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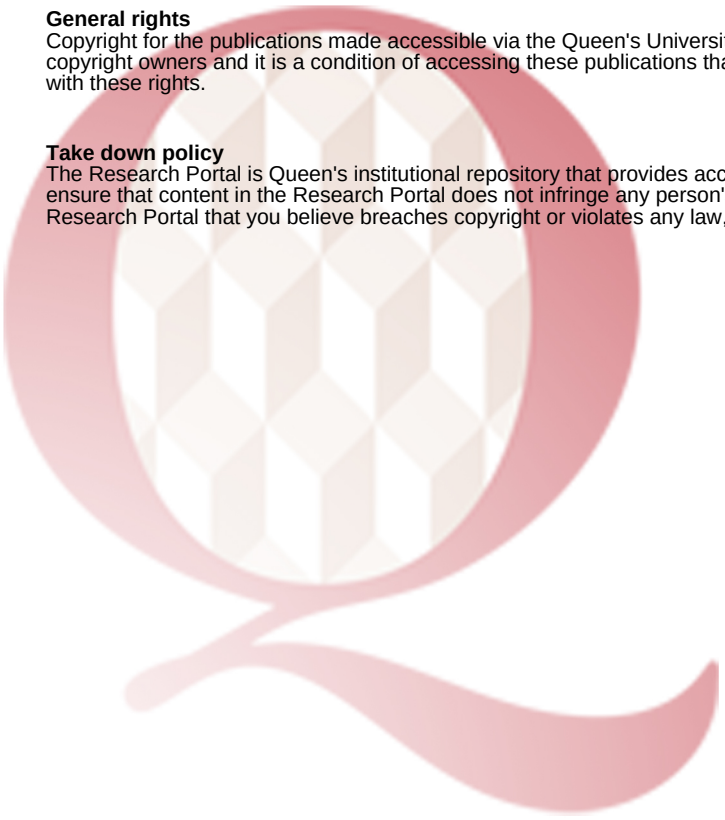

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A Test for Mucus Removal in the Chiton *Lepidochitona cinerea* (Linnaeus, 1767) (Polyplacophora: Chitonida: Ischnochitonidae)

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Abstract. Several methods have been proposed to ''clean'' the soft tissues of molluscs of mucus, so that the surface cilia can be examined microscopically. We report the first empirical test of the effectiveness of methods for removing mucus in the pallial cavity surface of chitons. Three methods were compared, at several time intervals: the enzyme hyaluronidase, the mucolytic agent N-acetyl cysteine (NAC), and seawater washing via the natural action of cilia in excised tissue. Treatment in NAC for 10 min produced the best results, and we recommend this protocol as a starting point for further investigation on mucus removal in a broader suite of taxa. We present the first description of the pallial surface cilia in the chiton *Lepidochitona cinerea*. During the course of this study, we also determined that these chitons were frequently infested with a ciliate protozoan parasite, *Trichodina* sp., which have been historically reported from chitons but never studied in detail. The parasites were absent where antimucus treatments were effective, but their abundance and large size (about 30-μm diameter) in less successful treatments obscured the view of the pallial cavity surface.

INTRODUCTION

Traditionally, molluscan systematics has relied largely on analyses of shell anatomy. More recently, with the improvement of visualization techniques such as SEM imaging, shell features have been extended to microstructural elements in gastropods (e.g., Geiger, 2012), bivalves (e.g., Turner, 2008) and chitons (e.g., Sigwart & Sirenko, 2012). However, another potentially rich source of information that has still not fully exploited for systematics is microanatomy of the soft parts of the body. Previous work on the visualization of soft parts in other molluscan taxa using a scanning electron microscope (SEM) has required the removal of mucus (e.g., Nezlin et al., 1994), but there has been no definitive protocol reported for use on adult chitons, nor any comparison of methods in any taxon. To determine the best way forward to visualize cilia in the pallial cavity of chitons, we reviewed published techniques for a variety of invertebrates and directly tested the effect on the chiton *Lepidochitona cinerea*.

The enzyme hyaluronidase has been reported to digest mucus and similar materials in a variety of taxa including bivalves (Calabro et al., 2005), prosobranchs (Nezlin et al., 1994), caenogastropods (Ronkin, 1952), and onychophorans (Mayer & Harzsch, 2007). However, the efficacy of proteolytic enzymes has been refuted (e.g., Stamm & Docter, 1965), and

hyaluronidase has not been widely used. N-acetyl cysteine (NAC) has also been reported to function as a mucolytic agent in both vertebrate and invertebrate animals, including a marine bivalve (Moraes & Lopes, 2003), a sacoglossan (Pierce et al., 1996), and planarians (Bocchinfuso et al., 2012), but its primary use has been in mammals (e.g., Stamm & Docter, 1965). In the absence of a chemical treatment, motor cilia may continue to beat in excised tissue. So by excising tissue blocks and maintaining them in cold seawater, the mucus could clear "naturally" (SC, personal observation). This is also analogous to washing in seawater or phosphate-buffered saline, which is another treatment suggested previously (e.g., Buckland-Nicks, 1993). In order to test and optimize a treatment effective for chitons, we experimentally compared these three approaches.

MATERIALS AND METHODS

Fourteen treatment regimes were tested for their efficacy in mucus removal. These were all variants on the three techniques outlined above (hyaluronidase, NAC, and surgical separation) using different time and temperature intervals. Live specimens of *Lepidochitona cinerea* collected in Strangford Lough (County Down, Northern Ireland) were used for each treatment. All animals were held in aquaria flow-through seawater at the Queen's University Marine Laboratory (QML), where one of the authors (JS) has successfully kept long-term captive specimens. All experiments were performed on site in laboratory facilities at QML. Following treated incubation with gentle agitation, specimens were flushed with a pipette. All specimens were fixed in 4% glutaraldehyde in cacodylate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide in cacodylate buffer in preparation for SEM visualization. Tissues were then dehydrated in acetone and prepared for histology as described by Ruthensteiner (2008). Specimens were dried overnight using hexamethyldisilazane (HMDS, Sigma-Aldrich), sputter-coated with gold using a Polaron E5100 SEM Coating System (Quorum Technologies) and visualized in a FEI Quanta 200 scanning electron microscope operating at 10 kV.

Bovine hyaluronidase (Sigma-Aldrich) was applied at 4.46 e^{-3} mM in low-calcium artificial seawater (420 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 10 mM Trizma, 10 mM MnCl). Specimens were incubated for periods of 5 min, 10 min, or 1 hr, and at temperatures of either 10°C or room temperature (six treatment regimes, two specimens each). Another set of specimens were treated with 500 mM NAC in an 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (50 mM MES, 10 mM sucrose, 90 mM sorbitol, all Sigma-Aldrich, pH titrated to 5.5 using NaOH, adapted from Pierce et al., 1996). These specimens were incubated for intervals of 5 min, 10 min, or 30 min, and also at temperatures of either 10°C or room temperature (six treatment regimes, two specimens each). Four *L. cinerea* were decapitated and kept in seawater at 5°C for either 1 hr or for 24 hr before fixation (two treatment regimes, two specimens each), a method to induce "natural" clearing of the tissue by the action of still-active cilia, which has given good results in *Tritonia diomedea* (SC, personal observation). Finally, one specimen of *L. cinerea* was fixed without mucus removal treatment, as a control.

RESULTS AND DISCUSSION

The development of SEM techniques has been a crucial step forward in the study of microanatomy across all taxa, yet recent attempts to characterize the pallial cavity of another chiton, *Leptochiton rugatus* via SEM were hampered by excessive mucus obscuring the surface epithelium (JS, SC, personal observation). The SEM images here (Figure 1) show that the pallial cavity of *Lepidochitona cinerea* is carpeted with microvilli, with large tufts of cilia (6 μm in diameter) of approximately 15–20 μm in length at regular intervals (approximately 25 μm apart, Figure 1A, B). This pattern persisted throughout the pallial cavity and into the gill row with no visible change in density or frequency. These are the first images to illustrate the surface features of the pallial cavity in *L. cinerea*.

The "success" of our treatments was assessed qualitatively by visualization under SEM, to determine whether cilia were clearly visible, or whether tissue appeared badly dehydrated, or covered in mucus sheets obscuring some or all of the cilia in the pallial cavity. Specimens were examined whole; that is, the entire pallial cavity on both sides was visually examined under SEM although only small portions are illustrated herein (Figure 1). Temperature did not have any substantive influence on tissue quality, and we conclude that performing these chemical treatments at room temperature does not decrease quality.

The different treatments for mucus removal had varying rates of success, with NAC generally producing the best results. Incubation of specimens with hyaluronidase did not effectively remove mucus from the pallial cavity when incubated for less than an hour (Figure 1C, D), but after longer incubations both cilia and microvilli were visible and only small spheres of mucus were present. NAC preserved cilia and microvilli in good condition, and left little mucus when applied for 10 min or more (Figure 1A, B). In addition, the use of hyaluronidase appears to have disrupted the tissue, with cracks appearing in the epithelium in hyaluronidase-treated samples (not shown), this effect was not present under NAC treatment despite identical dehydration and preparation for SEM.

The difference in mucus coverage observed between the decapitated specimens (i.e., natural clearing of the tissue)

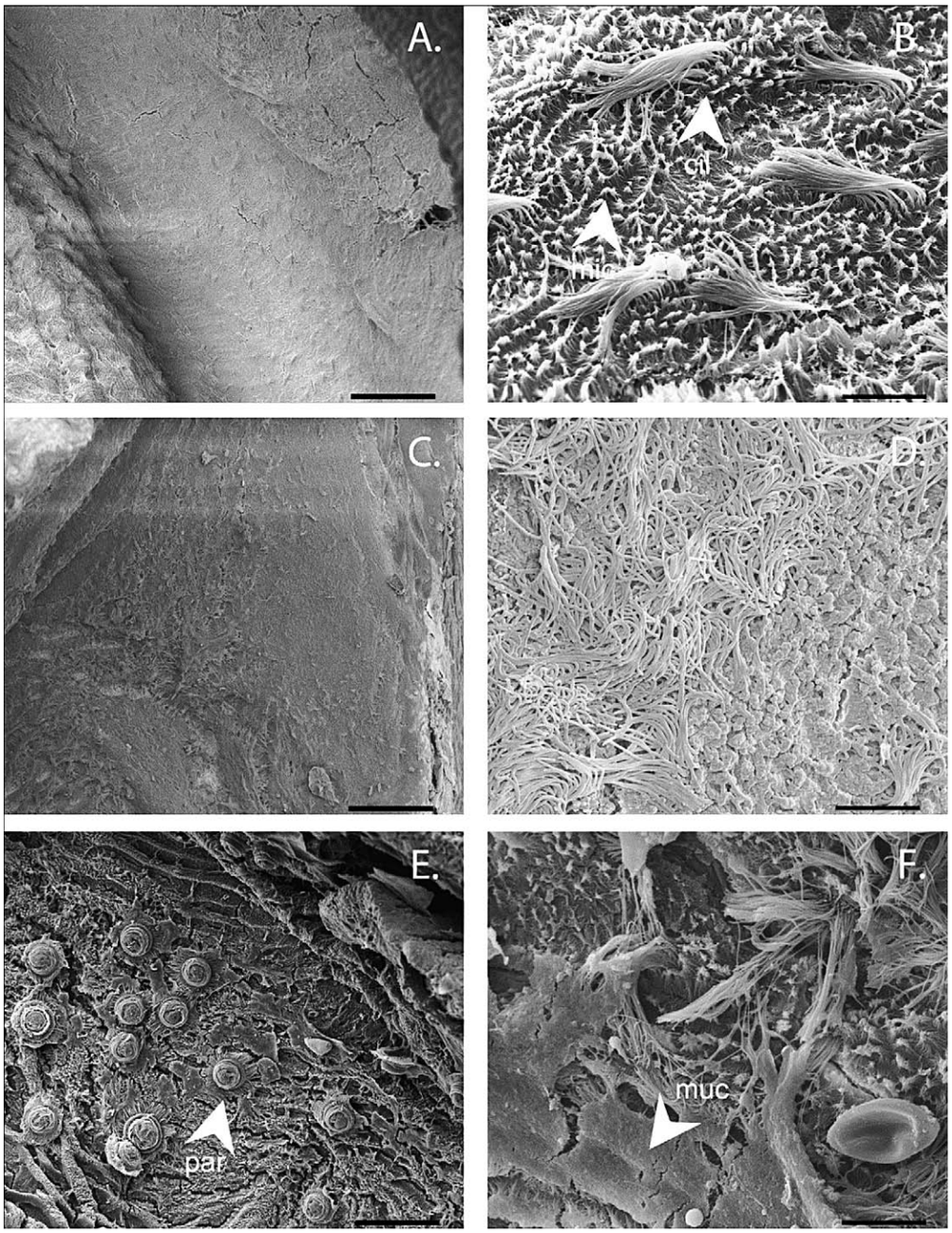

Figure 1. SEM images of the anterior pallial cavity of treated *L. cinerea*. **A**, **B**, NAC, 10°C, 10 min; **C**, **D**, Hyaluronidase, 10°C, 5 min; **E**, **F**, Decapitated, 1 hr. Abbreviations: cil, cilia tuft; mic, microvilli; par, *Trichodina* sp. parasite; muc, mucus. Scale bars: **A, C, E** = 100 μm; **B, D, F** = 10 μm.

and the untreated control specimen was minimal. In both cases, the cilia could not be clearly observed and the discrete cilia tufts, visible in other specimens, could not always be clearly identified beneath the mucus "sheet" (Figure 1E, F).

An additional novel observation is the presence of a ciliate protozoan parasite, *Trichodina* sp., which also obscured the view of the pallial cavity surface. These protozoa, visible at low magnification, had not been noted

during specimen preparation, but on subsequent investigation were found to infest the majority of specimens available for study. Therefore, we do not believe the influence of the parasite confounded our results here by influencing either mucus production by the chitons or its removal in our experiments. Under treatment conditions where the mucus was completely or partially removed, these parasites were also cleared. However, they were observed in the untreated, decapitated, and the shorter hyaluronidase-treated specimens and caused a significant obstruction to our view of the pallial cavity due to their large size (around 30 μm across). Their presence here is particularly notable as they have only been reported twice in the literature for chitons: once in our study species, *L. cinerea* (Plate, 1898) and one other report in the Pacific chiton *Katharina tunicata* (Kozloff, 1961).

Although the NAC treatment was the most effective for *Lepidochitona*, it may not be a panacea and its effectiveness requires further investigation across a broad range of molluscan taxa. Two additional specimens of *Leptochiton asellus* (Leptochitonidae) were treated with NAC at room temperature for 30 min as described above. Unlike *Lepidochitona cinerea*, which is a member of the suborder Chitonida, *Leptochiton asellus* belongs to the other major clade of living chitons, Lepidopleurida. However, in this case, mucus deposits continued to obstruct the structures. Ronkin (1952) noted that the susceptibility of mucus to digestion by hyaluronidase varied in different areas in *Busycon canaliculatum*, and postulated that variation in chemical composition might explain this. It may be that the taxonomic differences between chitons extend to the composition of mucus, with dissimilar proportions of glycoproteins and proteoglycans being affected differently by the NAC and hyaluronidase chemical treatments, and thus giving results of variable quality. Although the applicability of this treatment requires further investigation, our recommended treatment on the basis of our empirical tests is NAC, effective after 10 min as a starting point for other investigators.

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