J. mar. biol. Ass. U.K. (1995), **75**, 363-372
Printed in Great Britain

PERIOSTRACAL ADVENTITIOUS HAIRS ON SPAT OF THE MUSSEL *MYTILUS EDULIS*

D.R. DIXON*, A.M. SOLE-CAVA†, P.L. PASCOE* AND P.W.H. HOLLAND‡[]

*Plymouth Marine Laboratory, Citadel Hill, Plymouth, PL1 2PB. †Departmento de Genetica, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Bloco A - CCS - Ilha do Fundao, 21941 Rio de Janeiro-RJ, Brazil. ‡Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS. []Present address: School of Animal and Microbial Sciences, University of Reading, Whiteknights, Reading, RG6 2AJ

Morphological and genetic evidence is presented which supports the existence of periostracal adventitious hairs on spat of the mussel *Mytilus edulis*. This character appears not to have been reported previously for *Mytilus*, and was thought to be restricted to a closely-related genus, *Modiolus*. The species identity of hairy mussel spat was confirmed by PCR (polymerase chain reaction) amplification of a diagnostic portion of the nuclear ribosomal DNA repeat unit (i.e. the ITS-2 region). Size-frequency analysis of spat, sampled in mid-September 1993, from rock pools and from the byssus of a nearby adult mussel bed, showed that hairy spat (mean shell length 1·87 mm, SE 0·17) were significantly (t=7·74; P<0·001) smaller than smooth-shelled spat (mean shell length 2·77 mm, SE 0·28), although not all small-sized individuals displayed this character. These findings suggest that there is a gradual loss of hairs (through abrasion or by 'programmed' loss) as the animal grows. We suggest that this character has some adaptive significance since it probably reduces predation by boring gastropods (e.g. juvenile *Nucella lapillus*) and may inhibit fouling, particularly by conspecifics, during the primary settlement phase.

INTRODUCTION

Mollusc shells are generally composed of a thin outer, organic layer, the periostracum, which stabilizes and protects a more massive, inner, mineralized portion (Clark, 1976). Based on dry weight, the inorganic part of the shell in some bivalves and gastropods represents over 99% of its total mass (Clark, 1976; Hughes, 1970); this compares with a lower percentage in other, hard-shelled invertebrates (e.g. Dixon, 1980). Commonly the shell is elaborated into a series of ridges or spines used for defence or in burrowing (see Stanley, 1988, for a review). Given its variety of form and extreme resistance to decay, it is not surprising that these calcareous shell characters feature significantly in systematic descriptions of both living and extinct mollusc groups (Soot-Ryen, 1955; Yonge, 1976). In contrast, those less resistant and more fragile structures associated with the organic part of the shell feature much less in molluscan systematics.

Projecting periostracal structures (adventitious hairs, shingles and thorns) are a feature of several bivalve superfamilies, namely the Arcoida, the Mytiloida and the Veneroida (Watabe, 1988). Here we report the discovery of adventitious hairs (i.e. derived from the periostracum, see Carter & Aller, 1975) on mussel spat (*Mytilus edulis*

L.), a feature which appears not to have been reported previously for this species. Adventitious hairs are, however, a well known characteristic of a closely-related genus, *Modiolus* (Soot-Ryen, 1955; Tebble, 1966; Carter & Aller, 1975).

MATERIALS AND METHODS

Source of animals

A collection of mussel spat and adults was made from Whitsand Bay, south-east Cornwall, on 23 September 1993. Whitsand Bay is a wave-exposed shore consisting of a sloping sandy beach with large rocky outcrops which support an extensive mussel bed. Initially the hairy spat were found attached to the fronds of coralline algae (*Corallina* sp.) fringing the sides of rock pools at approximately mid-tide level. However, similar spat were later discovered attached to the byssus mats of the nearby mussel bed (*Mytilus edulis*). For comparative purposes, adults of *M. edulis*, *Modiolus modiolus* (L.) and *Modiolus barbatus* (L.) were also collected from the Isle of Man.

Animal handling and DNA extraction

After measurement to the nearest 0·1 mm with a calibrated graticule eyepiece, specimens of hairy and smooth-shelled spat were placed in individual 1·5-ml microfuge tubes containing 500 µl of a buffer compatible with both proteinase K and *Taq* DNA polymerase activity (50mM KCl, 10mM Tris-HCl pH 8·3, 2·5mM $MgCl_2$, 0·1 mg ml^{-1} gelatin, 0·45% Nonidet-P40, 0·45% Tween-20; Higuchi, 1989). The shells were broken, using a clean, blunt mounted needle, before adding 5–10 µl of 10 mg ml^{-1} proteinase K, depending on the size of the spat. The samples were incubated at 55°C for 3–4 h and heated at 95°C for 10 min. Polymerase chain reaction analysis was conducted using 1–4 µl of sample per reaction. Gill tissue was removed for DNA extraction from several adult specimens of *Mytilus edulis*, *Modiolus modiolus* and *M. barbatus*. High-molecular-weight DNA was purified by overnight digestion with proteinase K followed by repeated phenol/chloroform extractions (Sambrook et al., 1989). After ethanol precipitation, DNA was vacuum dried and dissolved overnight in TE (10mM Tris, 1mM EDTA, pH 8), and stored at 4°C.

Polymerase chain reaction amplification. Two oligonucleotide primers, 'PHI9' (positive strand primer) and 'ITS2' (negative strand primer) were used for PCR amplification of the internal transcribed spacer-2 (ITS-2) separating the 5·8S and 28S ribosomal genes. These were designed as 'versatile' PCR primers, with the potential to amplify the ITS-2 region of most eukaryotes.

The primer sequences are:

PH19 (5' to 3'): CATCGACACTT(T/C)GAACGCA

ITS2 (5' to 3'): AATCCTGGTTAGTTTCTTTTCCTCCGCT.

Amplification reactions were performed in a volume of 15–20 µl using standard conditions (Holland, 1993). Products of PCR were visualized on 2% agarose gels and

fragment sizes were estimated graphically based on the running distances of a standard 1 kb molecular-weight marker (Gibco BRL).

Scanning electron microscopy. Spat shells were fixed in 70% ethanol, dehydrated with acetone, substituted with liquid CO_2 and then critical-point dried. Shells were coated with gold before viewing and photographing using a JEOL JSM 35C microscope.

RESULTS

The mussel spat used in this study were originally collected for a genetic survey of nuclear DNA variation in *Mytilus edulis* in the UK (Sole-Cava et al., in preparation). Some of the mussel spat collected from rock pools in Whitsand Bay, south-east Cornwall, were found to possess prominent adventitious hairs on their shell surfaces. Given the tendency for the horse mussel, *Modiolus modiolus* and the bearded mussel *M. barbatus*, to extend their range up the sea-shore by inhabiting intertidal rock pools (Lewis, 1964; Tebble, 1966; Seed & Brown, 1975), our first impression was that the 'hairy' spat may be the young of either of these two species. However, we also found hairy spat adhering to the byssus mats of a nearby, aerially exposed mussel bed (*Mytilus edulis*), suggesting that we could be dealing with an unusual variety of juvenile *M. edulis*.

Figure 1 shows details of the shells of hairy and smooth-shelled mussel spat from Whitsand Bay, south-east Cornwall, plus an enlargement of a small group of adventitious hairs to show their typical form and structure (see also Bottjer & Carter, 1980); this contrasted with the more uniform diameter and extremely variable size of the byssus threads. A byssus thread can be seen attached to the smooth-shelled spat in Figure 1B.

To determine the taxonomic identity of the hairy and smooth spat, we used genetic techniques. Due to their small size, mussel spat do not lend themselves to conventional genetic analysis using allozyme electrophoresis, unless laborious laboratory culture techniques are first employed (e.g. Beaumont, 1991). Instead, therefore, we attempted to identify the unusual spat through analysis of nuclear DNA variation, detected using the sensitive polymerase chain reaction (PCR; Saiki et al., 1988). Polymerase chain reaction is well suited for analysing minute amounts of DNA and has been used previously to study the DNA of early life-history stages of marine invertebrates (Olson et al., 1991; Côrte-Real et al., 1994b; Dixon et al., 1995).

First we used PCR to determine the length of a diagnostic segment of the nuclear ribosomal DNA (rDNA) repeat, the ITS2 region, in the genome of adult *Mytilus edulis*, *Modiolus modiolus* and *M. barbatus*.

Figure 2 shows PCR amplification products (i.e. ITS-2 region) based on genomic DNA extracted from replicated specimens of the northern horse mussel, *Modiolus modiolus*, the bearded horse mussel, *M. barbatus*, and the edible mussel, *Mytilus edulis*. These specimens were all identified based on their gross shell characteristics (Soot-Ryen, 1955; Tebble, 1966).

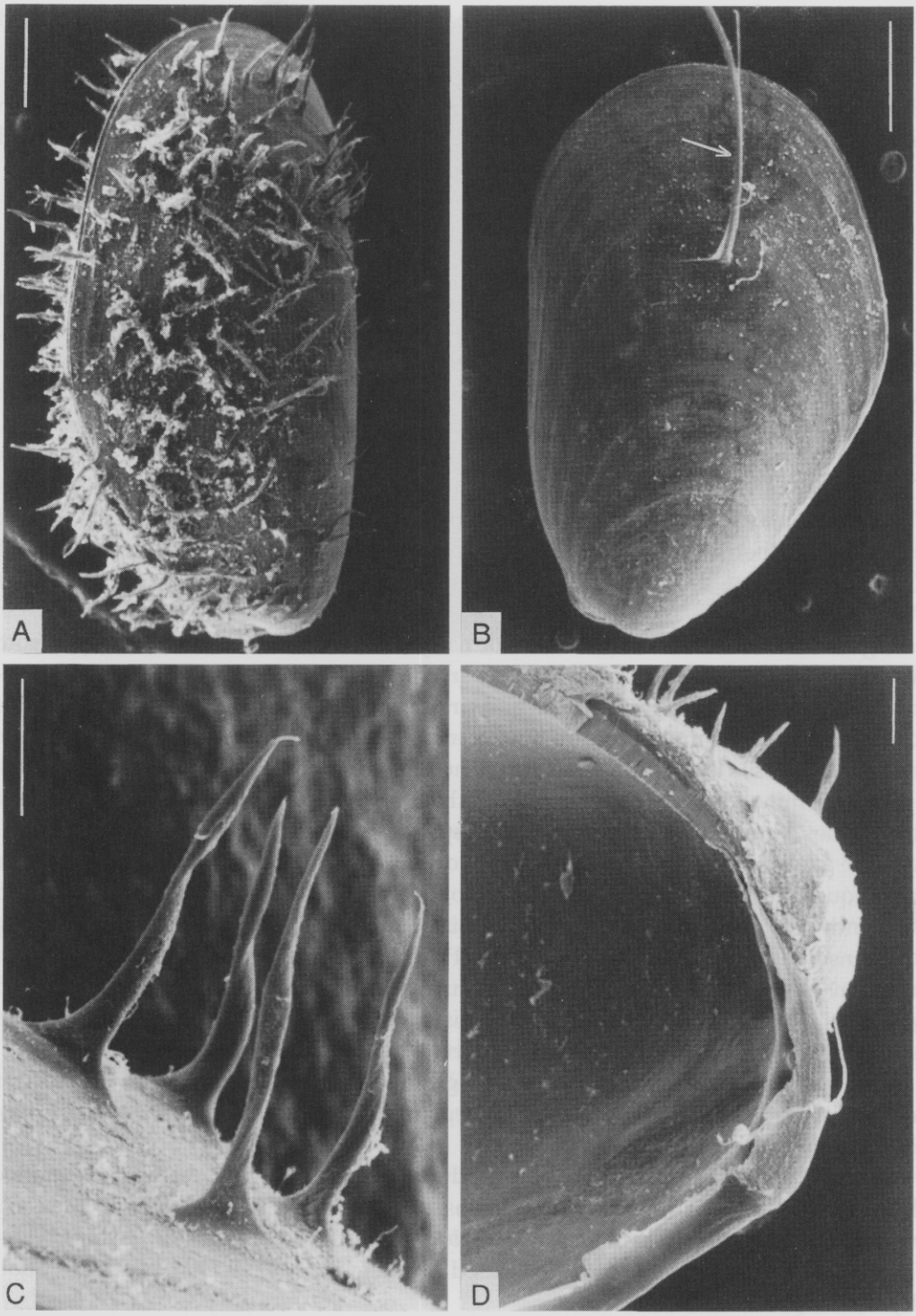

Figure 1. (A & B) External views of the shells of hairy and smooth-shelled spat from Whitsand Bay, south-east Cornwall. The smooth shell valve has a byssus thread (arrowed) attached to it.

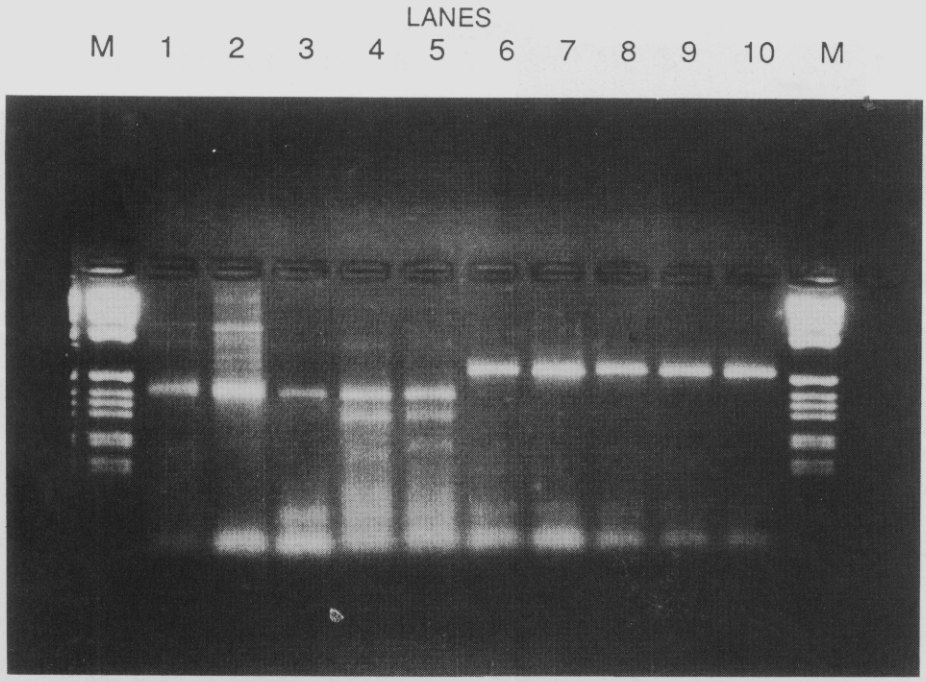

Figure 2. Polymerase chain reaction amplification of ITS2 region from adult bivalves. Agarose gel stained with ethidium bromide and fluoresced with ultra-violet light to show PCR products. Lanes 1–3, *Modiolus modiolus*; 4–5, *M. barbatus*; 6–10, *Mytilus edulis*. All were adult specimens from the Isle of Man. M, molecular weight markers (Gibco BRL 1 kb ladder).

The DNA from each animal yielded at least one strong band after PCR amplification using the ITS-2 primers (Figure 2). There were significant differences between all three species both in the number and size of the amplified fragments. However, there was good agreement between individuals within species with respect to the banding patterns observed.

Since the ITS2 PCR amplification pattern is diagnostic for each species, we applied the technique to DNA from approximately 50 hairy and 100 smooth-shelled spat from Whitsand Bay. Figure 3 shows representative results for the PCR amplifications carried out on adult specimens of *Modiolus modiolus* and *Mytilus edulis* and on representative hairy and smooth-shelled spat. Unlike the previous experiment, the adult mussels used here came from Whitsand Bay, the same locality which yielded the hairy spat. There was no difference in the size (~520 bp) of amplification products obtained for *M. edulis* from Whitsand Bay and Port Erin (cf. Figures 2 & 3). Nor was there any difference between the size of this band and the products obtained for the hairy and smooth-shelled spat (Figure 3). This clearly demonstrates that the hairy spat are *Mytilus* and not *Modiolus*.

(C) Scanning electron photomicrograph of a small group of adventitious hairs. (D) Scanning electron microscope enlargement of the hinge and umbone region of a hairy mussel spat shell. Scale bars: A & B, 0·5 mm; C & D, 0·1 mm.

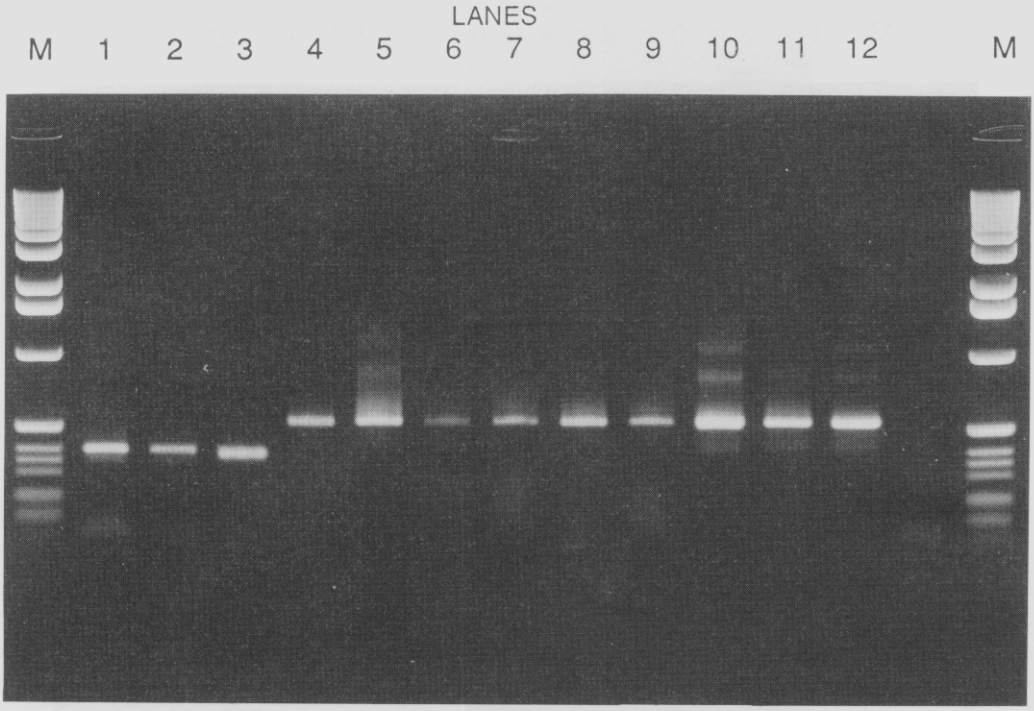

Figure 3. Polymerase chain reaction amplification of ITS2 region from adult bivalves and mussel spat. Lanes 1–3, *Modiolus modiolus*; 4–6, hairy mussel spat; 7–9, smooth-shelled mussel spat; 10–12, Whitsand Bay adult *Mytilus edulis*. M, molecular weight markers (Gibco BRL 1 kb ladder).

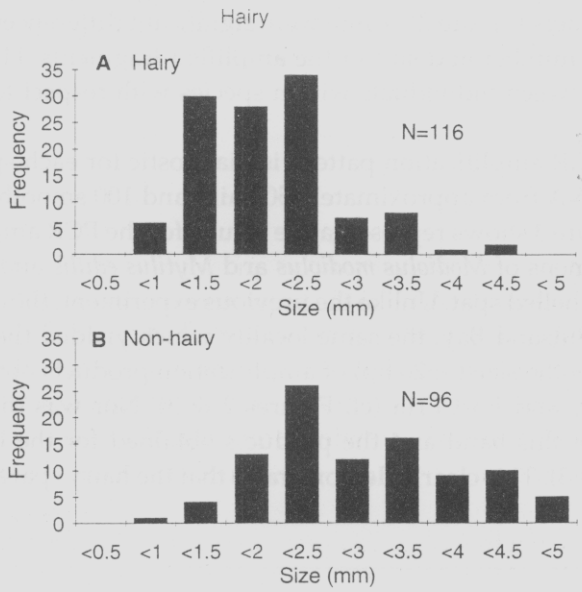

Figure 4. Size-frequency distributions for (A) hairy and (B) smooth-shelled mussel spat sampled in September 1993.

Figure 4 shows size-frequency histograms based on a sample size of 212 hairy and non-hairy mussel spat collected at random from a rock pool and from the byssus threads of an adjacent mussel bed. A preliminary investigation revealed no difference in the frequency of hairy spat in rock pools compared with the main mussel bed. In a recent study it was estimated that approximately 70% of the mussel spat at Whitsand Bay are hairy; this frequency varies in different locations (J. Wills, personal communication). There was a significant difference (t=7·74; P<0·01), however, in the size-frequency distribution of the two types of spat (Figure 4). While not all the smallest spat showed signs of hairs, the distribution data clearly indicate that it was the smallest individuals which were the more affected. Clearly the spat population in Whitsand Bay comprises two distinct phenotypes.

DISCUSSION

The species identity of hairy mussel spat (*Mytilus edulis*) has been confirmed using PCR-amplification of a diagnostic region of the nuclear rDNA repeat unit. Sequencing of internal transcribed spacer regions of rDNA has proved a successful approach to species identification particularly in cases where morphological confusion has existed previously (e.g. with rock oysters, Anderson & Adlard, 1994). Our findings relating to length differences in the ITS2 region of *Mytilus* and *Modiolus* spp. show the great potential of PCR-based strategies for the identification of early life-history stages of marine invertebrates (see also Côrte-Real et al., 1994b; Dixon et al.,1995). Bivalves such as *Mytilus* are extremely variable in their shell morphology (e.g. Beaumont et al., 1989) and there is a need for new (e.g. molecular) methods for resolving species identities. Polymerase chain reaction is becoming increasingly suited to this purpose with the advent of portable instruments, and the availability of non-destructive sampling methods (e.g. Côrte-Real et al., 1994a).

Possible functional role of hairs

Recently Harper & Skelton (1993) proposed a defensive role for the thickened periostracum in the Mytiloidea, particularly as a defence against boring predation by muricid gastropods (*Nucella lapillus* and *Morula musiva*), and Wright & Francis (1984) have experimentally demonstrated that the periostracal awns (long hairs) of *Modiolus modiolus* inhibit pedal attachment of *N. lapillus*. Bottjer & Carter (1980) listed the possible roles of periostracal and adventitious 'hirsute' structures in molluscs as follows: (1) protection of the shell from encrusting and boring organisms, (2) stabilization of the shell in the substratum in burrowing forms (e.g. *Modiolus*), (3) camouflage, and (4) protection of the mantle margins and extension of the range of mantle tactile perception. To these we would add: (5) a buoyancy aid for spat during secondary settlement, (6) a spacing mechanism to reduce competition during filter feeding, and (7) a mechanism to prevent fouling by byssus threads produced by conspecifics.

It is already recognized that many mussel populations undergo two distinct settlement phases as part of their normal reproductive cycle (Bayne, 1964; King et al., 1989;

reviewed by Seed & Suchanek, 1992). Primary settlement commonly takes place on the fronds of filamentous red algae some distance from the main mussel bed (e.g. King et al., 1989), whence they are transported to their final settlement site by water currents (hence the need for buoyancy). Secondary settlement could not proceed if the small mussels were to become too firmly anchored at their primary settlement site. While spat appear to have no difficulty in freeing themselves from their own byssus threads, this does not apply to the byssus threads secreted by other (particularly larger) mussels (Figure 1B). Our recent studies have shown a higher frequency of byssus threads attached to smooth-shelled spat compared to hairy spat (Dixon et al., in preparation).

Taxonomic significance of hairs

Soot-Ryen (1955), in his comprehensive review of the systematics of the Mytilidae, makes no reference to adventitious hairs in the genus *Mytilus*. More recently Bottjer & Carter (1980) did, however, mention adventitious hairs in relation to *M. edulis*, but an earlier reference which they cite in support of this statement (Carter & Aller, 1975) makes no mention of this phenomenon! The fossil record for *M. edulis* goes back less than two million years (Seed, 1976), and the genus is thought to have evolved from a more primitive infaunal or semi-infaunal modiolid stock (Soot-Ryen, 1969; Stanley, 1972; Bottjer & Carter, 1980; Seed, 1990). Adventitious hairs remain a conspicuous shell character within the genus *Modiolus*, where they function in both the juvenile and adult stages to stabilize the shell in soft substrates (e.g. Tebble, 1966). Our findings indicate that, despite its preference for hard substrates, adventitious hairs have not been lost entirely by *Mytilus edulis*, but have become restricted to (some) spat where, among other things, they may reduce predation by juvenile *N. lapillus* and reduce fouling in the period between primary and secondary settlement.

We consulted a number of UK mussel experts as to their views on 'hairy' mussel spat. Several replied that they had not seen this character, "despite having examined thousands of mussel spat", while others had seen it but had thought the spat were *Modiolus*. These comments, albeit anecdotal, indicate that there could be significant variation in hairiness amongst mussel spat in different parts of the UK; our recent work has shown this to be the case (Dixon et al., in preparation).

It should be noted that not all the small mussel spat we examined from Whitsand Bay showed signs of adventitious hairs (see Figures 3 & 4). Since south-eastern Cornwall lies within the hybrid zone for *Mytilus edulis/M. galloprovincialis* genotypes in south-western England (see Skibinski et al., 1978; Beaumont et al., 1989; Gosling, 1992, for a review), it would be interesting to know how this shell character relates to those other morphological characters which have already been used to separate these two forms of mussel (e.g. Beaumont et al., 1989).

We are grateful to Steve Hawkins (Port Erin Marine Laboratory) for providing mytilids from the Isle of Man; to Linda Dixon for skilled technical assistance; to David Nicholson for photographic assistance; and to those anonymous 'mussel experts' who spoke candidly about their experiences with the 'hairy' mussel. We are also grateful to Rupert Lewis for providing information on the distribution and genetics of *Modiolus* in the Isle of Man. Professor A.J. Southward

kindly commented on an earlier draft of this paper, This work was supported in part by a NERC GAMES research grant awarded to D.R.D. and P.W.H.H., and by a grant awarded to A.M.S.C. by Conselho Nacional de Pesquisas, CNPq, Brazil.

REFERENCES

Anderson, T.J. & Adlard, R.D., 1994. Nucleotide sequence of a rDNA internal transcribed spacer supports synonymy of *Saccostrea commercialis* and *S. glomerata*. *Journal of Molluscan Studies*, **60**, 196–197.

Bayne, B.L., 1964. Primary and secondary settlement in *Mytilus edulis* L. (Mollusca). *Journal of Animal Ecology*, **33**, 513–523.

Beaumont, A.R., 1991. Genetic studies of laboratory reared mussels, *Mytilus edulis*: heterozygote deficiencies, heterozygosity and growth. *Biological Journal of the Linnean Society of London*, **44**, 273–285.

Beaumont, A.R., Seed, R. & Garcia-Martinez, P., 1989. Electrophoretic and morphometric criteria for the identification of the mussels *Mytilus edulis* and *M. galloprovincialis*. In *Reproduction, genetics and distributions of marine organisms* (ed. J.S. Ryland and P.A. Tyler), pp. 251–258. Denmark: OIsen & Olsen. [Proceedings of the 23rd European Marine Biology Symposium, University of Wales, Swansea.]

Bottjer, D.J. & Carter, J.G., 1980. Functional and phylogenetic significance of projecting periostracal structures in the Bivalvia (Mollusca). *Journal of Palaeontology*, **54**, 200–216.

Carter, J.G. & Aller, R.C., 1975. Calcification in the bivalve periostracum. *Lethaia*, **8**, 315–320.

Clark, G.R., 1976. Shell growth in the marine environment: approaches to the problems of marginal calcification. *American Zoologist*, **16**, 617–626.

Côrte-Real, H.B.S.M., Dixon, D.R. & Holland, P.W.H., 1994a. Intron-targeted PCR: a new approach to survey neutral DNA polymorphism in bivalve populations. *Marine Biology*, **120**, 407–413.

Côrte-Real, H.B.S.M., Holland, P.W.H. & Dixon, D.R., 1994b. Inheritance of a nuclear DNA polymorphism assayed in single bivalve larvae. *Marine Biology*, **120**, 415–420.

Dixon, D.R., 1980. The energetics of tube production by *Mercierella enigmatica* (Polychaeta: Serpulidae). *Journal of the Marine Biological Association of the United Kingdom*, **60**, 655–659.

Dixon, D.R., Jollivet, D.A.S.B., Dixon, L.R.J., Nott, J.A. & Holland, P.W.H., 1995. The molecular identification of early life-history stages of hydrothermal vent organisms. In *Hydrothermal vents and processes. Proceeding of a BRIDGE symposium, April 1994.* (ed. L.M. Parson et al.), pp. 343–350. London: Geological Society. [Special Publication no. 87.]

Gosling, E., ed., 1992. *The mussel* Mytilus: *ecology, physiology, genetics and culture*. Amsterdam: Elsevier.

Harper, E.M & Skelton, P.W., 1993. A defensive value of the thickened periostracum in the Mytiloidea. *Veliger*, **36**, 36–42.

Higuchi, R., 1989. Simple and rapid preparation of samples for PCR. In *PCR Technology*, (ed. H.A. Erlich), pp. 31–38. New York: Stockton Press.

Holland, P.W.H., 1993. Cloning genes using the polymerase chain reaction. In *Essential developmental biology: a practical approach* (ed. C.D. Stern and P.W.H. Holland), pp. 243–255. IRL Press at Oxford University Press.

Hughes, R.N., 1970. An energy budget for a tidal-flat population of the bivalve *Scrobicularia plana* (Da Costa). *Journal of Animal Ecology*, **39**, 357–381.

King, P.A., McGrath, D. & Gosling, E.M., 1989. Reproduction and settlement of *Mytilus edulis* on an exposed rocky shore in Galway Bay, west coast of Ireland. *Journal of the Marine Biological Association of the United Kingdom*, **69**, 355–365.

Lewis, J.R., 1964. *The ecology of rocky shores*. London: Hodder & Stoughton.

Olson, R.R., Runstadler, J.A. & Kocher, T.D., 1991. Whose larvae? *Nature, London*, **351**, 357–358.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science, New York*, **239**, 487–491.

Sambrook, J., Fritsch, E.F. & Maniatis, T., 1989. *Molecular cloning: a laboratory manual*, vol. 1, 2nd ed. Cold Spring Harbour Laboratory Press.

Seed, R., 1976. Ecology. In *Marine mussels: their ecology and physiology* (ed. B.L. Bayne), pp. 13–65. Cambridge University Press.

Seed, R., 1990. Taxonomic and evolutionary relationships within the genus *Mytilus*. In *The Bivalvia – Proceedings of a Memorial Symposium in honour of Sir Charles Maurice Yonge 1986* (ed. B. Morton), pp. 97–110. Hong Kong University Press.

Seed, R. & Brown, R.A., 1975. Growth as a strategy for survival in two marine bivalves, *Cerastodenna edule* and *Modiolus modiolus*. *Journal of Animal Ecology*, **47**, 283–292.

Seed, R. & Suchanek, T.H., 1992. Population and community ecology of *Mytilus*. In *The mussel* Mytilus*: ecology, physiology, genetics and culture* (ed. E. Gosling), pp. 87–169. London: Elsevier.

Skibinski, D.O.F., Ahmad, M. & Beardmore, J.A., 1978. Genetic evidence for naturally occurring hybrids between *Mytilus edulis* and *Mytilus galloprovincialis*. *Evolution*, **32**, 354–364.

Soot-Ryen, T., 1955. A report on the family Mytilidae (Pelecypoda). *Allan Hancock Pacific Expeditions*, **20**, 1–174.

Soot-Ryen, T., 1969. Treatise on invertebrate palaeontology. Part N. *Mollusca*, **6**.

Stanley, S.M., 1972. Functional morphology and evolution of bysally attached bivalve mollusks. *Journal of Palaeontology*, **46**, 165–212.

Stanley, S.M., 1988. Adaptive morphology of the shell in bivalves and gastropods. In *The Mollusca*. Vol. 11. *Form and function* (ed. E.R. Trueman and M.R. Clarke), pp. 105–141. London: Academic Press.

Tebble, N., 1966. *British bivalve seashells: a handbook for identification*. London: The British Museum (Natural History).

Watabe, N., 1988. Shell structure. In *The Mollusca*. Vol. 11. *Form and function* (ed. E.R. Trueman and M.R. Clarke), pp. 69–104. London: Academic Press.

Wright, M.M. & Francis, L., 1984. Predator deterrence by flexible shell extensions of the horse mussel *Modiolus modiolus*. *Veliger*, **27**, 140–142.

Yonge, C.M., 1976. The 'mussel' form and habit. In *Marine mussels: their ecology and physiology* (ed. B.L. Bayne), pp. 1–12. Cambridge University Press.

Submitted 1 July 1994. Accepted 21 October 1994.