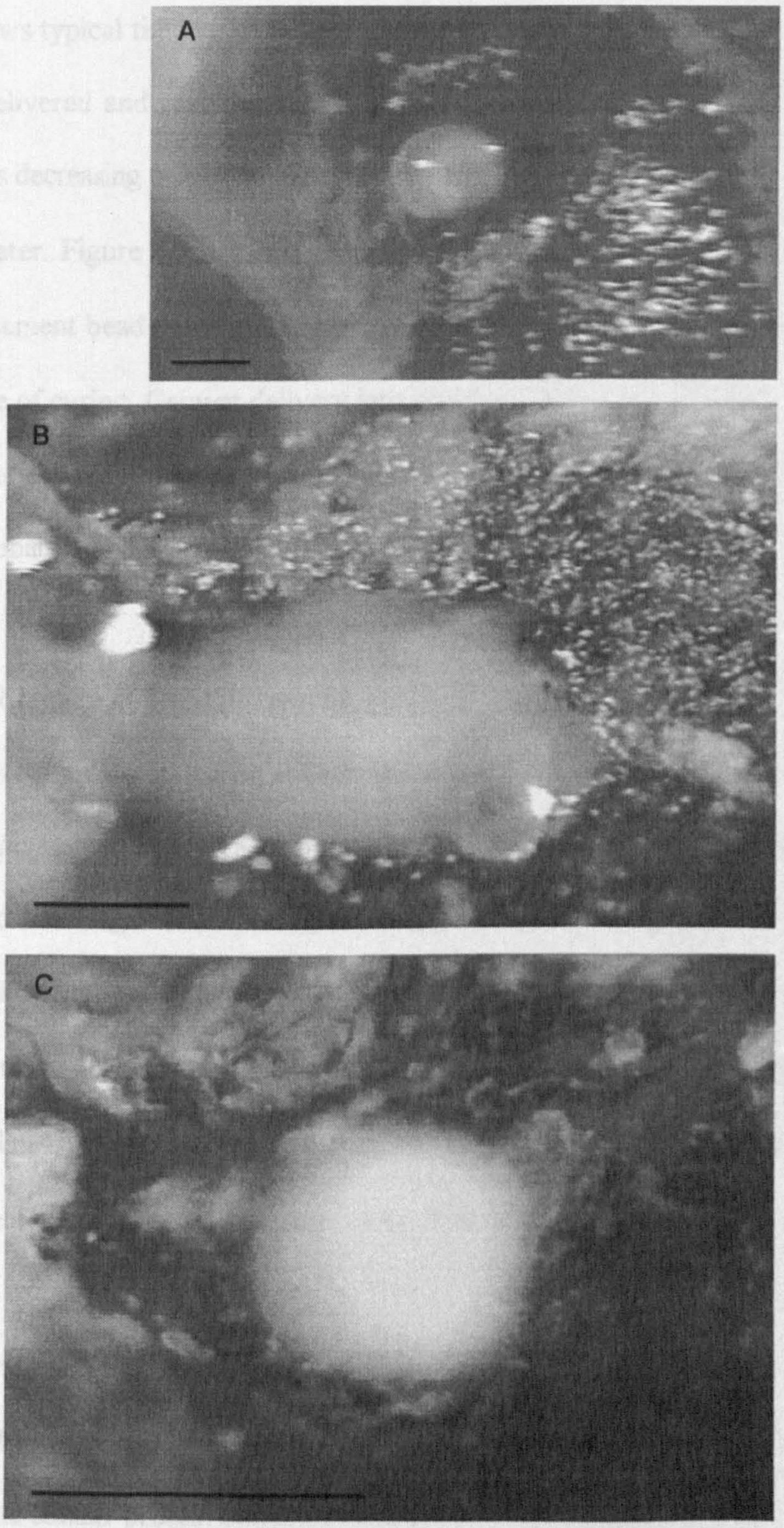


Figure 5: Typical time lapse recorded images of *Pollicipes pollicipes* cement. A, a fully translucent bead of cement delivered and cured under flowing nitrogen. Scale bar is 100 μm. B, a view of a milky bead of cement delivered and part cured for 1 h under seawater showing developing opacity throughout the mass. Scale bar is 1 mm. C. a zoned bead of cement delivered and cured in seawater. Scale bar is 500 μm.

Figure 5 shows typical time lapse images of cement beads. In Figure 5A the cement droplet was delivered and cured under N_2 and remains fully translucent. Figure 5C, however, shows decreasing opacity towards the perimeter in a cement bead delivered and cured in seawater. Figure 5B is somewhat intermediate, showing opacity developing throughout a cement bead delivered and partially cured in seawater and indicating no visible initial site of curing. Cement delivery into distilled water was only ever observed once, the droplet exuded (with a refractive index inferred to differ from that of distilled water) turned opaque and white on curing as for cement delivered in seawater.

Carbon and Nitrogen content of cement

Table 1 shows the measured carbon and nitrogen content for the cements of *Capitulum mitella* and *Pollicipes pollicipes*. The weight range of samples of *C. mitella* cement was 81-432 µg and the range for *P. pollicipes* samples was 32-164 µg. The cement dry weight for both species is highly correlated with measured element content (Table 1). Thus, the percentage dry weight of each element, for each species, was estimated using least squares linear regression of predicted element content with dry weight. Any predicted element content beyond the range of the calibration curve for the analyser was disregarded.

The carbon and nitrogen contents of both species cements were similar (Table 1) at 48-56 % and 17-21 % respectively. Lacking amino acid profiles, the nitrogen content of the cements indicate similar protein constituents of 100 % for *P. pollicipes* and *C. mitella* (using 6.25 as a conversion factor following Saroyan et al. 1970a, Walker, 1972 and Lindner and Dooley, 1973). The carbon content of *P. pollicipes* cement, is not

Species	element	r	df	p	%dry wt.
C. mitella	C	0.999	6	<0.001	53.0(±2.5)
	N	0.996	4	<0.001	19.1(±2.3)
P. pollicipes	C	0.999	5	<0.001	50.6(±2.5)
	N	0.999	5	<0.001	17.9(±0.9)

Table 1: Pearson's correlation coefficients (r, with degrees of freedom df) for measured carbon and nitrogen content of *Capitulum mitella* and *Pollicipes pollicipes* cements with dry weight and % dry weight content for each element estimated from least squares linear regression (with 95 % confidence interval).

significantly different to that of *C. mitella* (Table 1) and the C:N ratios for each species are also similar at 2.6-3.1 and 2.4-3.3 respectively. It is therefore unlikely that there are major differences in the composition of their cements.

Variable space within cement

Figure 6 shows the wet weight (µg) of *Capitulum mitella* and *Pollicipes pollicipes* cement with dry weight (µg) for samples naturally cured (in seawater) and after heat treatment. Table 2A shows Pearson's correlation coefficient (r) between wet and dry weights for treated and untreated samples of cement from both species. A significant relationship is indicated in all cases. The slope coefficient and constant for each relationship from least squares linear regression are also shown in Table 2A. Table 2B shows the result of an analysis of variance of wet weight with dry weight (covariate) for each relationship shown in Table 2A. A significant relationship between wet and dry weights ($F_{[1,68]} = 753$, $p < 0.001$) and a significant difference between at least 2 slope coefficients ($F_{[3,68]} = 26.67$, $p < 0.001$) are indicated. Table 2C details the result of a Tukey-Kramer procedure to determine the minimum significant difference (MSD)

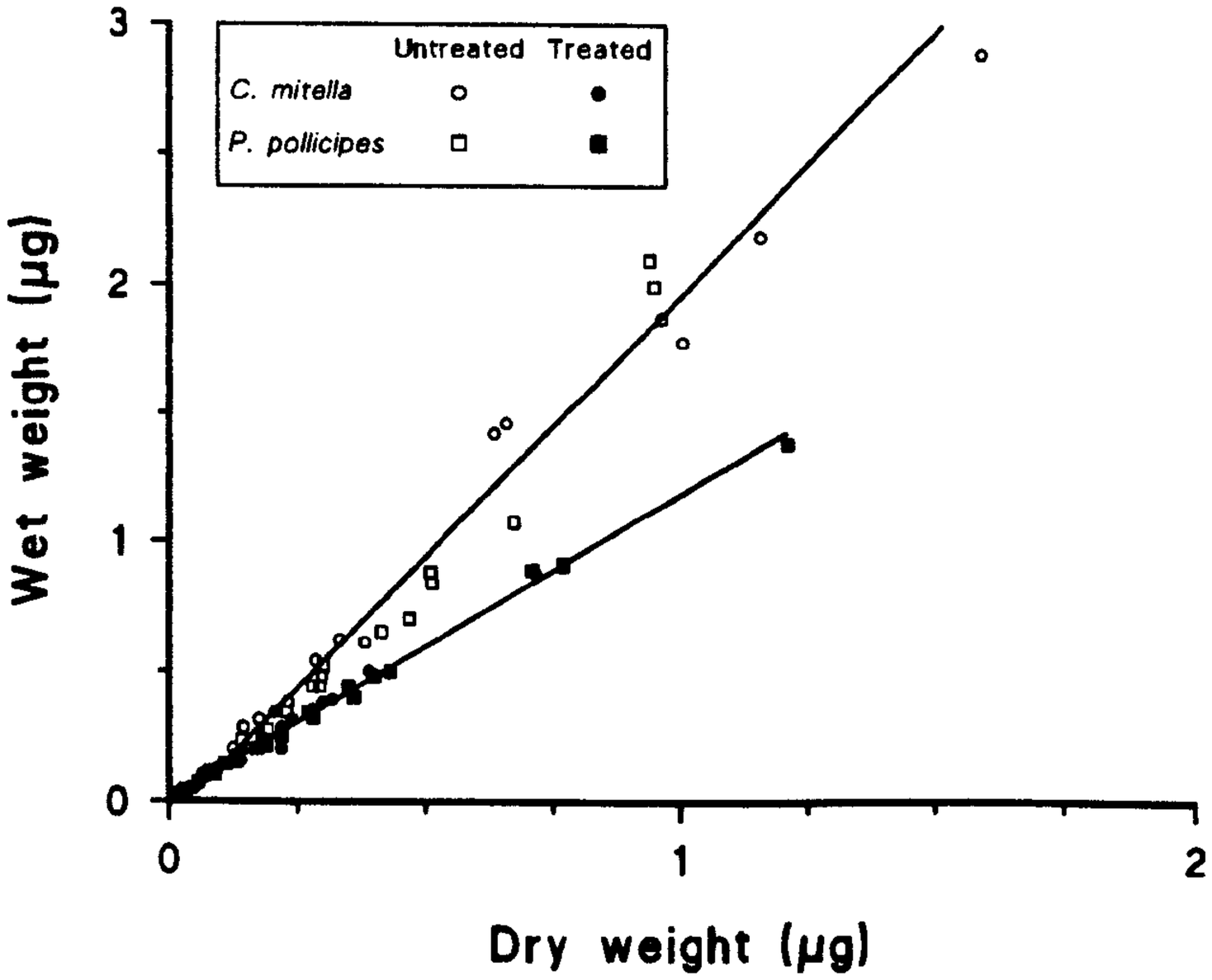


Figure 6. The capacity of the cements of *Pollicipes pollicipes* and *Capitulum mitella* to absorb water in their natural state and after heating to dryness at 90 °C from a small volume of distilled water.

A. Species	No treatment				Heat treated			
	slope(±95%CI)	int(±95%CI)	r	df	slope(±95%CI)	int(±95%CI)	r	df
C. mitella	2.02(±0.08)	0.04(±0.14)	0.997	18	1.22(±0.06)	0.002(±0.02)	0.995	17
P.pollicipes	2.06(±0.19)	0.10(±0.08)	0.984	18	1.17(±0.03)	0.005(±0.02)	0.998	15

B. Analysis of variance of wet weight for groups (from A.) with covariate (Dry Wgt.)

Source	df	SS (seq.)	SS (adj.)	MS (adj)	F	p
Dry Wgt.	1	216.303	12.661	12.661	752.78	<0.001
Group	3	0.665	0.065	0.022	1.29	0.286
Group x Dry Wgt.	3	1.346	1.346	0.449	26.67	<0.001
Error	68	1.144	1.144	0.017		
Total	75	219.458				

C. Tukey-Kramer procedure for minimum significant difference (MSD) for pairwise differences of slope coefficients. * = significant difference at 95%.

		Untreated		Heat treated
		C.mitella	*P.pollicipes*	*C.mitella*
Untreated	*P.pollicipes*	0.121		
Heat treated	*C.mitella*	0.121*	0.105*	
	P.pollicipes	0.105*	0.087*	0.087

D. Combined species linear relationships.

	slope(±95%CI)	int(±95%CI)	r	df
Not treated	2.03(±0.05)	-0.07(±0.07)	0.997	38
Heat treated	1.17(±0.03)	0.01(±0.01)	0.997	34

Table 2: Analysis of relationships between wet and dry weights (µg) for *Capitulum mitella* and *Pollicipes pollicipes* cement in their natural state and after drying at 90°C from a minimal quantity of distilled water (seq. = sequential, adj. = adjusted for entry order).

required for a significant difference between unplanned pairwise slope coefficients. Results indicate that there was no significant difference between the slope coefficients for species within a treatment but that a significant difference between slope coefficients for treatments was consistent for both species (Table 2A and C). Table 2D shows the relationships, using least squares linear regression, of wet and dry weights for untreated and treated cement of both species combined. The relationships are also shown in Figure 6. The ratio of wet:dry weight for untreated cement was approximately 2 (Table 2D). Therefore untreated cement absorbs roughly its own weight in water. The ratio of wet:dry weight for heat treated cement was approximately 1.2 (Table 2D) hence the heat

treated cement was significantly less porous than the untreated cement, only absorbing 14-20 % of its weight of water. Such a reduction in porosity must be due to an effect of the heat treatment preventing the ingress of water.

Cement masses of *P. pollicipes* and *C. mitella* are opaque and white when viewed under incident light and slightly translucent and brown when viewed with transmitted light. Figure 7 shows a thick (*ca.* 300 μm) section of *P. pollicipes* cement viewed with incident light. Typically, two major zones are apparent; an opaque white inner zone (iz) and a translucent outer zone (oz). The translucent outer zone is that which formed the interface with the medium in which it was exuded and set (seawater). No such zone was ever seen at the cement/adherand (basal integument) interface. A smaller translucent cement bead (tb) within the larger section was exuded and cured under flowing nitrogen prior to the delivery and curing of the larger bead in seawater.

Figure 8A,B shows 2 sections of *P. pollicipes* cement before (A) and after (B) heating to dryness, from a small volume of distilled water at 90 °C, viewed with transmitted light. In Figure 8A two zones are clearly evident whilst Figure 8B shows that heat treatment has resulted in an homogeneous mass which appears fully translucent in both incident and transmitted light. The effect was permanent, reimmersion had no effect on the resultant homogeneous translucence. Pieces of cement that were allowed to dry in air or in a desiccator also became fully translucent but, on rewetting, reverted to the normal state shown in Figure 8A.

Figure 9 shows a cryostat section (15 μm thick) of *C.mitella* cement where the (opaque) inner zone (iz) is evident from a greater granularity in comparison to the finer grained (translucent) outer zone (iz) formed at the seawater interface (si) on solidification.

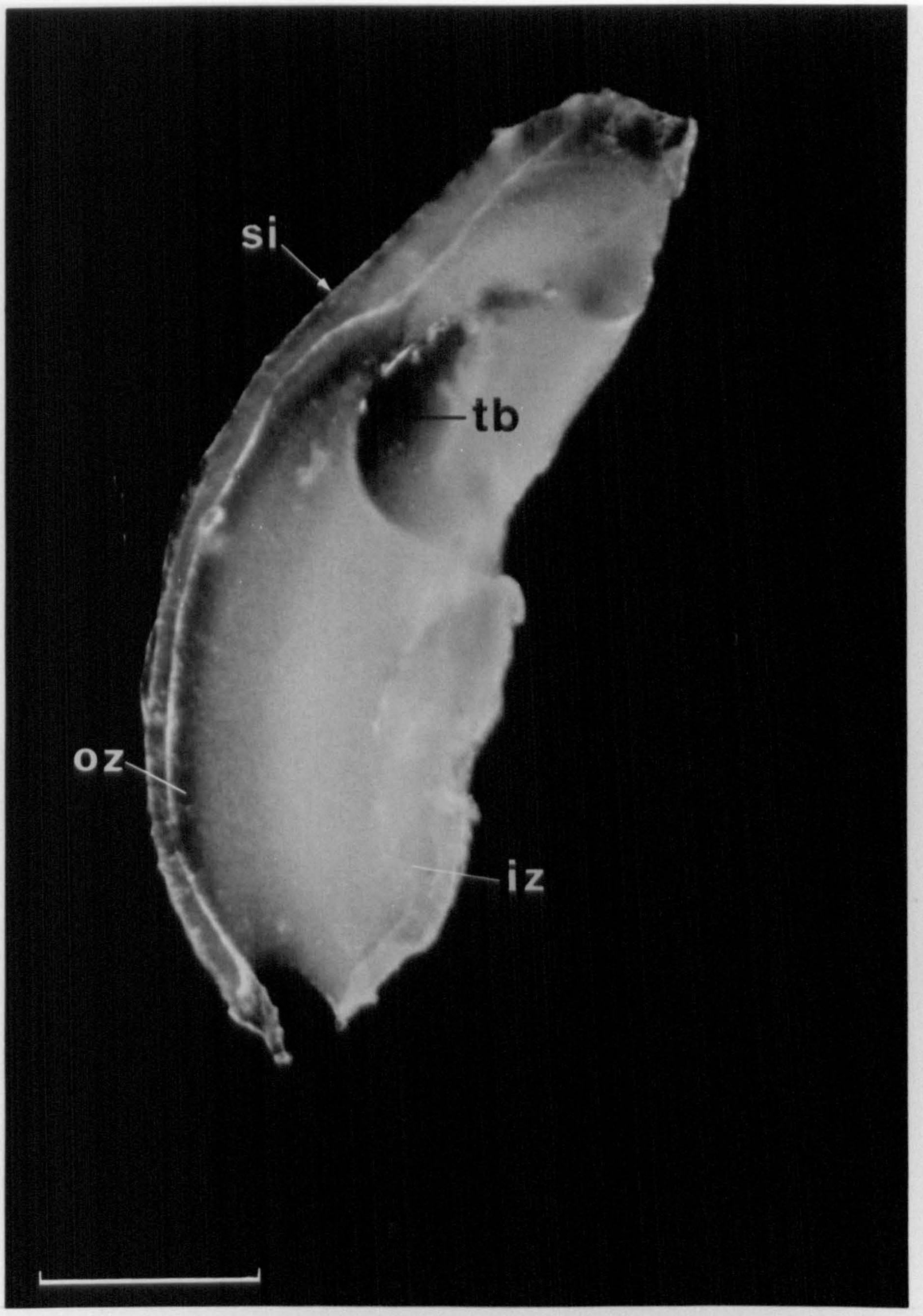


Figure 7: A 300 thick µm section of *Pollicipes pollicipes* cement, exuded and cured in seawater, viewed with incident light showing zones of differing translucency. An opaque inner zone (iz) and an outer zone (oz) which is translucent possibly due to less water within the cement. A translucent bead (tb) was exuded and cured under flowing nitrogen before the larger bead was formed. Scale bar is 200 µm.

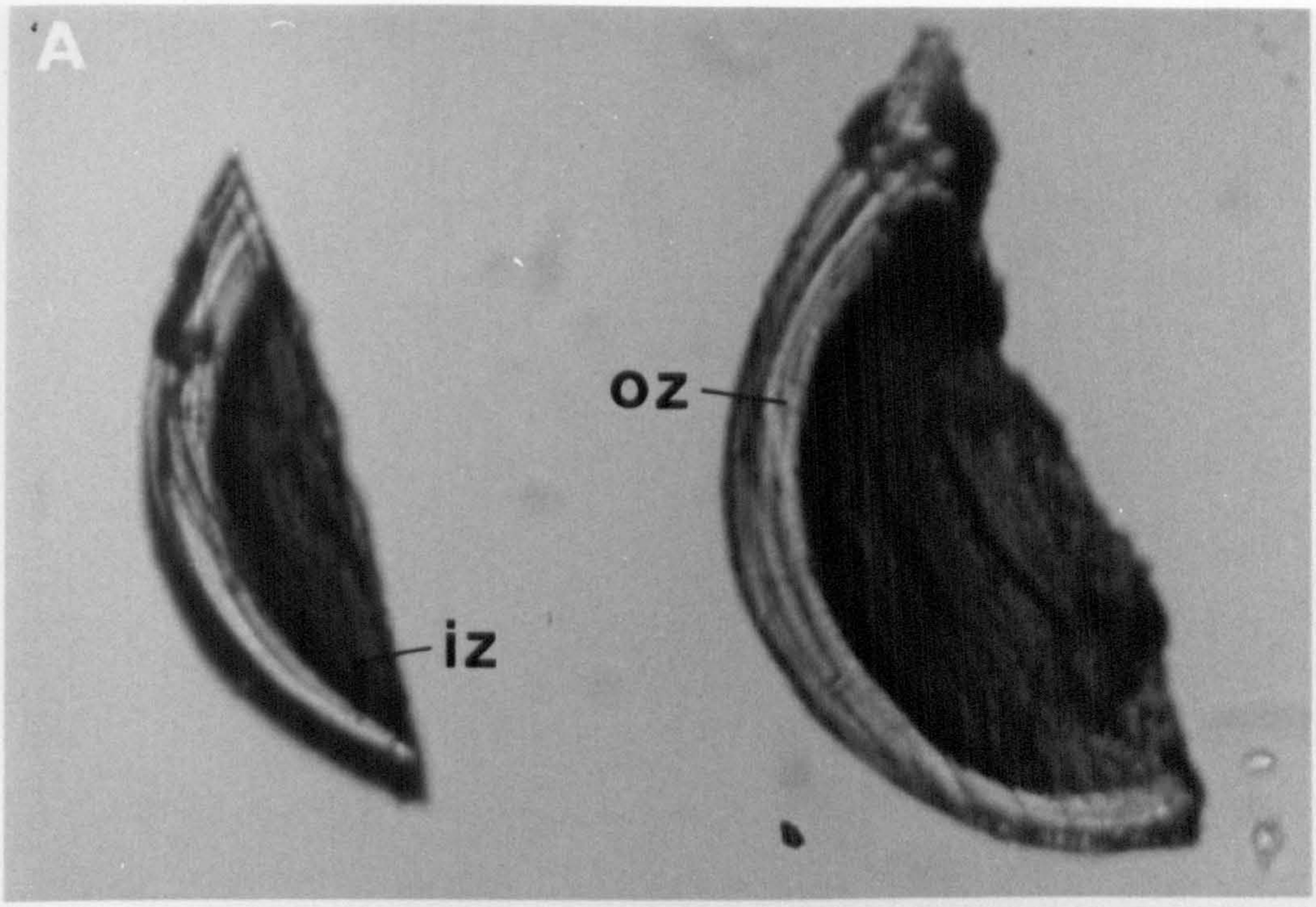


Figure 8: A; hand cut sections of *Pollicipes pollicipes* cement viewed with transmitted light. The cement is translucent to transmitted light but zonation is obvious. The outer zone (oz) is more translucent than the inner zone (iz) probably due to less light refraction from absorbed water. B; the same sections after heat treatment (see text) resulting in an homogeneous, fully translucent appearance. Scale bar is 200 µm.

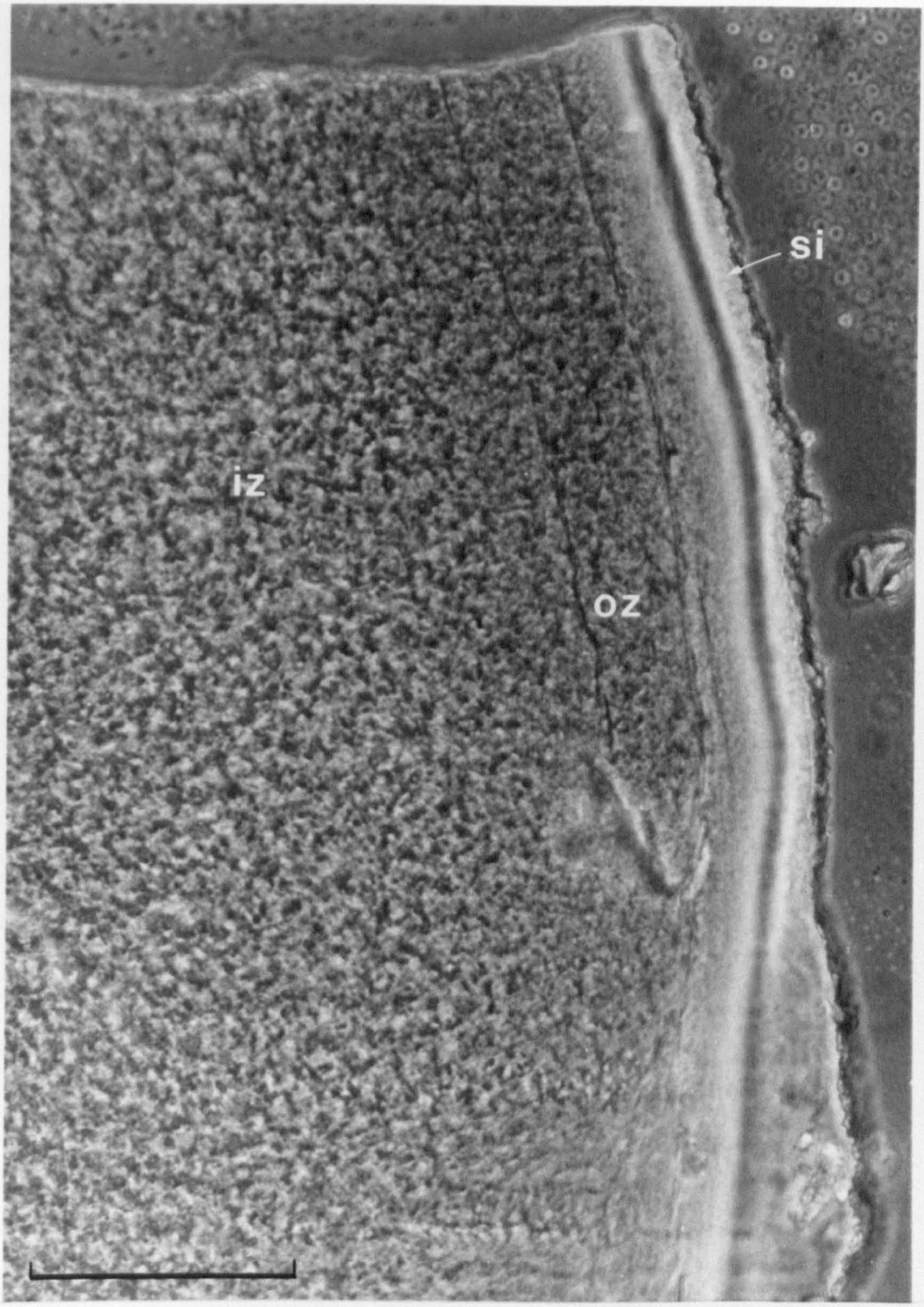


Figure 9: A 15 μm thick cryostat section of *Capitulum mitella* cement showing the fine grained translucent outer zone (oz) towards the seawater interface (si) in which the cement was cured. The greater granularity of the inner zone (iz) corresponds with the white opacity of this zone to incident light. Scale bar is 60 μm.

Evidence then clearly indicates that the opacity of the inner zone of thick sections or whole beads of wet cement is due to interconnected pockets of space within the zone which contain water that reflects/disperses light to confer opacity. The translucent outer zone, by comparison, has less space and the inner zone can be permanently transformed to this condition by heating whilst still retaining a degree of porosity.

Figure 10 shows a photomicrograph of a cryostat section (12 μm thick) of *C. mitella* cement with four, thin, translucent zones. Large beads of cement were often observed with such demarcation lines, indicative of curing at the seawater interface, which must result from additional cement delivered through previously cured yet still porous cement. The small translucent bead of cement, exuded and cured under flowing nitrogen within a larger bead delivered later and cured in seawater from the same pore, shown in Figure 7, must also have been porous to the cement delivered in seawater.

Figure 11 shows the appearance of cement sections under incident light from *P. pollicipes* and *C. mitella* after rehydration following drying, from a small volume of distilled water, at various temperatures. A significant change in (nominal) translucency (Spearman's $r_s = 0.779$, $df = 29$, $p < 0.001$) with temperature of drying indicates that the space within cement reduces in proportion to the temperature. No visible effect on translucence occurred below *ca.* 53 °C (Fig. 11) and full translucence (minimum porosity) was conferred at evaporating temperatures above 70 °C (Fig. 11).

Cement samples with CTC (carbon tetrachloride, at 74.4 °C) and ethanol (absolute, at 84.7 °C) as solvents in the heat treatment process were fully translucent after drying, as for samples dried in air at ambient temperature. However, on rehydration (with distilled water) the inner zone of samples returned to the opaque condition whilst

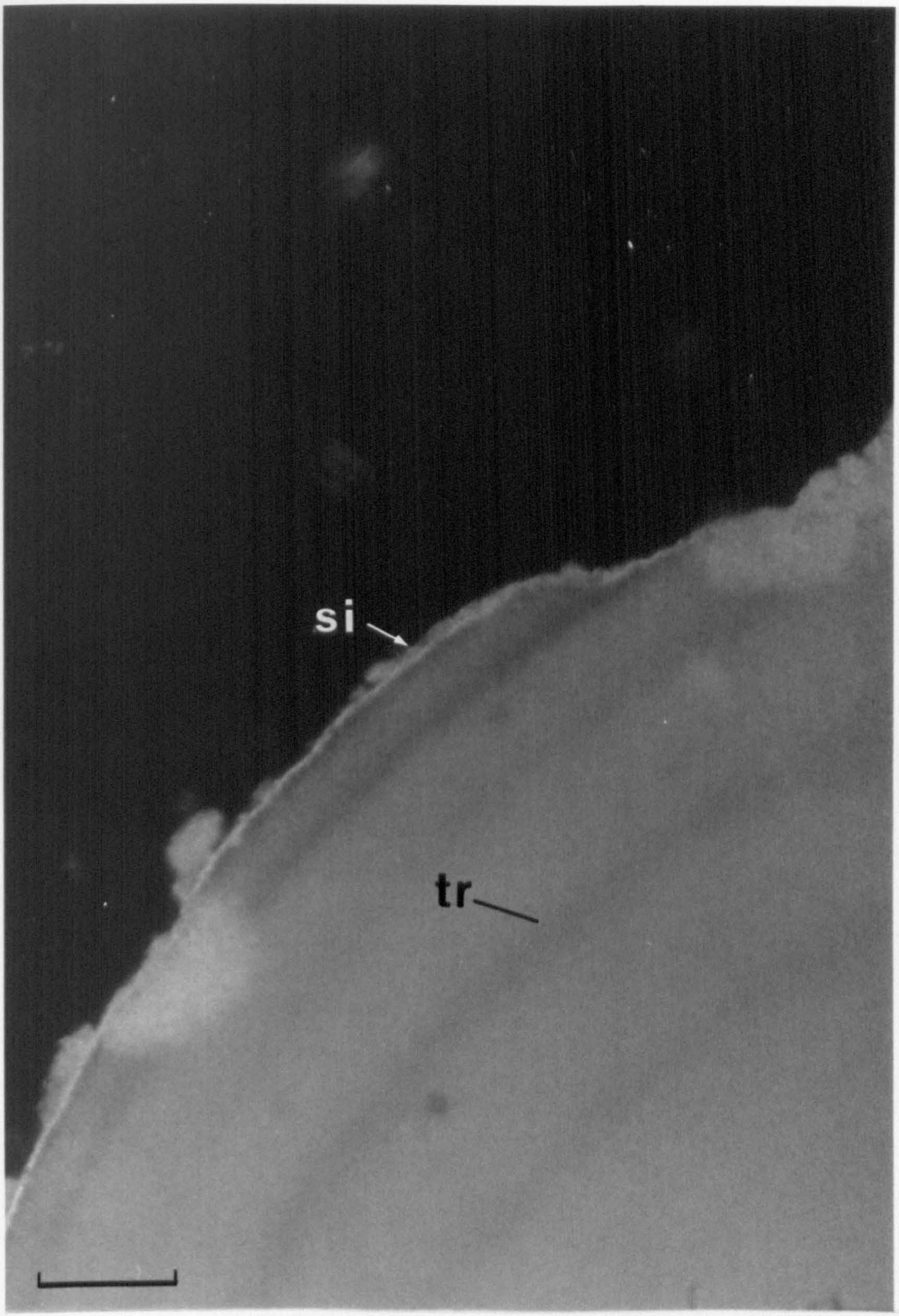


Figure 10: A 12 µm thick cryostat section of *Capitulum mitella* cement. Concentric translucent rings (tr) of presumed low porosity cured cement demonstrate the delivery of cement through previously cured, yet obviously still porous cement. Each ring marks the seawater interface (si) at the time of cement delivery. Scale bar is 100 µm.

simultaneously run control samples at the same temperature, remained permanently, fully translucent. It is therefore clear that the action of heat alone is insufficient to reduce porosity, water must also be present. Drying from ethanol did not affect the ability of such sections to become permanently translucent when later subjected to the same treatment with distilled water as the solvent.

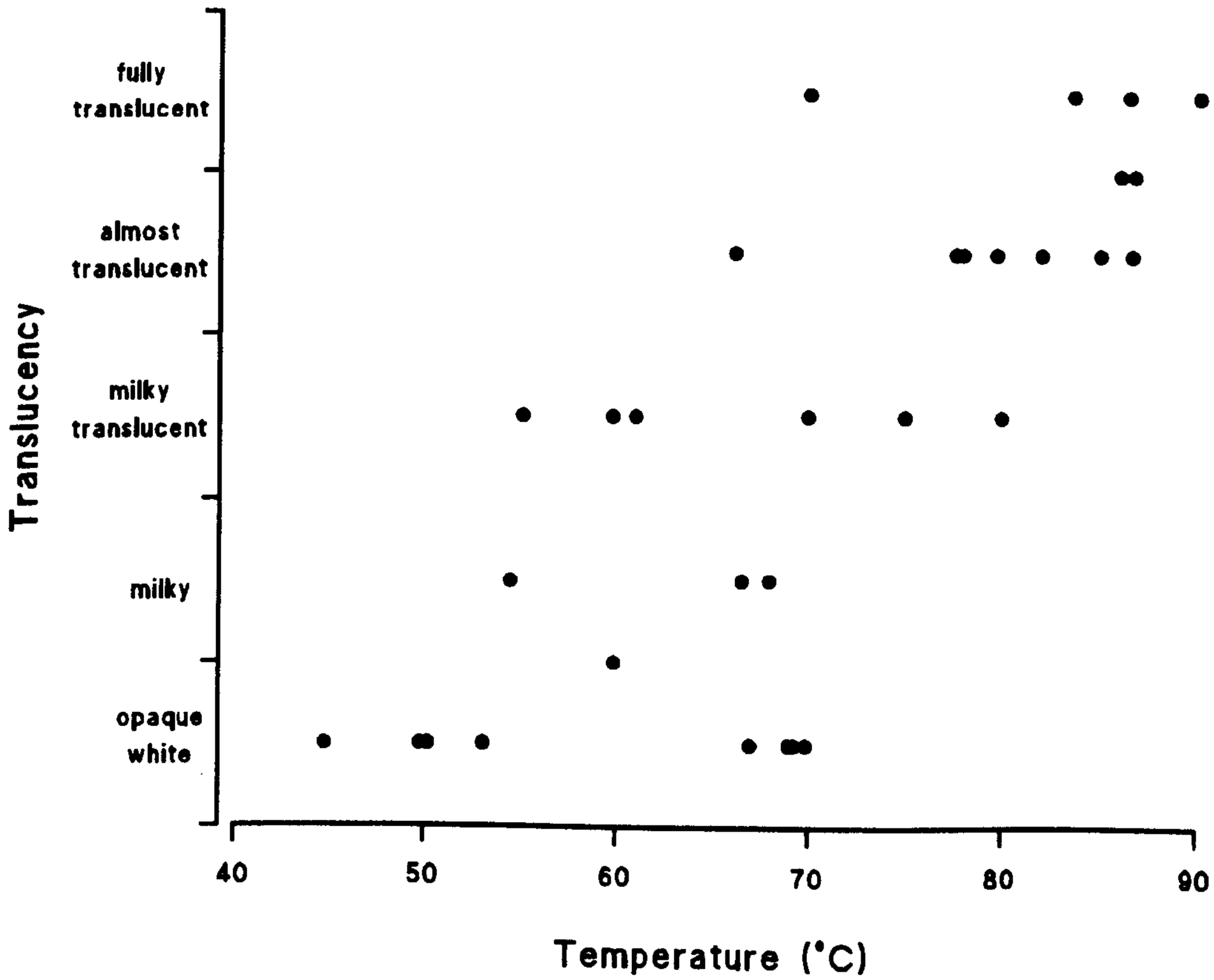


Figure 11: Opacity reduction in *Pollicipes pollicipes* and *Capitulum mitella* cement dependent on temperature at which water was evaporated from the cement. Translucence (permanent) of samples was nominally graded from the full opacity of the inner zone to the full translucence of the outer zone of untreated hydrated cement.

Similar treatment of sections using deoxygenated water gave the same result as that for aerated water. Such treated sections, together with control samples remained fully translucent on rehydration following drying from a minimal quantity of solvent at 75 °C. There was therefore no evidence of a requirement for oxygen in the porosity reduction process.

Histochemical treatment of cement

Bromphenol blue stain for protein. Thin, hemispherical, cryostat sections of *P. pollicipes* cement were stained a dark sky blue. Darker staining of thin concentric rings were visible in many larger sections indicating a greater protein density in areas with, consequently, less space (*c.f.* Fig. 10). Thicker cryostat sections were stained a darker blue-purple with darker concentric rings more obvious than in thinner sections. A zone to the centre of each section was always devoid of such rings, indicative of the first volume of cement delivered.

Modified alkaline tetrazolium reaction. Stained sections were an even light purple indicating an even distribution of -SS and/or -SH groups. Deeper staining to the ring structure seen with bromphenol blue was also evident in the larger sections, again indicating a greater concentration of protein and less free space. Larger sections of cement subjected to porosity reducing treatment prior to staining were evenly stained with no darker stained ring structures.

Nitroprusside reaction. A lack of colour development in untreated sections indicated no (or very few) available -SH groups. The procedure was therefore not applied to porosity reduced sections. Sections with -SS groups reduced to -SH groups in thioglycollic acid gave a positive result. Such treated sections developed a strong even

pink colour which rapidly faded, indicating an homogeneous density of reduced cystine.

Iodine oxidation and iodoacetic acid blocking of SH. Sections subjected to iodine oxidation required increased washing time to remove absorbed iodine which imparted a dark brown colour to sections. Both control and blocked sections developed permanent translucence after heat treatment indicating no visibly determinable effect from any available -SH groups.

Nitrosonaphthol stain for tyrosine. No visible difference between zones in untreated cement or between untreated and heat treated cement sections were seen. In all cases, faint green-yellow fluorescence was observed which was much less intense than expected from tyrosine. None of the cement sections showed an unequivocal fading of fluorescence over *ca.* 4 minutes, as would have been expected from tyrosine.

Tyrosyl-OH block and iodination of tyrosine. No difference could be seen between control and treated cement sections. All remained permanently translucent after heat treatment and rehydration, suggesting no involvement of tyrosine in the porosity reduction process.

DISCUSSION

The nitrogen content of *Pollicipes pollicipes* and *Capitulum mitella* whole cement (17-21 % dry weight) is slightly greater than the single values reported by other workers for various cirripede cements using microcombustion techniques. Saroyan *et al.* (1970a), Walker (1972), and Lindner and Dooley (1973) reported nitrogen content in the range 13-14 % for balanomorph barnacles. Barnes and Blackstock (1974) and Walker and Youngson (1975) reported 11 % and 16 % nitrogen in the cements of *Dosima fascicularis* and *Lepas anatifera* respectively. The greater nitrogen content of *P. pollicipes* and *C. mitella* cements here, in comparison to that of most other cirripedes cements, is reflected in both a higher carbon content (48-56 % dry weight compared to 41-50 %) and, of course, a greater estimated protein constituent of the cements (100 % compared to 76-96 %). Walker (1972) demonstrated that values for the estimated protein constituent using microcombustion and microKjeldahl techniques differed by up to 20 % dry weight for *Balanus crenatus* and *Chirona hameri* cements with estimates of 97-100 % protein based on the microKjeldahl technique. Nevertheless, Walker and Youngson (1975) estimated the protein constituent of *L. anatifera* cement at 100 % dry weight, using microcombustion, which agrees with the value for the scalpellids cements here (100 %). Furthermore, Naldrett (1993) reported finding only protein in the cement of *B. crenatus*. Variability in the amino acid composition of cirripede cements is common (e.g. Walker, 1972, Lindner and Dooley, 1973, Barnes and Blackstock, 1974, Walker and Youngson, 1975, Naldrett, 1993) and such variability will be reflected in the carbon and nitrogen content.

The zoned appearance of *P. pollicipes* and *C. mitella* cement is similar to that reported for the permanent cement of *Semibalanus balanoides* cyprids by Walker (1971) and for the cement of *B. crenatus* adults by Naldrett (1993). Walker (1971), using TEM, noted that cypris cement had a thin outer zone in the form of a close reticulum, a thin mid zone of moderately electron dense material whilst the bulk of the material had the form of a loose reticulum. Naldrett (1993), also using SEM, found the outer layer of adult barnacle cement to be 'smooth' whilst the bulk of the material contained interconnected spaces. Results here clearly demonstrate that the cements of *P. pollicipes* and *C.mitella* are surprisingly porous, able to absorb their own weight in water. It is likely that balanomorph cement is similarly porous contrary to Naldrett's (1993) contention concerning the outer zone, considering the similar nature of lepadomorph and balanomorph cements.

Results of the bromphenol blue and modified alkaline tetrazolium stains, together with the CN analysis serve to confirm that the cements of *P. pollicipes* and *C. mitella* are (as has been well established for other barnacles) predominantly protein.

The curing time for the cement of adult *P. pollicipes* was estimated at *ca.* 2 h. Saroyan *et al.* (1970b) report fresh cement hardening within fifteen minutes of detached barnacles (probably *B. crenatus*) being replaced on a surface. Cheung *et al.* (1977), using more rigorous observations, found the liquid cement of *B. eburneus* to solidify after several hours in air or in water (salt and distilled). Yule and Walker (1987) measured the tenacity of *Semibalanus balanoides* cypris permanent cement finding that the strength reached an asymptotic maximum of *ca.* $9x10^5$ Nm^{-2} after 2-3 hours from settlement. They interpreted the increase in strength with time as the curing time of the cement which concurs with the evidence here and that of Cheung *et al.* (1977).

Evidence is therefore steadily mounting that both the adult and cypris permanent cements of barnacles in general may differ only in the proportion of some amino acids, being essentially similar proteins, undergoing the same curing process.

The space (porosity) within the cements of *C. mitella* and *P. pollicipes* can be reduced by up to 39-45 % by saturating with distilled water and heating to dryness. No reduction in such porosity can be demonstrated using ethanol or CTC as the solvents. Porosity reduction can be induced, even after ethanol treatment by resaturating with distilled water indicating the mechanism of porosity is not simply the result of a heat induced conformational change in the cement protein.

Darker staining of concentric rings in the cement by bromphenol blue and modified alkaline tetrazolium indicates areas of greater protein density. These rings correspond to the translucent zones in hydrated, unstained cement which are considered less porous by virtue of a lower volume of interconnected space. The homogeneous appearance of heat treated hydrated or stained (modified alkaline tetrazolium) cement demonstrates the reduction of space within the normally more porous zone indicating full curing on high temperature drying comparable to full curing in air or nitrogen.

The zoned appearance of cement delivered and cured in seawater, compared to the homogeneous translucence seen in cement cured in air or nitrogen, strongly suggests a differential degree of curing between the zones of cement cured in seawater. Nevertheless, such cement retains the ability to become fully cured (no return to opacity on rewetting) when it is dried from water saturation at temperatures greater than *ca.* 70 °C. The more porous inner zone is thus prevented from complete curing in the presence of water or seawater. Although only one observation of delivery and curing in distilled water was made here, Cheung *et al.* (1977) also found cement to cure to an opaque mass

in water. The large variability found by Yule and Walker (1987) in the tenacity measurements of cypris permanent cement can perhaps be explained (in part) by variable cement strength due to differential porosity/solidification of very small volumes in the light of the present results.

Walker (1971) suggested that the zoning of cyprid cement may be the result of a requirement for oxygen from the environment or that seawater may have had an effect on the outer layers. The evidence presented currently indicates that the protein mixture fully cures in a thin outer zone at the seawater (or distilled water) interface. Water would be easily displaced from this zone to the surrounding medium. However, full curing of cement in the inner zone is prevented, possibly in a purely mechanical manner by enclosing water in increasingly small spaces within the solidifying cement whereby the increasing resistance to the physical displacement of the water is ultimately balanced with *partial curing*.

Although evidence indicates that seawater inhibits the inner zone of cement from fully curing, water is still essential for curing. No heat induced, permanent, reduction in porosity was obtained when cement was saturated with ethanol or CTC. Unless the presence of water was crucial to the process, some permanent change in porosity at 75 °C would have been expected (see Fig. 11). For cement masses cured in air or nitrogen, sufficient water must have been available for full solidification either as vapour with the gas or, more likely, as the solvent for the pre-polymerised cement moieties. Cement must therefore be delivered requiring no exogenous agents to solidify. The delivery environment, however, may affect the degree of curing.

The short delivery time of cement (5-20 mins.) compared to the relatively long curing time (*ca.* 2 h) together with the porous nature of the cement negates the necessity of a

flushing mechanism and polymerisation initiation point postulated by Saroyan *et. al.* (1970b). The increasing porosity of cement towards the basal integument means the pores can never be clogged. Indeed, in large cement masses, translucent concentric rings of more dense, yet still porous, cement are evidence of spasmodic delivery through the same pore in excess of curing time (e.g. Figure 10). Reasonable muscular action alone could ensure little or no liquid cement residue in the collecting canals or principal ducts. Assuming that water is the solvent for the pre-cured cement moieties, polymerisation initiation at or close to the time of delivery is unlikely since results show that water alone is required for curing. Cement in the ducts should experience the least degree of solidification since the distances involved for water displacement are the greatest. Naldrett (1993) suggested that solidification of cement may be caused by delivery into a salt solution. He further inferred that cement would not solidify in the duct system since it would be secreted in a low salt or salt free liquid (water). Such is clearly not so since the present results show full curing in air and nitrogen. Cheung *et al.* (1977) also found balanomorph cement to solidify in water. Delivery into an ionic medium is therefore not necessary, cement is delivered in a state that has the potential to cure fully with no environmental interaction. A similar conclusion was made by Cheung *et al.* (1977) finding that liquid cement contained all the necessary components to solidify.

Naldrett (1993) failed to find any evidence for phenolic tanning of adult barnacle cement using NMR spectroscopy. It has been clearly shown that the adhesive in mussel byssus plaques is quinone tanned requiring oxygen (see review by Rzepecki and Waite, 1991). Unlike *P. pollicipes* and *C. mitella* cements, tanning of mussel byssus does not occur in N_2 scrubbed seawater (Ravera, 1950, cited in Waite, 1985). Curing of barnacle cements without oxygen confirms Naldrett's (1993) rejection of phenol tanning and

demonstrates a fundamental difference between the proteinaceous cements of barnacles and mussels.

Staining with nitrosonaphthol did not reveal more tyrosyl-OH groups in partially cured cement compared to fully cured cement and blocking of tyrosine by either nitrobenzoylation or iodination did not prevent part cured cement becoming fully cured. Sundler *et al.* (1976) demonstrated that the nitrosonapthol staining method requires p-hydroxylated phenolic compounds and that the minimum detectable amount of p-tyrosine and tyrosine dipeptides was 0.1-0.3 μg (on filter paper). The amount of tyrosine in the cement of various cirripede species reported from the literature ranges from 0.1-6 %, with protein contributing at least 80 % of the cement mass. Such low levels of tyrosine could explain the lack of a strong fluorescence. It could, of course, also be the case that no p-hydroxylated tyrosine was present. Nevertheless, assuming that the tyrosine blocking of any tyrosine present was successful, the involvement of tyrosine in cement curing is negated, which further supports the rejection of quinone tanning.

The acceptance of phenol tanning for cyprid cement by Naldrett (1993) on the evidence of Walker's (1971) determination of phenols and polyphenol oxidase in the glands and secreted cement warrants further investigation. The zonation seen in and the curing time estimated for lepadomorph cement are remarkably similar to that observed for balanomorph, permanent, cypris cement (*c.f.* Walker, 1971 and Yule and Walker, 1987). Crisp *et al.* (1985) estimated the tensile strength of cypris permanent cement of *Semibalamus balanoides* at 9.7×10^5 Nm^{-2}. Yule and Walker (1984b) estimated the tensile strength of 4-19 month old *S. balanoides* adults cement at 9.25×10^5 Nm^{-2}. Evidence then indicates similar physical properties for both cyprid permanent cement and adult cement. Furthermore, Walker (1973) demonstrated that the adult cement glands

of *S. balanoides* were derived from the cypris cement gland cells following metamorphosis.

The results of specific blocking and staining protein groups present some difficulty in interpretation. Whilst positive results are unequivocal, negative results could be attributed to poor methodology and/or lack of sensitivity. Positive results from controls are more suggestive of a real negative result or at worst a lack of sensitivity.

The positive staining of reduced disulphide does indicate the presence of a greater concentration of disulphide over sulphhydryl groups in partially and fully cured cement. The resistance to dissolution in thioglycollate was also reported by Lindner and Dooley (1973) for balanomorph cement and clearly indicates that the solidified state of the cement is not due to disulphide bonds alone. The negative result for sulphhydryl groups could be due to the sensitivity of the stain. Hammet and Chapman (1938) showed that the nitroprusside procedure employed can be sensitive to 5×10^{-6} M in solution but reported that such would only be an approximation for sensitivity in tissue. However, assuming the negative result indicates the virtual absence of -SH groups, blocking of any available -SH with iodoacetic acid and iodine oxidation of -SH (producing -SS and/or $-SO_3H$, Pearse, 1985) had no effect on further curing of partially cured cement and that thioglycollate reduced cement was not solubilised, all strongly suggest that disulphide bonding plays no part in curing after delivery. Naldrett (1993) arrived at a similar conclusion, from NMR studies, suggesting that disulphide bonds were formed before the cement was exuded from the pores.

The failure to interfere with the further curing of part cured lepadomorph cement by modifying radical groups that are involved in disulphide bonding and phenol tanning, together with the demonstration that cement needs only water to solidify, lends support

to evidence from Barnes and Blackstock (1976), Yan and Pan (1981) and Naldrett (1993) in that barnacle cement is not solidified by covalent bonds, but more likely by individually weaker, hydrophobic and perhaps electrostatic interactions between relevant protein groups.

The relatively large cement masses of *P. pollicipes* and *C. mitella* have provided an opportunity to investigate properties of the cement that have been apparently more difficult using, comparatively, much smaller samples usually collected from balanomorphs. The differentially cured zones of cement cured in seawater and the ability to completely cure the inner zone by heating have provided a novel opportunity to investigate the free radicals of partially and completely cured cement. It is expected that advanced analytical techniques, such as FTIR spectroscopy and, perhaps, further histochemical studies, will help to elucidate differences between the partially, yet stable and fully cured cement, possibly solving the enigma of the curing process.

CHAPTER 6

General discussion

The aims of the current work were to contribute to evidence indicating lepadomorph cement to be essentially the same as that of balanomorphs based on the biochemical analyses of *Dosima fascicularis* (Barnes and Blackstock, 1974, 1976) and *Lepas anatifera* (Walker and Youngson, 1975) whilst appraising properties of the cement that have recieved scant attention (i.e. long term stability in seawater and zonation/porosity) and to address the lack of information on ecological aspects of adhesion in lepadomorphs. The latter was directed by the suggested ability for relocation in pollicipid lepadomorphs (Hoffman, 1984, 1989) and by the lack of literature concerning the adhesion of lepadomorph cyprids, particularly the potentially endangered *Pollicipes pollicipes*, which has chiefly been the result of an inability to culture the settling stage larva in large numbers for study (see Lewis, 1975a,b and Molares *et al.*, 1994).

No evidence was found to suggest that the cements of *P. pollicipes* and *C. mitella* are grossly different to balanomorph cement. Lepadomorph cement resistance to degradation by marine bacteria and low dissolution rates concur with the reported resistance to dissolution in various solvents (Lindner and Dooley, 1973) and the suggested resistance to bacterial degradation (Naldrett, 1993) for the cement of balanomorph barnacles. The similarly zoned appearance of naturally cured cement of *P. pollicipes* and *C. mitella* here to that of balanomorph cement (Walker, 1971 and Naldrett, 1993), together with both the same ability to cure in air, water and seawater and the similar curing period (Cheung *et al.*, 1977) all support the biochemical evidence of Barnes and Blackstock (1974, 1976) and Walker and Youngson (1975) and the conclusion of Yule and Walker (1987) that the cements of lepadomorphs and balanomorphs are essentially the same.

Lipid	Carbohydrate	Species	Source
27.4	2.6	*Balanus crenatus*	Cook (1970)
0.95	1.0	*Chirona hameri*	Walker (1972)
0.69	1.05	*Balanus crenatus*	Walker (1972)
8.2	2.0	*Dosima fascicularis*	Barnes & Blackstock (1974)
<1	*ca*.1	*Lepas anatifera*	Walker & Youngson (1975)
0.01	0.6-1.2	*Balanus amaryllis*	Yan and Tang (1981)

Table 1: Lipid and carbohydrate content (% dry weight) of barnacle cements.

The nitrogen content of *P. pollicipes* and *C. mitella* cement does not indicate the presence of significant quantities of lipids or carbohydrates in the cement, but this, of course, is subject to the general applicability of the commonly employed conversion factor (6.25) of nitrogen to protein. Walker (1972), and Walker and Youngson (1975) give nitrogen values that could indicate only protein for the cements of *Chirona hameri* and *Lepas anatifera* respectively depending on the method employed. Furthermore, Naldrett (1993), using advanced analytical techniques, reported only protein comprising the cement of *Balanus crenatus*. However, lipids and carbohydrates are commonly reported components of the cements of various barnacle species. Table 1 shows the quantities of lipid and carbohydrate reported by various workers. Conversely, Saroyan *et al*. (1970a) and Lindner and Dooley (1973) found no lipid in the cement of *B. crenatus* and Walker (1970) also found no evidence of lipid in the cement gland secretions of three other balanomorph species. The presence (and hence rôle) of lipid and carbohydrate, whether real with interspecific variability or a result of contaminant due to the relatively small quantities of cement employed in such analyses, may be better addressed using the much greater quantities of cement easily obtained from lepadomorph barnacles since evidence now clearly indicates a great degree of similarity between the cements of balanomorphs

and lepadomorphs. Indeed, Walker (1972) noted that balanomorph cement contaminated by the method of collection may have contributed to the inorganic residue he found in cements of *C. hameri* and *B. crenatus*. Nevertheless, a uniform distribution of calcium, sulphur and phosphorus in the cements led Walker (1970) to suggest that these elements may be an essential part of the cement matrix. In the light of present results the porous nature of cement could be responsible for such a uniform distribution of contaminants and careful preparation and analysis of larger masses of lepadomorph cement should again help to resolve this hitherto unattended detail. Whether barnacle cement contains specific elements, lipid or carbohydrate as other than contaminants has not mitigated against the conclusion that lepadomorph and balanomorph cements are essentially the same and, therefore, solidify by the same process.

The work of Barnes and Blackstock (1976), Yan and Pan (1981), Yan *et al.* (1983) and Naldrett (1993) demonstrated that barnacle cement could be dissolved in a solution of the anionic detergent SDS (sodium dodecyl sulphate) with a reducing agent 2Me (2-mercaptoethanol). Naldrett (1993) pointed out that dissolution in such a solvent together with the cement's resistance to dissolution in thioglycollic acid (Lindner and Dooley, 1973) does not favour a curing process mediated by covalent bonding.

Naldrett (1993) concluded from the solubility of cured cement and NMR spectroscopy studies that soluble yet previously polymerised proteins, through cysteine residues, were solidified through hydrophobic complexing after delivery. The real, yet low rate of dissolution (in seawater) of lepadomorph cement shown in the current study, together with an apparent resistance to bacterial degradation, evidence

that water inhibits full curing (of the inner zone of cement beads at least) yet is necessary for solidification (no further curing in ethanol or CTC, see chapter 5) and the confirmation that -SS groups and few or no -SH groups are present in partially cured cement all support the hypothesis for solidification through hydrophobicity. Interestingly, the presence of the reductant 2Me appears necessary for full dissolution of barnacle cement over a short period (Barnes and Blackstock 1976, Yan and Pan, 1981, Yan *et al.*, 1983 and Naldrett, 1993). The explanation could be that the formation of a highly hydrophobic core, resistant to the action of SDS alone, required disruption of the protein structure/matrix by the reductant to facilitate dissolution at a greater rate than could otherwise be achieved. Indeed, Naldrett (1993) noted how *B. crenatus* cement needed to be ground into fine particles to facilitate dissolution in SDS-2Me and some protein moieties even appeared to 'repolymerise'.

A protein which possesses 30 % of amino acids with non-polar side chains may achieve high stability, and hence resistance to dissolution, providing the water molecules surrounding the residues can be released (Pearse, 1985). Results here

hydrophobic residues %	Species	Source
34.5	*Balanus crenatus*	Cook (1970)
27.2	*Chirona hameri*	Walker (1972)
26.8	*Balanus crenatus*	Walker (1972)
37.2	*Dosima fascicularis*	Barnes and Blackstock (1974)
36.2	*Lepas anatifera*	Walker and Youngson (1975)
40.0	*Dosima fascicularis*	Barnes and Blackstock (1976)
28.7	*Balanus amaryllis*	Yan and Tang (1981)
28.1-28.2	*Balanus perforatus*	Naldrett (1993)
30.2-30.4	*Balanus crenatus*	Naldrett (1993)

Table 2: Percent hydrophobic amino acid residues in barnacle cement.

comply with such a mechanism in that the presence of water can reduce the degree of curing but that curing can proceed if water is driven out by heat. Furthermore, Table 2 shows the percentage residues of the hydrophobic amino acids phenylalanine, leucine, isoleucine, methionine, valine, alanine and tryptophan from various workers amino acid profiles for various barnacle species. Although variation in amino acid composition between and even within barnacle species appears to be common (e.g. see Cook, 1970, Walker, 1972, and Naldrett, 1993 for variation in the composition of *B. crenatus* cement), all species consistently contain around 30 % hydrophobic amino acid residues even though they may contain varying amounts of each. Naldrett (1993) further noted that *B. crenatus* cement was the least soluble, attributing this to the higher percentage of cysteine residues. Table 1 shows that the insolubility of *B. crenatus* cement may more likely be attributed to the higher percentage of hydrophobic residues within balanids.

The weight of evidence now strongly favours hydrophobic complexing as a major factor in the solidification of barnacle cement, but the role of hydrogen or 'weak' (other electrostatic) bonds as suggested by Barnes and Blackstock (1976) has yet to be determined.

Mobile organisms in the marine environment generally use fluid, viscous, proteinaceous (e.g. barnacle cyprids, Walker and Yule, 1984 and anemones, Young *et al.* 1988) or mucopolysacharride (e.g gastropods, Grenon and Walker, 1980, 1981 and Miller, 1974), ultra-Stéfan type adhesives. The demonstrated ability of *Pollicipes pollicipes* and *Capitulum mitella* to actively relocate predominantly by growth, leaving tracks of cement, shows that peeling, through pedal waves or walking, is not employed, thus an ultra-Stéfan type adhesive would serve no purpose concurring

with Yule and Walker's (1987) rejection of such. A solid cement with sufficient cohesive/adhesive strength and elasticity to resist breaking/yielding under the forces encountered is thus suitable for lepadomorphs and balanomorphs.

The inability of *Lepas anatifera* to actively relocate appears to be reflected in its settlement strategy. Evidence of settlement site, from cypris antennules, was only ever found close to the base of adults or directly to the substratum where competition for space and food, other than by conspecifics, is much less than in the more diverse intertidal zone inhabited by *P. pollicipes* and *C. mitella*. *P. pollicipes*, at least, utilises its ability to relocate in its settlement strategy, but more information is necessary to determine whether *C. mitella* does likewise.

A stimulus for directed relocation was not determined but constant unidirectional flow and gravity were rejected. *P. pollicipes* in the laboratory or field attached to larger conspecifics always moved down the host peduncle toward the substratum irrespective of adult orientation. Solitary *P. pollicipes* attached to plastic plates merely reorientated with respect to the flow or moved small distances in apparently random directions. *C. mitella* fixed in close proximity to other individuals generally moved together forming a tight cluster, *P. pollicipes* were not fixed in this manner so it is not known whether they would respond in the same way. Clearly, *P. pollicipes*, and perhaps *C. mitella*, were directed towards the substratum or another adult but whether the stimulus was chemical or physical in nature remains to be determined. The ability to settle large numbers of scalpellid cyprids would be useful in further studies of the relocation phenomenon, particularly to elucidate the stimulus(i), growth of the principal duct and formation of pores at the leading edge of travel, the mechanism of sloughing basal material at the trailing edge of travel and, for *C.*

mitella, the involvement of muscular activity in laying the peduncle to the substratum.

The culture of large quantities of *P. pollicipes* cyprids has been achieved during the present study (*c.f.* Lewis, 1975a,b and Molares *et al.* 1994) but the provision of conditions conducive to settlement of more than a relatively small number of the larvae was not attained. The conditions provided here were similar to those that have proved successful for balanomorph barnacles (e.g. see Crisp and Meadows, 1962, 1963).

Neal and Yule (1992) demonstrated a link between the tenacity of temporarily attached *Semibalamus balanoides* cyprids and their propensity to settle. Goodrick (1994) found the mean tenacity of *P. pollicipes* cyprids of 2-8 days in age was *ca.* 3 x 10^4 N m^{-2} on various substrata, including conspecific integument and arthropodin treated slate, somewhat lower than that for three balanomorph species at 4-14 x 10^4 N m^{-2} on similar substrata. *P. pollicipes* inhabit areas of strong wave action thus one might expect that given a suitable stimulus they would exhibit much greater tenacity and, hence, propensity to settle. Pineda's (1994) work suggests that strong flow with suitable surface topography may be important for the settlement response of the closely related *P. polymerus* (Darwin, 1851, Newman, 1987, Foster and Buckeridge, 1987) in the field. Factors that influence the fixation of the cyprids of balanomorph barnacles include light intensity, water flow, surface texture, microbial and microalgal films and chemical cues (Walker, 1995). The cyprids of balanomorph barnacles are thus highly developed for selection of a suitable settlement site using many cues (see Walker, 1995) and it is not inconceivable that

the more primitive pollicipids may use fewer and/or more easily determined cues such as flow.

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APPENDIX

Data are contained in MS-Dos text files on the accompanying disc in column format. The following describes the contents of each file where C1 denotes column 1 *etc*.

FILE01.DAT: Measured dimensions of laboratory cultured *Pollicipes pollicipes* larval stages:
C1, Cephalic shield width (μm)
C2, Total length (μm)
C3, Metanauplii cephalic shield length (μm)
C4, Predicted stage after reiterated discriminatory analysis

FILE02.DAT: Measured dimensions of stage II and III *P. pollicipes* larvae identified through shape and antennule setation formula.
C1, Cephalic shield width (μm)
C2, Total length (μm)
C3, Stage

FILE03.DAT: Measured dimensions of stage I and II *P. pollicipes* larvae hatched from a field population on the Algarve coast.
C1, Cephalic shield width (μm)
C2, Total length (μm)
C3, Stage

FILE04.DAT: Capitulum dimensions of adult *P. pollicipes* attached to plastic plates employed in the study of active relocation.
C1, Rostro-Carinal length (RC, mm)
C2, Width (W, mm)
C3, Thickness (T, mm)
C4, Final RC length (mm)
C5, Final W (mm)
C6, Final T (mm)
C7, Animal identification n°.

FILE05.DAT: Repeated location measurement for *P. pollicipes* attached to plastic plates.
C1, Size class
C2, C4 and C6, Repeated x coordinate measurement
C3, C5 and C7, Repeated y coordinate measurement

FILE06.DAT: Measured locations over time of *P. pollicipes* attached to plastic plates which were maintained vertically and horizontally in a flume tank.
C1, Time (days)
C2, X coordinate (axis perpendicular to the water flow; positive values represent upward movement for vertically fixed animals)
C3, Y coordinate (positive values represent movement into the flow)
C4, Animal identification n°.
C5, Animal attitude; 0 = vertically fixed, 1 = horizontally fixed.

FILE07.DAT: Weight with time of *P. pollicipes* cement masses kept in a flowing seawater tank.
C1, Time (days)
C2, Wet weight (μg)

FILE08.DAT: Weight with time of sterile lepadomorph cement in repeatedly autoclaved seawater.
C1, Time (days)
C2, *P. pollicipes* cement wet weight (mg)
C3, *Capitulum mitella* cement wet weight (mg)
C4, Combined species cement wet weight (mg)

FILE09.DAT: Lepadomorph cement weights with time in the presence of bacterial isolates.
C1, Time (days)
C2, *C. mitella* cement wet weight (μg)
C3, Bacterial isolate
C4, Time (days)
C5, *P. pollicipes* cement wet weight (μg)
C4, Bacterial isolate

FILE10.DAT: CN autoanalyser calibration data.
C1, Acetanilide dry weight (μg)
C2, Formula calculated carbon dry weight (μg)
C3, Formula caculated nitrogen dry weight (μg)
C4, Peak area for carbon
C5, Peak area for nitrogen

FILE11.DAT: Lepadomorph cement mass weights used to measure carbon and nitrogen content.

C1, Cement mass dry weight (μg)

C2, Peak area for carbon content

C3, Peak area for nitrogen content

C4, Species; 1 = *C. mitella*, 2 = *P. pollicipes*.

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SHORT COMMUNICATIONS

MOBILITY IN LEPADOMORPH BARNACLES

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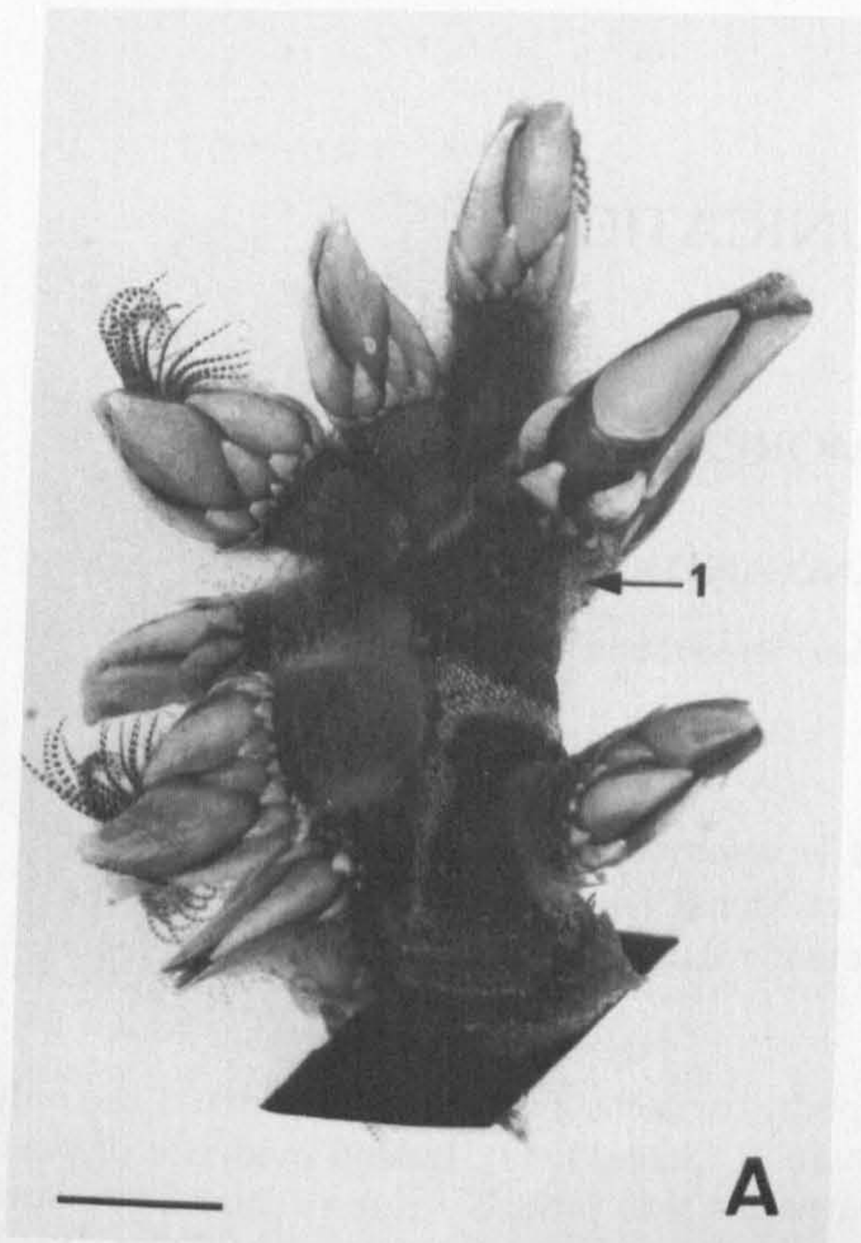
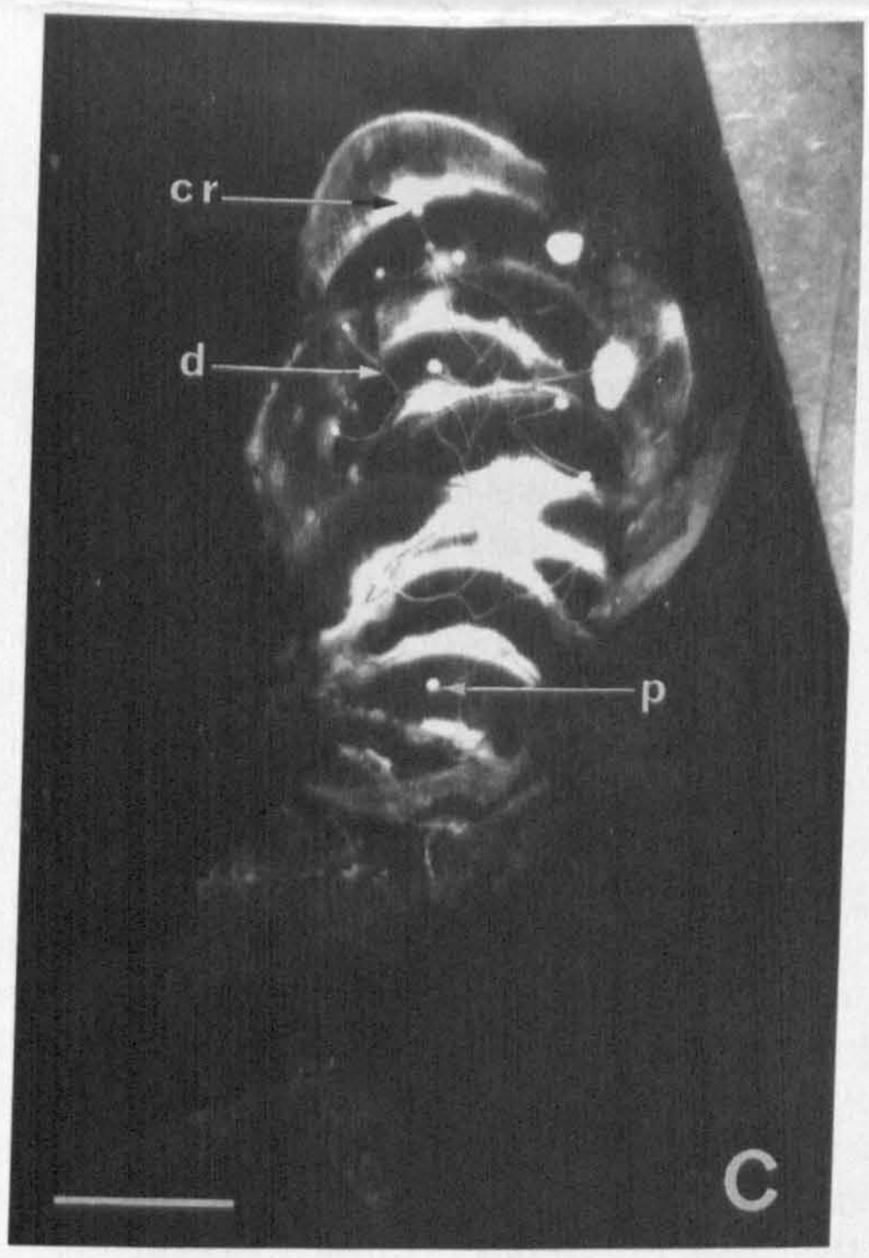
Circumstantial evidence of mobility by the lepadomorph barnacle genus *Pollicipes* in the literature is supported by observations at Menai Bridge which indicate that the phenomenon is real. Although slow, movement may be in response to gravity, as a positive taxis, or light, as a negative taxis.

Except for certain parasitic copepods and isopods, barnacles have been considered the only crustaceans that are sessile when adult (Waterman & Chace, 1960). Indeed evidence of post-settlement mobility in juvenile balanomorph barnacles was probably due to lateral pressure exerted by adjacent barnacles forcing flow in an essentially viscoelastic adhesive (Crisp, 1960). Lepadomorph barnacles, however, with flexible muscular stalks, have the potential to exhibit true mobility. Tracks of cement, indicative of such true mobility, were observed on adult *Pollicipes pollicipes* (Gmelin) bearing attached juveniles. These adults were collected from Portugal and maintained in tanks at Menai Bridge.

Indirect evidence for lepadomorph mobility has been reported in the literature. Hoffman (1984) found that *P. polymerus* juveniles (<7 mm total contracted length) settled on adults were distributed such that their relative position on the adult peduncle was directly proportional to their size. The smallest juveniles were always the most abundant and clustered close to the capitulum. He noted that such a distribution could result from vertical adult growth near the capitulum, but it would not explain how large juveniles were found attached to the basal disc, a region where small juveniles were only rarely found. Chaffee & Lewis (1988) found that the two most mitotically active regions in the peduncle of *P. polymerus* were immediately below the capitulum/peduncle junction and at the stalk base. Lateral extensions of the base are common in *Pollicipes* and rapid somatic growth is indicated within these extensions (Chaffee & Lewis, 1988). Such extensions were most obvious in juveniles attached to adult peduncles, but also present in adults within clusters. Only one extension per animal was reported. Possession of a peduncular extension and degree of disc thickening seemed to be proportional to wave action, suggesting that these basal modifications were adaptations to increase adhesion through a greater adherand surface area. Hoffman (1984) had earlier suggested that these extensions may be forced downward by peduncular haemolymph pressure to seek a new or enlarged attachment site. Evidence from Fyhn *et al.* (1973), Crenshaw (1979) and Walker & Anderson (1990) indicates that lepadomorph barnacles generally exhibit higher haemolymph pressure than acorn barnacles. Crenshaw (1979) noted how the peduncle of *P. polymerus* seemed well designed to sustain high internal pressure. He found the integument to stretch equally well in longitudinal and circumferential directions under relatively low, sustained stresses. Such properties would be necessary in the development of true mobility. Chaffee & Lewis (1988) noted that basal extensions thickened and seemed to release cement, but could not determine the cement's origin. The question must arise as to





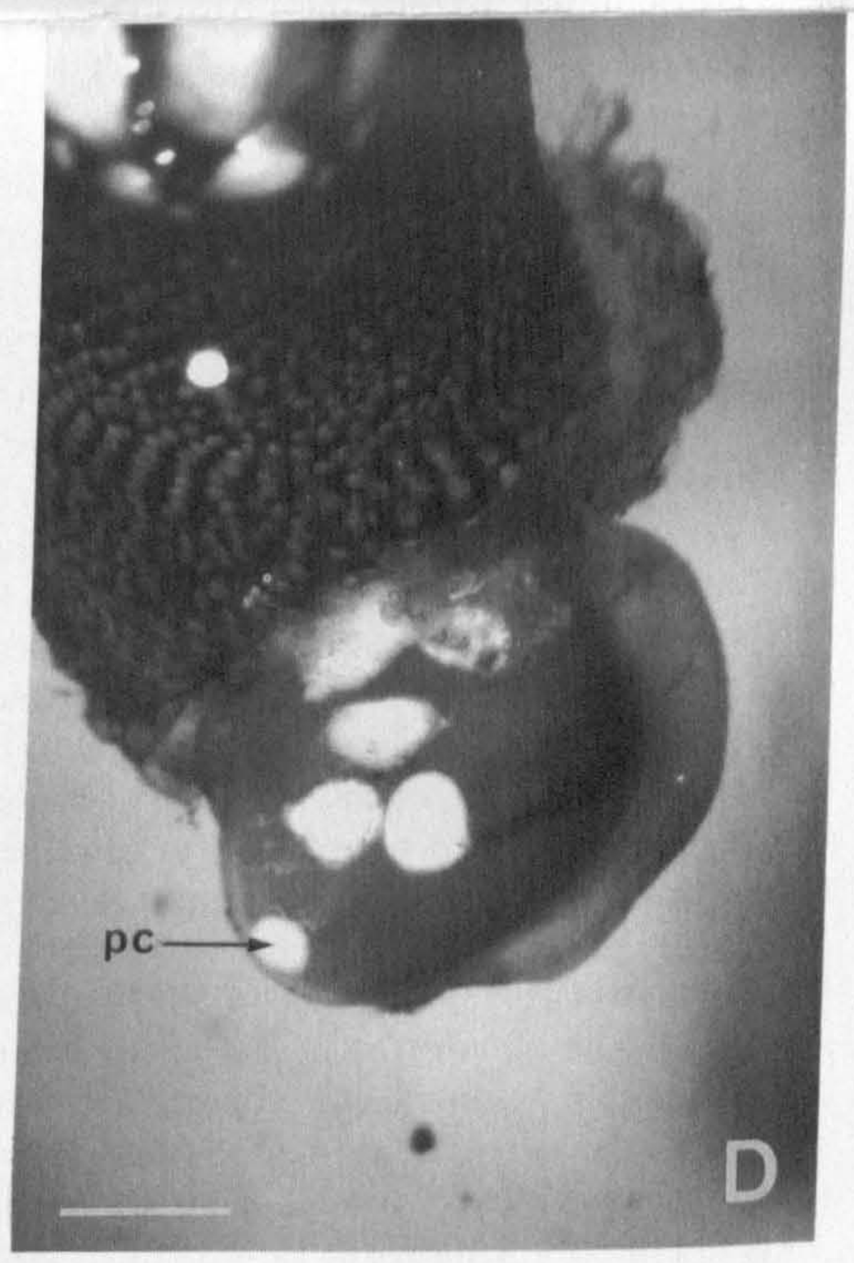


Figure 1. Mobility in *Pollicipes pollicipes*. (A) Rear view of an adult bearing younger mobile animals as indicated by tracks of residual cement. Arrow 1 indicates start of a track. Scale bar: 10 mm. (B) Front

whether the organism is indeed simply increasing its attachment area in response to environmental conditions, or whether there is directed mobility in a real, if slow, sense.

The clear tracks observed on animals kept at Menai Bridge and shown in Figure 1A,B, increasing in width (presumably with growth) with increasing distance from the start of the track, do indicate directed locomotion. The mechanism of mobility may then be an interaction of muscular activity and growth. The mitotically active basal region could produce new tissue, which may be distorted by high haemolymph pressure into the direction of travel. This directional outgrowth would then become attached to the substratum by the flow of proteinaceous cement and secondarily thickened. Directionality may be maintained through reduced growth at the trailing region of the base combined with muscular activity shearing old adhesive.

The direction taken by juveniles could be a response to gravity (positive taxis) or light (negative taxis) since tracks generally indicate movement of juveniles downward. For example a track begins from near the capitulum in Figure 1A (arrow 1), increasing in width to midway down the peduncle in Figure 1B (arrow 2). When the larger adults were removed from an upright position on the Portuguese shore and suspended upside down in tanks, attached juveniles showed a clear change in track direction (Figure 1B, arrow 3) toward the capitulum (also downward). The movement results in a more even distribution of like-sized young animals on the adult (Figure 1A,B) contrary to Hoffman's (1984) pattern for *P. polymerus*.

Early attempts to measure locomotion of animals initially fixed to glass plates with cyanoacrylate adhesive (Loctite) and epoxy resin (Araldite Rapid) have been less than conclusive. Two small specimens of *P. pollicipes*, rostro-carinal length 6·9 mm and 8·3 mm, maintained in a constant sea-water flow of ~8 *cm* s^{-1} in a flume, moved some 3-4·5 mm averaged over a 55-d period (0·06-0·08 mm d^{-1}). Insufficient data presently precludes precise calculation of mean speed or direction of travel, or whether locomotion is continuous or spasmodic. A more comprehensive study is currently under way. The number and distribution of cement ducts and pores in the basis is also under investigation with evidence so far suggesting that lateral extensions do carry cement ducts and exit pores. Figure 1C shows the dissected base of an animal with a network of ducts (d) and pores (p) with obvious indications of extensions with delivered cement, and Figure 1D shows a basal extension with six discrete masses of polymerized cement (pc) indicating six exit pores and associated ducts within the extension. Figure 1C further shows what appears to be a series of extensions, marked by ridges of cement (cr) indicating a directed path of travel from bottom to top of the picture. Whether the phenomenon of mobility is restricted to juveniles, and which stimuli initiate and control the direction of locomotion are also being considered.

The mobility of these lepadomorphs follows cypris settlement amongst the calcareous scales of the upper peduncle of an adult. Such a strategy would afford a degree of protection from predators and better extension into food-bearing water for the juveniles. As the juveniles grow the cirri will be maintained in the flow and the animals will eventually move off the host peduncle ensuring adults are not smothered by continued gregarious settlement and that recruited juveniles are not lost through death of the adults.

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view of the same animal showing direction change of an attached younger animal. Arrow 2 indicates direction of travel in natural environment, arrow 3 indicates direction after inversion of the group. Scale bar: 10 mm. (C) Dorsal view of base attached to black plastic (Darvic) with all tissue removed except ducts (d) and basal cuticle with pores (p). Polymerized cement appears bright white as cement ridges (cr), indicating development of lateral extensions in stages. Scale bar: 2 mm. (D) Dorsal view of an unattached basal extension with discrete masses of polymerized cement (pc). Scale bar: 2mm.

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