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# Cellular Mechanisms of Slime Gland Refilling in Pacific Hagfish (*Eptatretus stoutii*)

## **Comments**

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## RESEARCH ARTICLE

# Cellular mechanisms of slime gland refilling in Pacific hagfish (*Eptatretus stoutii*)

Sarah Schorno<sup>1,\*</sup>, Todd E. Gillis<sup>1</sup> and Douglas S. Fudge<sup>1,2</sup>

## ABSTRACT

Hagfishes use their defensive slime to ward off gill-breathing predators. Slime gland refilling is a surprisingly slow process, and previous research has shown that the composition of the slime exudate changes significantly during refilling, which likely has consequences for the functionality of the slime. This study set out to expand our understanding of slime gland refilling by examining the cellular processes involved in refilling of the glands, as well as determining where in the gland the main slime cells – the gland thread cells and gland mucous cells – arise. Slime glands were electrostimulated to exhaust their slime stores, left to refill for set periods of time, and harvested for histological and immunohistochemical examination. Whole slime glands, gland thread cell morphometrics and slime cell proportions were examined over the refilling cycle. Slime glands decreased significantly in size after exhaustion, but steadily increased in size over refilling. Gland thread cells were the limiting factor in slime gland refilling, taking longer to replenish and mature than gland mucous cells. Newly produced gland thread cells underwent most of their growth near the edge of the gland, and larger cells were found farthest from the edge of the gland. Immunohistochemical analysis also revealed proliferating cells only within the epithelial lining of the slime gland, suggesting that new slime cells originate from undifferentiated cells lining the gland. Our results provide an in-depth look at the cellular dynamics of slime gland refilling in Pacific hagfish, and provide a model for how slime glands refill at the cellular level.

**KEY WORDS:** Gland mucous cells, Gland thread cells, Histology, Proliferation

## INTRODUCTION

When attacked by predators, hagfishes respond by producing large volumes of slime within milliseconds from a series of slime glands along the length of their body (Fig. 1A) (Downing et al., 1981b; Koch et al., 1991; Fudge et al., 2003, 2005). Their slime acts to deter gill-breathing predators by clogging their gills (Lim et al., 2006; Zintzen et al., 2011). The two main cell types in the slime glands are gland thread cells and gland mucous cells. The gland thread cells produce a single, coiled proteinaceous thread formation known as a thread skein, whereas the gland mucous cells contain thousands of tiny, mucus-containing vesicles (Salo et al., 1983; Downing et al., 1984; Spitzer et al., 1984; Luchtel et al., 1991; Winegard et al., 2014).

The threads of the gland thread cells are composed mainly of intermediate filaments, but also contain microtubules during early phases of their development (Downing et al., 1981a,b, 1984; Winegard et al., 2014). The slime threads are an unusual case of a cytoskeletal protein being secreted. The ability of the slime thread's intermediate filament subunits to self-assemble, in combination with their impressive tensile strength, have made them an interesting biomimetic model for potentially producing protein-based textiles (Fudge et al., 2003, 2010; Negishi et al., 2012).

Ejection of exudate from a gland is powered by a thin layer of striated muscle (the *musculus decussatus*) that surrounds it (Downing et al., 1981a,b). Thread and mucous cells are released via holocrine secretion through the narrow gland pore, meaning that entire cells are released, and their plasma membranes are sheared off in the process (Fig. 1B,C) (Downing et al., 1981a,b). The mixing of the thread and mucous components with the surrounding seawater gives rise to the ultra-dilute fibrous mucus that clings to predators' gills and likely interferes with respiration (Fudge et al., 2003; Lim et al., 2006; Zintzen et al., 2011).

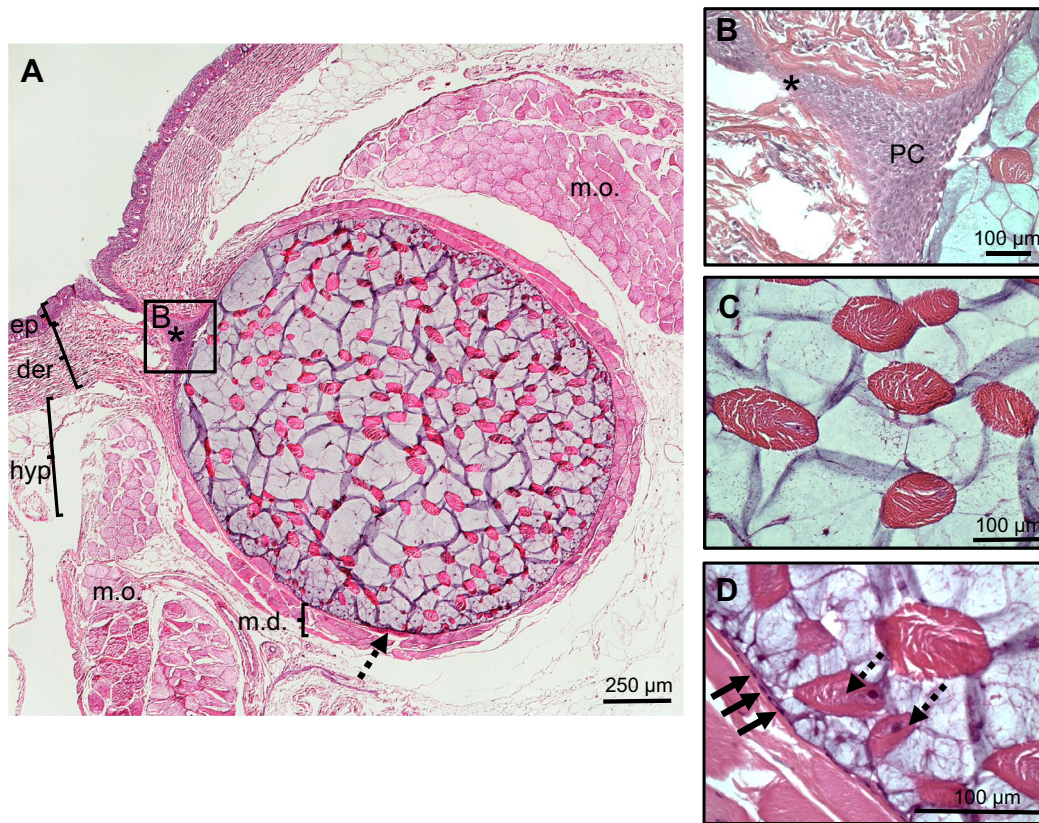
Recent work has shown that the hagfish slime glands are capable of refilling after ejecting their contents, but this is a surprisingly slow process, taking approximately 4 weeks in Pacific (*Eptatretus stoutii*) and Atlantic (*Myxine glutinosa*) hagfishes (Schorno et al., 2018). Additionally, the glands are never completely 'empty', with exhausted slime glands retaining a small percentage of slime cells, which may be a result of the limitations of muscle shortening of the *musculus decussatus* (Schorno et al., 2018). If exhausted glands are stimulated again prior to 4 weeks, they can still release some exudate, although the amount of exudate released, and the size of the thread skeins in the exudate, is reduced compared with that of full slime glands (Schorno et al., 2018). There are also differences in the mucous vesicle to thread skein ratio in the exudate of partially refilled glands that could impair the ability of the slime to clog gills (Schorno et al., 2018; Koch et al., 1991).

Other researchers have examined the histology and cell biology of hagfish slime glands (Newby, 1946; Downing et al., 1984; Spitzer et al., 1988; Winegard et al., 2014). Spitzer et al. (1988) showed that slime glands in the process of refilling contain smaller gland thread cells on average compared with full slime glands, and Winegard et al. (2014) elucidated some of the morphological changes that gland thread cells undergo during their development. However, where the gland thread cells and gland mucous cells originate is still not completely understood. Both cell types are thought to be derived from the undifferentiated cells in the epithelial lining of the slime gland (Fig. 1D), although evidence for this is scant (Newby, 1946; Winegard, 2012). Newby (1946) postulated that these undifferentiated cells are in turn derived from 'polyhedral cells' (Fig. 1B), which appear to be continuous with the epidermal cells that line the gland pore and the surface of the skin, but again no evidence was provided for this assertion.

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**Fig. 1. Hematoxylin and eosin stained cross-section of a Pacific hagfish slime gland.** (A) Slime glands release their contents through the gland pore (\*), and are surrounded by a collagenous gland capsule (dashed arrow) and striated muscle layer known as the musculus decussatus (m.d.). Slime glands lie below the epidermis (ep), dermis (der) and hypodermis (hyp), and are embedded within the body musculature (musculus obliquus: m.o.). (B) Polyhedral cells (PC) lie near the gland pore. (C) Gland thread cells stain pink within the gland, while gland mucous cells stain light purple. (D) Small, undifferentiated cells (solid arrows) are found along the gland epithelium lining. Immature gland thread cells (dashed arrows) and gland mucous cells are found near the edge of the slime gland.

The present study expands on the results of our previous study (Schorno et al., 2018) in which we examined slime gland refilling through an examination of the exudate alone. Here, we used histological and immunological techniques to elucidate the cellular mechanisms of slime gland refilling in Pacific hagfish, and determined how changes to the distribution of slime cells within the gland may affect the composition of the exudate during refilling. Our results reveal where new cells originate in the slime gland, as well as how the two main secretory cell types grow and move within the glands to ready them for ejection. We used this information to develop a model of the cellular events involved in slime gland refilling.

## MATERIALS AND METHODS

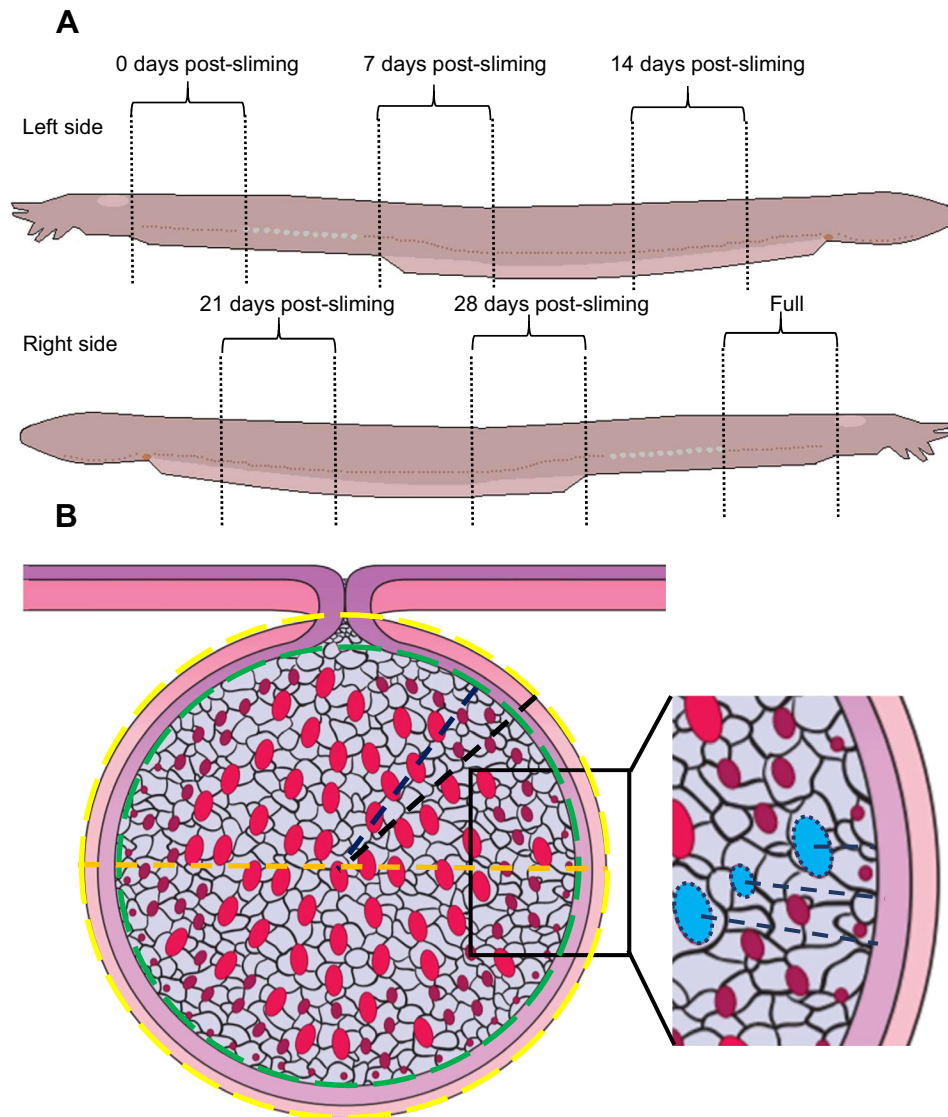
### Experimental animals

Pacific hagfish [*Eptatretus stoutii* (Lockington 1878)] were obtained from Bamfield Marine Station in Bamfield, BC, Canada, and housed at the Hagen Aqualab at the University of Guelph, Guelph, ON, Canada, in a 2000 liter environmentally controlled aquatic recirculating system filled with chilled artificial seawater (34 ppm, 10°C). The sex of the hagfish used in this study was not measured, and all hagfish utilized were adults but the exact age of each individual was unknown. Prior to experiments, hagfish were isolated in floating bins within the tank for a minimum of 1 month (30 days) to allow their slime glands to completely refill. Hagfish were fed a seafood medley (squid, shrimp and cuttlefish) to satiety once per month. All housing and feeding conditions were approved

by the University of Guelph Animal Care Committee (Animal Utilization Protocol no. 2519).

### Hagfish anesthesia and slime gland exhaustion

Pacific hagfish ( $n=5$ ; average mass= $65.12 \pm 4.41$  g) were anesthetized by placing them in 3 liters of artificial saltwater (Coralife, Energy Savers Unlimited, Inc., Carson, CA, USA) containing 3 ml of a clove oil (Sigma-Aldrich, Oakville, ON, Canada) anesthetic solution (1:9 clove oil to 95% ethanol). Hagfish were deemed anesthetized when they ceased to respond to touch. Anesthetized hagfish were removed from the anesthetic water, rinsed with deionized water, patted dry using Kim Wipes (Kimberly-Clark Corporation, Irving, TX, USA) and placed on a dissection tray. A Grass SD9 electric stimulator (60 Hz, 18 V; Grass Instruments, Quincy, MA, USA) was used to stimulate and exhaust individual slime glands. To collect glands in various stages of refilling from each of the five hagfish that were sampled, five slime glands from six different regions were assigned to one of six recovery treatments (0, 7, 14, 21 and 28 days post-sliming, and full). Slime glands from each of the regions were stimulated in anesthetized hagfish until exhaustion (no longer releasing exudate) (Schorno et al., 2018) at times that would result in the desired recovery time for those glands when the experiment was concluded (Fig. 2A). Experimental hagfish were isolated from the others by placing them in a smaller container floating in the tank. If a hagfish slimed unexpectedly at any point during its handling, it was excluded from the trials.



**Fig. 2. Examination of refilling slime glands harvested from Pacific hagfish.** (A) Each hagfish had various sections of slime glands along their body exhausted and then allowed to refill for a certain amount of time (0, 7 and 14 days post-sliming on the left side of the animal; 21 and 28 days post-sliming and full glands on the right side of the animal). Individual hagfish were kept isolated for 28 days during experiments, and were handled every week to empty the glands indicated. (B) Slime gland diameter was measured using caliper measurements of whole tissue (orange dashed line). Slime cell area in the gland (green dashed circle) and gland circumference (yellow dashed circle) were measured from histological sections. Thickness of the muscle layer was approximated by subtracting the internal gland radius (purple dashed line) from the whole gland radius (black dashed line). Inset shows gland thread cell cross-sectional area (blue circles) measurements, and distance from the edge of the slime gland (blue dashed lines) was measured by measuring the distance from the center of each cell to the nearest edge of the slime gland.

### Gland tissue preparation

Twenty-eight days after emptying of the first region of slime glands, hagfish were euthanized and slime glands from the six recovery treatments were harvested for histological analysis. Euthanasia was carried out via clove oil anesthesia (as described above) followed by decapitation. Hagfish were dissected according to a protocol developed by Winegard (2012) in which the skin with slime glands still attached at the gland pore is removed from the rest of the hagfish body using rat tooth forceps. Dissected slime glands were fixed in 4% paraformaldehyde solutions (cat. no. 19200; Electron Microscopy Sciences, Fort Washington, PA, USA) in preparation for paraffin embedding. A solution of 4% paraformaldehyde was prepared in a  $0.9 \text{ mol l}^{-1}$  sodium citrate (Fisher Scientific, Ottawa, ON, Canada),  $0.1 \text{ mol l}^{-1}$  PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid);

Sigma-Aldrich] stabilization buffer (pH 6.7) to reduce swelling of the gland mucous cells within the slime glands.

### Paraffin embedding and antibody staining of hagfish tissues

Fixed tissues were processed for paraffin embedding using a routine overnight protocol in the Ontario Veterinary College Veterinary Histology Unit. Slime gland diameter was measured and recorded using Mitutoyo digital calipers (Mitutoyo ABSOLUTE Digimatic Caliper, Takatsu-ku, Kawasaki, Kanagawa Prefecture, Japan) both before fixation and after fixation/processing of the slime gland tissue. The radius of the slime gland was then calculated, and these measurements were used to determine how many slices were required to section to approximately the middle of every slime gland. Tissues were then paraffin embedded using standard techniques.

Hagfish tissues were trimmed and longitudinally sectioned (5  $\mu\text{m}$  thick sections) to their approximate center (around the gland pore) using a rotary microtome, and then sections were placed on glass microscope slides (Fisher Scientific). Whole glands were stained with hematoxylin and eosin (H&E; Fisher Scientific), and slides were covered with cover glass (22 $\times$ 50 mm; Fisher Scientific) and sealed with Cytoseal XYL (Richard Allen Scientific, San Diego, CA, USA). The Cytoseal was left to dry overnight before visualization. Tissues for immunological staining were left unstained, placed on glass slides and de-waxed using standard protocols before antibody staining.

### Immunohistochemistry

Sites of proliferation within the slime glands were investigated via immunohistochemistry of the proliferating cell nuclear antigen (PCNA). Each slide contained two sections of a slime gland. Slides were de-waxed using standard protocols, and sodium citrate buffer (pH 6.0; Fisher Scientific) was used for heat-induced (95°C) antigen retrieval. Slides were rinsed 3 $\times$ 2 min with phosphate buffered saline (PBS) and blocked with 3% normal goat serum (catalog no. 16210072; Gibco™, Fisher Scientific) for 1 h at room temperature. The slides were then incubated with anti-PCNA primary antibody ( $\alpha$  rabbit polyclonal; 1:100; catalog no. sc-7907; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in a humidified chamber overnight at 4°C. Each slide had one section act as a negative control, in which no primary antibody was applied, to confirm specificity of the secondary antibody. The skin (still attached to the slime gland) acted as the positive control on each slide. Slides were then rinsed 3 $\times$ 2 min with PBS. Alexa Fluor 568 goat anti-rabbit IgG secondary antibody (1:200; catalog no. A11011; Life Technologies, ThermoFisher, Ottawa, ON, Canada) was applied to sections for 1 h at room temperature, and slides were again rinsed 3 $\times$ 2 min with PBS before being counter-stained with 4',6-diamidino-2-phenylindole (DAPI; 1:10,000; catalog no. D9542; Sigma-Aldrich) to highlight cell nuclei. Slides were given a final 3 $\times$ 2 min wash with PBS, then mounted with Fluoromount™ aqueous mounting medium (catalog no F4680; Sigma-Aldrich) and covered with cover glass (22 $\times$ 50 mm; Fisher Scientific).

### Brightfield and fluorescence imaging

Fluorescently labeled and histologically stained hagfish tissue slides were analyzed using a Nikon Eclipse 90i Epi-fluorescent microscope. Brightfield color images were taken using a Q-imaging EXi 12-bit color camera (Q-Imaging Surrey, BC, Canada) driven by NIS Elements AR software (Nikon Instruments, Inc., Melville, NY, USA). Epifluorescent monochrome images were taken on an inverted Nikon Eclipse epi-fluorescent microscope with a cooled monochrome camera driven by NIS Elements Software and a Leica DM5000B epifluorescence microscope with a Leica DFC350FX monochrome camera driven by Volocity software (Improvision, Lexington, MA, USA). Fluorescent signals from secondary antibodies were imaged using the Nikon epi-fluorescent microscope with a DAPI filter cube (excitation 340–380 nm) and a green fluorescent protein long-pass type filter cube (AT-GFP/F LP; excitation 480 nm and beyond) to image the DAPI and Alexa Fluor 568, respectively. Secondary antibodies were imaged using the Leica epifluorescence microscope with a DAPI filter cube (excitation 340–380 nm) and Texas Red filter cube (excitation 540–580 nm) to image the DAPI and Alexa Fluor 568, respectively. Brightfield images were captured using DIC optics and fluorescent channel images were overlaid on them. The monochrome fluorescent images were assigned color channels based on the emission wavelengths of the secondary antibodies and

were then assembled into multichannel images using Fiji ImageJ software (Abràmoff et al., 2004).

### Histology image analysis

Histological (H&E) sections of whole slime glands were analyzed for overall slime gland morphometrics as well as gland thread cell morphometrics (Fig. 2B). For whole slime gland analysis, total area of the slime gland occupied by slime cells ( $\mu\text{m}^2$ ) and circumference of the slime gland (including the muscle layer;  $\mu\text{m}$ ) were recorded using NIS Elements software. The thickness of the muscle layer (musculus decussatus) was calculated by subtracting the internal radius of the slime gland (center of gland to gland epithelium) from the radius of the whole slime gland (including muscle layer). For gland thread cell morphometrics, gland thread cell concentration (number of cells/total area of the slime cells; number  $\mu\text{m}^{-2}$ ), cell cross-sectional area ( $\mu\text{m}^2$ ) and distance from the edge of the slime gland ( $\mu\text{m}$ ) were all calculated. It is important to note that owing to the nature of sectioning an ellipsoidal cell such as the gland thread cell, smaller cross-sections may just be an artefact of the slicing process, where what appears as small cells may only be slices of the narrow end of a larger gland thread cell. Histograms of gland thread cell size were created to examine the frequency of cell sizes within the slime glands at each refilling time point (bin size=100  $\mu\text{m}$ ). Histograms of gland thread cell distance from the edge of the gland at each refilling time point were also created (bin size=25  $\mu\text{m}$ ).

Owing to the difficulty of determining the cell edges of gland mucous cells, only gland thread cell morphometrics within the gland were examined. However, the total cross-sectional area of the slime gland occupied by gland mucous cells was calculated by subtracting the total area occupied by gland thread cells from the total area of the slime cells. These values were then used to determine the percent area of the slime gland that each cell type occupied. We assumed that the area occupied by anything other than the two main slime cells was negligible.

### Statistical analysis

All statistical analyses were run using SPSS v. 25.0 (SPSS Inc., IBM, Armonk, NY, USA) with  $\alpha=0.05$ . Non-positive values (0 or negative values) and outliers identified by SPSS software as being 1.5 $\times$  outside of the interquartile range (IQR) were also excluded from analyses and figures. For whole slime gland analysis, a one-way ANOVA with a Tukey's *post hoc* honestly significant difference (HSD) test was conducted to determine the effect of days post-sliming on the diameter of the slime gland. To determine the relationship between circumference of the muscle layer and its thickness, a non-linear regression analysis was performed on these data. For gland thread cell morphometrics, a one-way ANOVA with a Tukey's *post hoc* least significant difference (LSD) test was conducted to determine the effect of days post-sliming on the concentration of gland thread cells within a slime gland. A one-way ANOVA with a Tukey's *post hoc* HSD test was conducted on the percent area gland mucous cell and percent area gland thread cell data to determine the effect of days post-sliming on these two variables. To test for differences between percent area of gland mucous cells and percent area of gland thread cells at each refilling time point, independent sample *t*-tests were conducted. A one-way ANOVA with a Tukey's *post hoc* HSD test was conducted on the gland thread cell cross-sectional area and gland thread cell distance from edge of the slime gland data to determine the effect of days post-sliming on these two variables. A power curve was fit to the gland thread cell cross-sectional area versus distance from the edge of the gland data, and non-linear regression analysis was also

performed to determine the relationship between these two variables at each refilling time point.

## RESULTS

### Gross morphological differences among slime glands during refilling

Slime gland diameter (measured on whole, fixed slime glands using digital calipers) increased significantly during refilling (one-way ANOVA: d.f.=5, 23,  $F=5.53$ ,  $P<0.01$ ; Figs 3 and 4). *In situ* slime glands still attached to the epidermis demonstrate how much the refilling slime glands were reduced in size compared with full slime glands (Fig. 3A). Slime glands during the first 3 weeks of refilling (0, 7, 14 and 21 days post-sliming) ranged in diameter from  $1.77\pm 0.14$  to  $1.98\pm 0.06$  mm. Slime glands at 28 days post-sliming were not quite as large as full glands, but there was no significant difference in gland diameter between the two time points (Tukey *post hoc* HSD:  $P=0.52$ ; Fig. 3B). Slime glands at 28 days post-sliming were on average  $2.28\pm 0.23$  mm in diameter, whereas full slime glands were  $2.62\pm 0.17$  mm in diameter. As the slime glands increased in size with refilling, the musculus decussatus layer increased in circumference and decreased in thickness (Fig. 4). An exponential curve was fit to the muscle layer thickness versus circumference data, and non-linear regression analysis revealed that

circumference of the muscle layer had a significant effect on its thickness ( $R^2=0.34$ ,  $F=14.50$ ,  $P<0.001$ ).

The number of gland thread cells in slime gland cross-sections increased significantly with refilling, with 0 days post-sliming glands containing  $243\pm 32$  gland thread cells increasing to  $682\pm 90$  cells in full glands (one-way ANOVA: d.f.=5, 19,  $F=11.44$ ,  $P<0.001$ ). Although the absolute number of gland thread cells in the cross-sections increased significantly with refilling, the concentration of gland thread cells [number of cells/area of slime gland ( $\mu\text{m}^2$ )] remained mostly constant (Fig. 5A). However, there was a significant increase in the concentration of gland thread cells at 7 days post-sliming compared with other time points (one-way ANOVA: d.f.=5, 20,  $F=3.09$ ,  $P<0.05$ ; Tukey *post hoc* LSD:  $P<0.05$ ), with a gradual return to the baseline concentration in the subsequent refilling time points. The number of gland mucous cells could not be accurately discerned because the borders between individual cells were often indistinguishable.

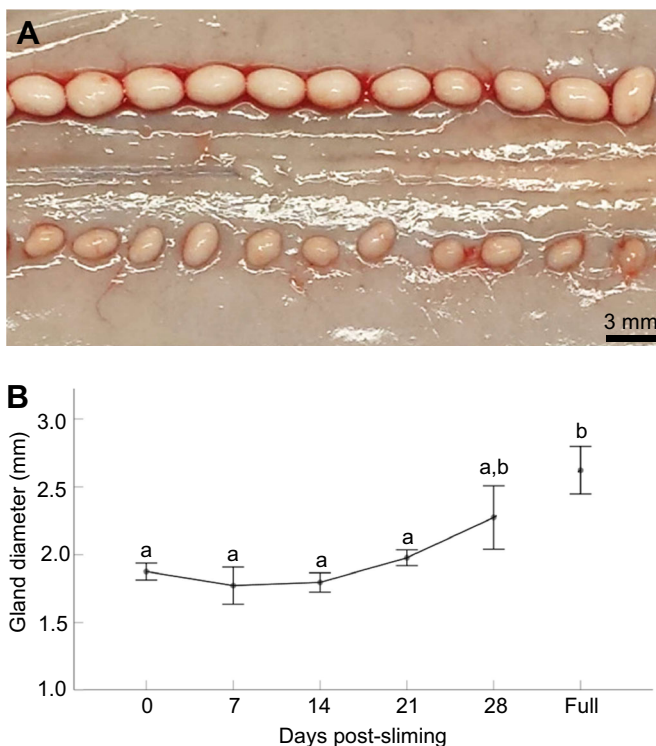
The area occupied by gland mucous cells was calculated by subtracting the total area occupied by gland thread cells from the total area of slime cells within the gland. Gland mucous cells occupied significantly more area of slime glands compared with gland thread cells during the first 3 weeks of refilling (independent-sample *t*-tests: d.f.=8,  $P<0.05$ ). Gland mucous cells on average occupied  $83.5\pm 2.3\%$  of the slime gland area during the first 3 weeks of refilling (0, 7, 14 and 21 days post-sliming), whereas gland thread cells occupied approximately  $16.5\pm 2.3\%$  of the slime gland area. However, by 28 days post-sliming and in full glands, gland thread cells occupied a larger proportion of the glands relative to earlier refilling time points. By 28 days post-sliming, gland mucous cells occupied  $64.7\pm 9.6\%$  of the gland area whereas gland thread cells occupied  $35.3\pm 9.6\%$ . In full slime glands, gland mucous cells and gland thread cells each occupied nearly equal proportions of the slime gland area ( $56.6\pm 11.9\%$  for gland mucous cells,  $43.4\pm 11.9\%$  for gland thread cells).

### Immunofluorescence for proliferation in refilling slime glands

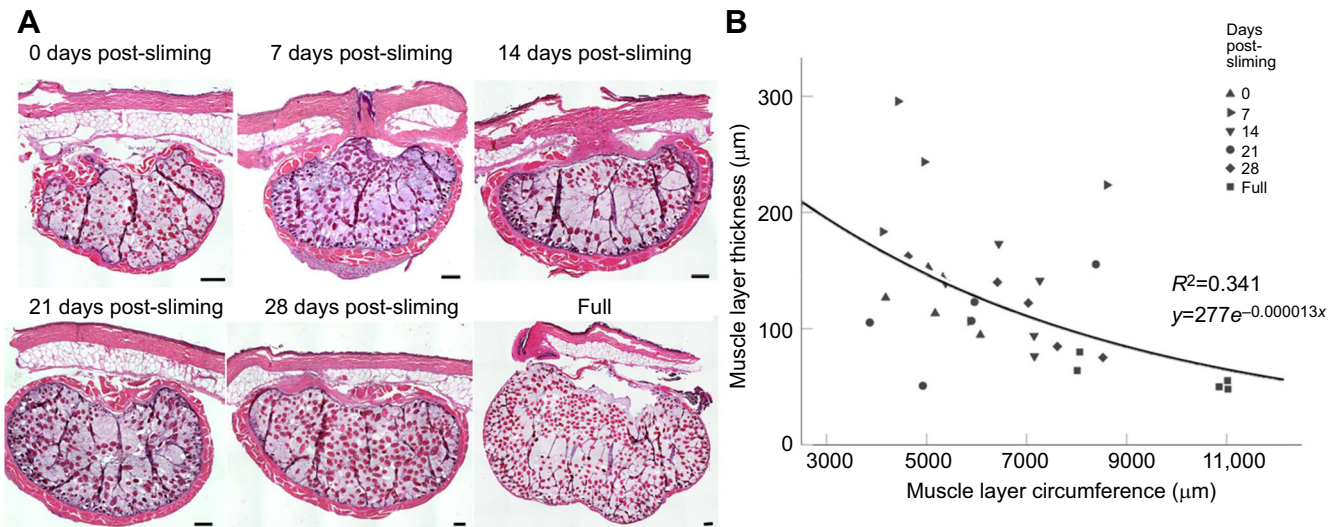
Cells were counted as PCNA positive (proliferative cells) when their nuclei exhibited strong Texas Red and DAPI fluorescence. Each slide had one section that acted as a negative control, in which no primary antibody was applied to confirm specificity of the secondary antibody; these sections showed no PCNA-positive fluorescence. The skin (still attached to the slime gland) acted as the positive control on each slide, and had PCNA-positive cells in the epidermis and bottom layer of the dermis (Fig. 6A). Epithelial cells and polyhedral cells at the neck of the gland pore showed no signs of PCNA-positive cells (Fig. 6B). PCNA positive cells were observed scattered within the epithelial lining of the slime gland, and were most often seen next to small slime cells close to the edge of the gland (Fig. 6C,D). No PCNA-positive cells were seen in the gland interior.

### Changes in gland thread cell size and location within the slime gland during refilling

Gland thread cells increased significantly in cross-sectional area during refilling (one-way ANOVA: d.f.=5, 10,010,  $F=143.55$ ,  $P<0.001$ ). Gland thread cells were significantly smaller on average in exhausted (0 days post-sliming) and 7 days post-sliming glands compared with those in full slime glands (Tukey HSD:  $P<0.05$ ). The largest gland thread cells in exhausted glands were up to approximately  $6500\ \mu\text{m}^2$  in cross-sectional area, whereas the largest gland thread cells in full slime glands were up to approximately  $7800\ \mu\text{m}^2$  in cross-sectional area. The difference in size of the largest gland thread cells from the exhausted versus the full slime



**Fig. 3. Changes in Pacific hagfish slime gland diameter during refilling.** (A) Slime glands from locations anterior to the gill pouches, still attached to skin, illustrate the size difference between full slime glands (top row) and slime glands immediately after being exhausted via electrical stimulation (bottom row). (B) Slime gland diameter significantly increased during refilling (one-way ANOVA: d.f.=5, 23,  $F=5.53$ ,  $P<0.01$ ). Slime glands gradually increased in diameter, and by 28 days post-sliming, slime glands were not significantly different in diameter from full glands (Tukey *post hoc* HSD:  $P=0.52$ ), but also were not significantly different from the exhausted glands. The apparent lack of complete refilling in 28 days may have been a result of frequent handling of the hagfishes used in this experiment. Error bars are s.e.m.,  $n=5$  for each time point.



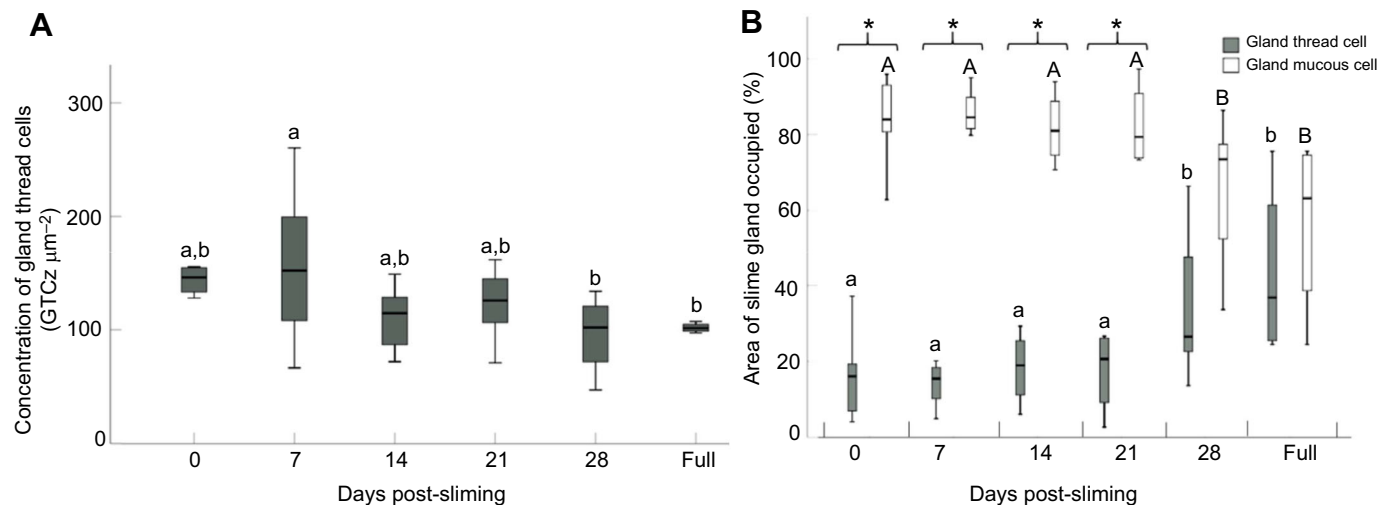
**Fig. 4. Changes in Pacific hagfish whole slime gland morphometrics during refilling.** (A) Slime glands increased in terms of size and number of slime cells present within the glands during refilling. The trabecular network (appears as dark purple lines in gland sections) is more pronounced in refilling slime glands compared with full glands. Scale bars, 200 μm. (B) The striated muscle layer (musculus decussatus) becomes thinner as the gland refills and increases in size.  $n=5$  for each time point.

glands is likely the result of preferential release of large gland thread cells from full glands during emptying of the gland. Histograms of gland thread cell cross-sectional area (Fig. 7) showed an increase in the frequency of small (<1000 μm<sup>2</sup>) gland thread cells at 7 days post-sliming. As refilling progressed, the frequency of larger gland thread cells increased. At 28 days post-sliming, there are two peaks in frequency of gland thread cell cross-sectional areas, one around 700–800 μm<sup>2</sup> and one around 2300–2400 μm<sup>2</sup>. Full slime glands exhibited even more large cells and a flatter distribution of cell sizes than in other treatments.

Gland thread cells were also found significantly farther from the edge of the slime gland as refilling progressed (one-way ANOVA:

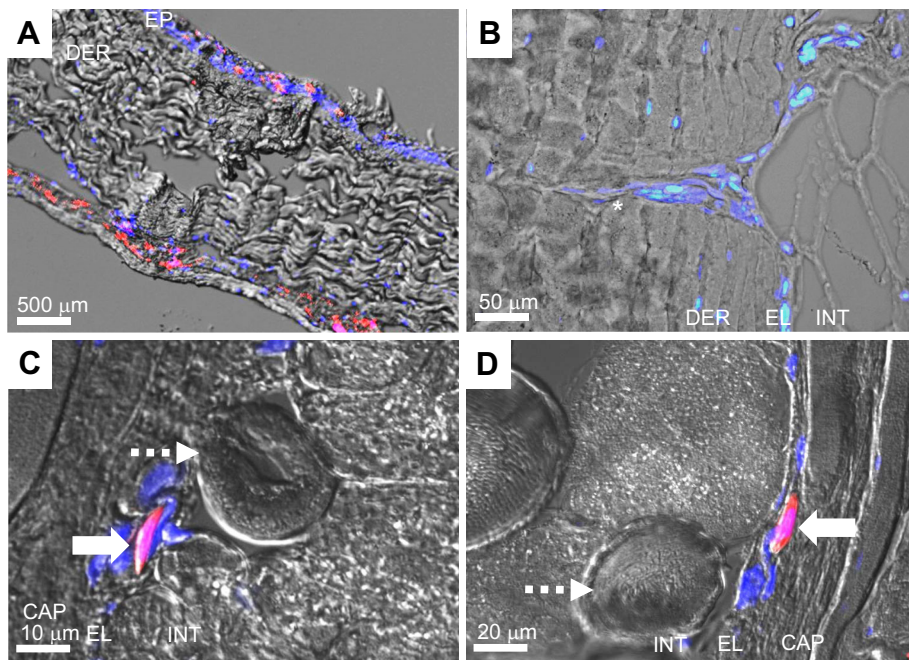
$d.f.=5$ , 10,010,  $F=341.74$ ,  $P<0.001$ ), with distance from the gland epithelium ranging from approximately 9 μm up to 815 μm in exhausted slime glands, and approximately 9 μm up to 1700 μm in full glands. These results are not surprising given that gland size limits how far a gland thread cell can be from the edge, and full glands were substantially larger than glands in other treatments. Histograms of gland thread cell distance from the gland edge (Fig. 8) showed an increase at 7 days post-sliming in the frequency of gland thread cells very close to the edge (25–50 μm). As refilling progressed, the frequency of gland thread cells farther from the edge increased.

Scatterplots were created to visualize the relationship between gland thread cell area and distance from the edge of the slime



**Fig. 5. Pacific hagfish slime cell concentrations and proportions during refilling.** (A) Gland thread cell concentration significantly changed during refilling (one-way ANOVA:  $d.f.=5$ , 20,  $F=3.09$ ,  $P<0.05$ ), with the concentration highest at 7 days post-sliming (Tukey *post hoc* LSD:  $P<0.05$ ). This indicates a potential increase in proliferation of the gland thread cells. Different letters indicate significant differences in gland thread cell concentration among glands allowed to recover for varying time periods. (B) Gland mucous cells occupied significantly more area of slime glands compared with gland thread cells during the first 3 weeks of refilling (independent-sample *t*-tests:  $d.f.=8$ ,  $P<0.05$ ). At 28 days post-sliming (independent-sample *t*-test:  $d.f.=8$ ,  $P=0.06$ ) and in full glands (independent-sample *t*-test:  $d.f.=8$ ,  $P=0.46$ ), gland mucous cells and gland thread cells occupied approximately equal area of the slime glands. Asterisks (\*) indicate significant differences between percent area of slime gland occupied by gland mucous cells versus gland thread cells at a given time point. Different letters indicate significant differences in percent area occupied between time points.  $n=5$  for each time point for both panels.





**Fig. 6. Fixed Pacific hagfish slime gland sections stained for nuclei with DAPI (blue) and immuno-stained for the proliferative marker PCNA (red).** Proliferative cells exhibit magenta nuclei, owing to co-localization of the DAPI and PCNA fluorescent antibody. (A) Proliferating cells were found in the epidermis (EP) and dermis (DER) of hagfish skin. (B) The slime gland pore (\*) contained many polyhedral cells, but no cells in this region were found to be proliferating. (C) Proliferating cell (solid arrow) in the gland capsule (CAP) and gland interior (INT) are also indicated. A small gland thread cell (dashed arrow) can be seen near the epithelial lining. (D) Another proliferating cell (solid arrow) in the gland epithelial lining (EL) of a gland at 14 days post-sliming, with a small gland thread cell (dashed arrow) nearby.

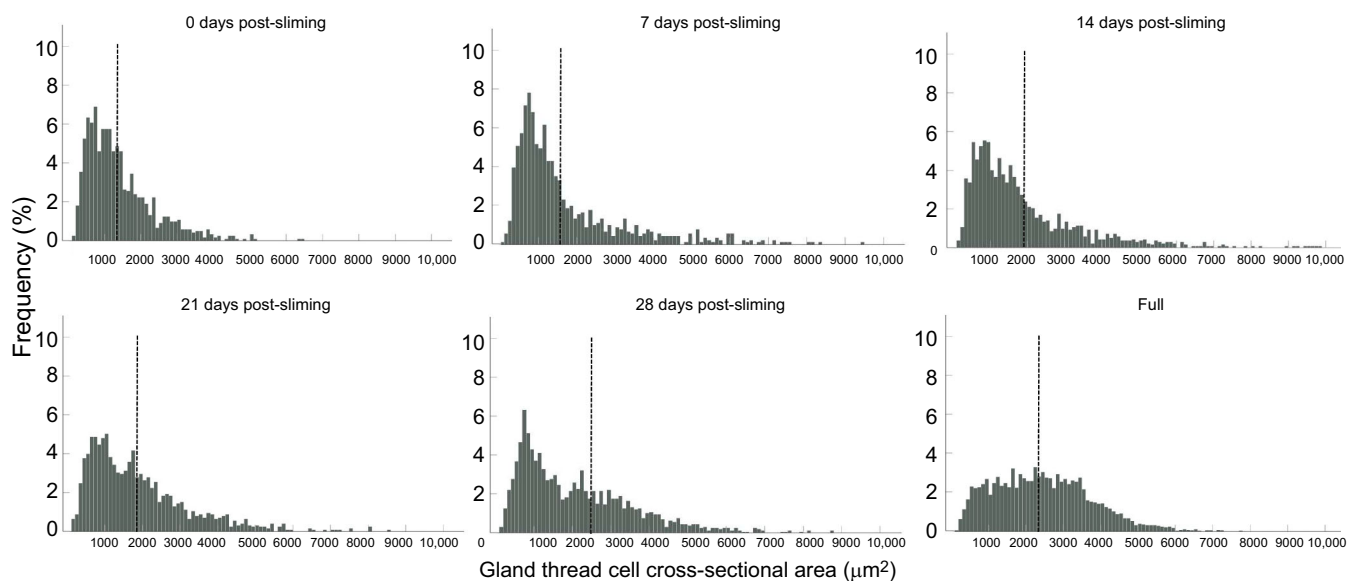
gland, and whether this relationship changed with refilling (Fig. 9). On average across all time points, smaller gland thread cells were found closer to the edge of the slime gland, whereas larger gland thread cells were found farther from the edge. Slime glands from all time points showed a strong relationship between gland thread cell distance from the edge of the gland and gland thread cell cross-sectional area (non-linear regression;  $P < 0.001$ ). The power curves plateaued at surprisingly small distance values, indicating that most of the increase in gland thread cell size likely occurs near the end of the slime gland. At 7 days post-sliming,

most of the gland thread cells within the slime gland were of a smaller size ( $< 2000 \mu\text{m}^2$ ) and were found closer to the edge of the slime gland ( $< 25 \mu\text{m}$ ) (Tukey *post hoc* HSD:  $P < 0.001$ ). Gland thread cells slowly increased in size and distance from the edge over the refilling cycle.

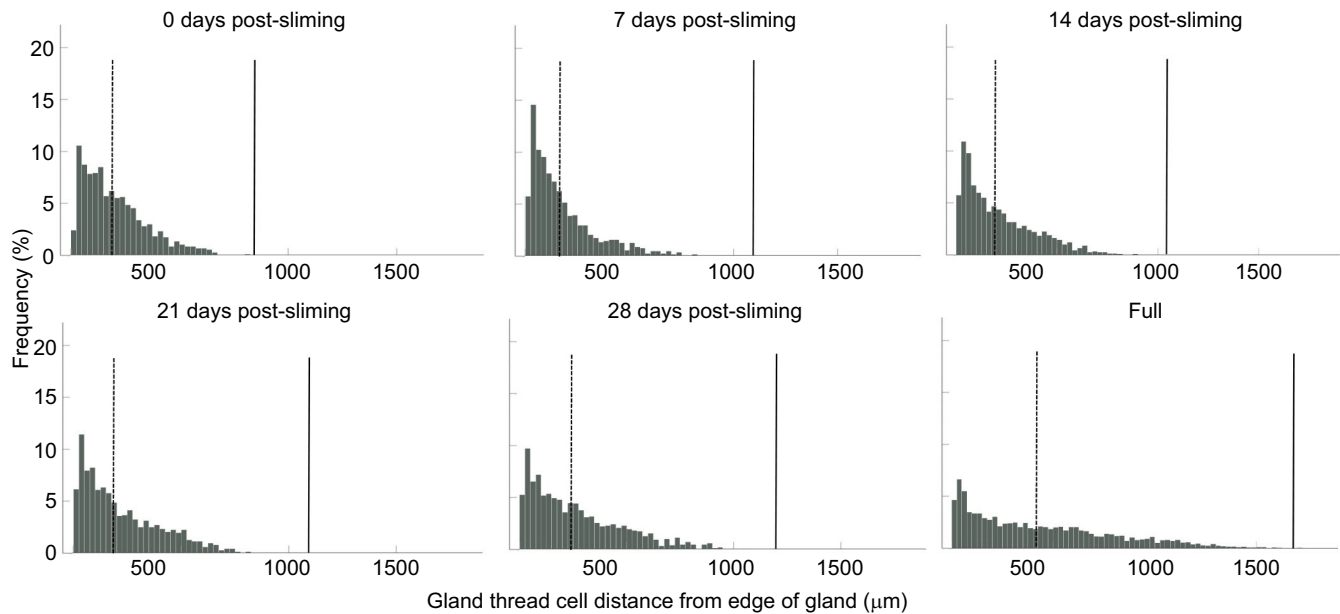
## DISCUSSION

### Emptying of Pacific hagfish slime glands

As the slime gland empties and the muscle layer around the gland contracts, the diameter of the slime gland becomes smaller and



**Fig. 7. Frequency of Pacific hagfish gland thread cell sizes in emptied, refilling and full glands.** Gland thread cell cross-sectional area differed significantly with days post-sliming (one-way ANOVA: d.f.=5, 10,010,  $F=143.55$ ,  $P < 0.001$ ). Gland thread cells were smaller on average in exhausted slime glands compared with full slime glands. At 7 days post-sliming, there was an increase in the frequency of small gland thread cells, but as refilling progressed, the frequency of larger gland thread cells increased. The initial increase in small gland thread cells may indicate an increase in proliferation and production of new cells, which is consistent with the idea that slime cells begin to be replaced shortly after a slime gland is stimulated to release exudate. Dashed line indicates the average cross-sectional area of gland thread cells for each refilling time point.  $n=5$  for each time point.



**Fig. 8. Frequency of Pacific hagfish gland thread cell distances from edge of the slime gland.** Gland thread cell distance from the edge of the slime gland differed significantly with days post-sliming (one-way ANOVA: d.f.=5, 10,010,  $F=341.74$ ,  $P<0.001$ ). Gland thread cells were found closer to the edge of the slime gland on average in exhausted slime glands compared with full slime glands. At 7 days post-sliming there was an increase in the frequency of gland thread cells found near the edge of the slime gland, but as refilling progressed, more cells were found farther from the edge. The early increase in the frequency of gland thread cells near the edge of the slime gland may indicate an increase in proliferation and production of new gland thread cells near the gland epithelium. Dashed line indicates the average distance of gland thread cells from the edge of the gland for each refilling time point; solid line indicates the maximum radius of slime glands for each refilling time point.  $n=5$  for each time point.

thus the circumference of the muscle layer (musculus decussatus) decreases. As would be expected, with decreasing circumference of the muscle layer, there was a concomitant increase in its thickness. In a previous study (Schorno et al., 2018), we compared the size of exhausted and full slime glands, and found that more exudate is squeezed out of the glands than can be accounted for by a 10% contraction of the muscle fibers in the musculus decussatus, which is typically the upper limit for contraction of striated muscle (Rassier et al., 2003; Peterson et al., 2004; Herzog et al., 2008). We thus proposed that ejection of exudate from slime glands may also involve relaxation of series elastic elements that are stretched during refilling (Schorno et al., 2018). Ejection may also be aided by the action of adjacent body muscles, such as the musculus obliquus, which is in close proximity to the slime glands.

In a previous study (Schorno et al., 2018), we showed that a single slime gland can release multiple boluses of exudate, with the mass of successive secretions decreasing exponentially. Compositional analysis of exudate from these successive stimulations of the glands revealed that skein volume remained mostly constant, from the first stimulation of the gland to the last, and that the skeins released were generally of a larger size ( $40 \times 10^3$  to  $60 \times 10^3 \mu\text{m}^3$ ) (Schorno et al., 2018). This is interesting considering that full slime glands can have smaller gland thread cells located close to the gland pore. We also saw that the largest gland thread cells in exhausted glands were much smaller than the largest gland thread cells in full glands (exhausted:  $6500 \mu\text{m}^2$ ; full:  $7800 \mu\text{m}^2$ ). This difference in size of the largest gland thread cells between the exhausted and full glands reinforces the slime glands' ability to eject uniformly large thread skeins despite a diversity of gland thread cell sizes present in a full slime gland. It is possible that there is a preferential release of larger gland thread cells closer to the center of the gland owing to stronger adhesion forces acting on the smaller cells in the gland. Stronger adhesion forces would keep smaller slime cells restrained during emptying of the slime gland. Alternatively, the

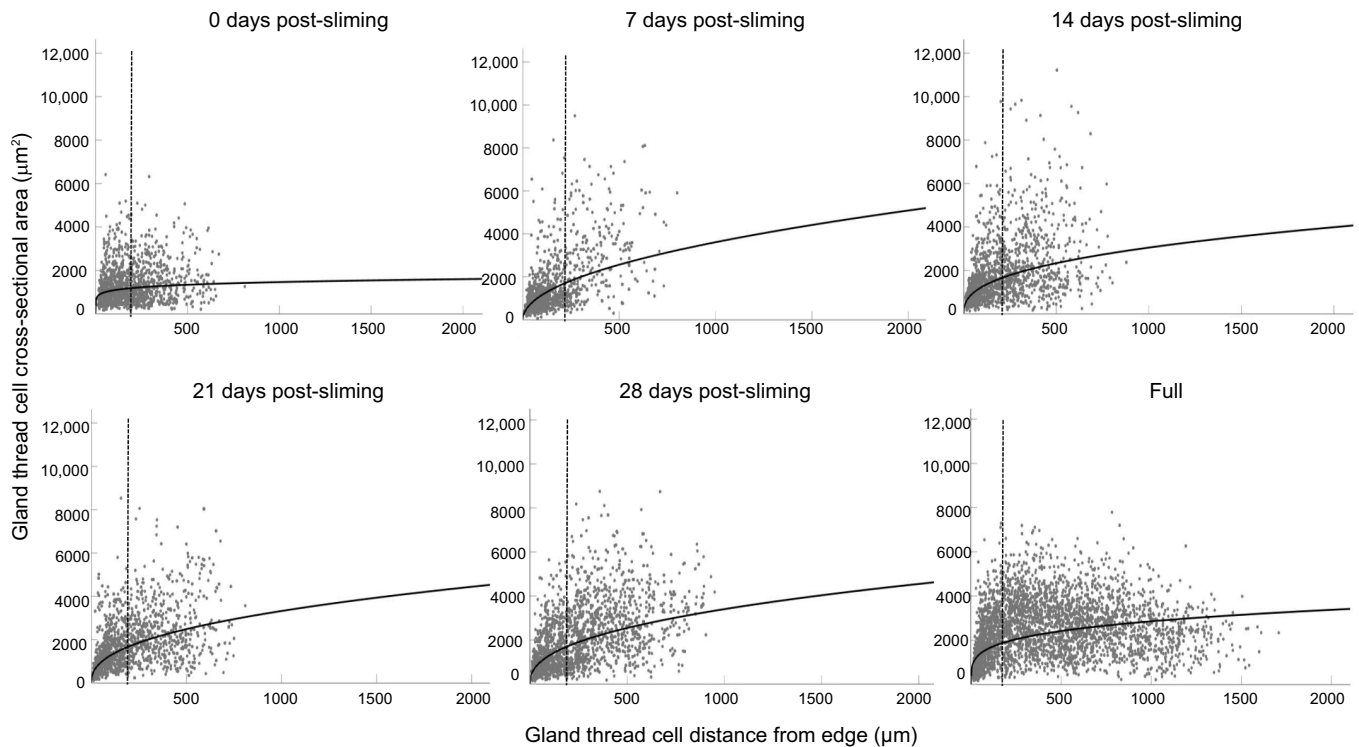
action of the musculus decussatus alone may simply not be enough to eject these smaller cells, which are closer to the edge of the gland.

#### Limiting factors in slime gland refilling

With its obvious utility in protecting hagfishes from fish predators, one might assume that selection for fast refilling of the slime glands would be strong, which makes the slow refilling we have demonstrated here and previously (Schorno et al., 2018) somewhat puzzling. Hagfishes have the lowest metabolic rates of any vertebrate (Forster, 1990), and this is likely a contributing factor, but there may be other explanations as well. The two main secretory products produced by the glands are mucus and threads. As stated in our previous study, it is difficult to imagine that production of the gland mucous cells is the limiting factor in slime gland refilling, considering that most mucus-producing epithelia continuously produce mucus (Schorno et al., 2018). In the present study, we saw that significantly more area of the slime gland is occupied by gland mucous cells than gland thread cells early in refilling (0–21 days post-sliming). Not until 28 days post-sliming and in full glands do the gland thread cells occupy approximately equal amounts of space within the slime gland as gland mucous cells. These observations indicate that refilling of the slime gland is likely limited by the production of gland thread cells, which is reasonable given the complexity of the skein and the remarkable length of the thread they each produce (Winegard et al., 2014; Fudge et al., 2005).

#### Changes in exudate composition correspond to changes in slime cells within the gland

From analyzing the exudate of refilling slime glands, we saw a higher thread skein to mucous vesicle ratio at 14 days post-sliming, and the gland thread cells that were released were, on average, significantly smaller (Schorno et al., 2018). We proposed that releasing higher quantities of small thread skeins could conserve the functionality of the slime. If this were the case, we would expect to



**Fig. 9. Scatterplots of Pacific hagfish gland thread cell size and distance from gland edge as a function of refilling state.** A significant relationship between gland thread cell distance from the edge of the slime gland and cross-sectional area was found at each refilling time point (non-linear regression:  $P < 0.001$ ). Fewer large gland thread cells were found within exhausted slime glands (0 days post-sliming), and those that were present were also closer to the edge of the slime gland compared with those in full slime glands. The initial steepness of the power curve fit to the data (between 0 and 200  $\mu\text{m}$ ; dashed line) illustrates a gradient of gland thread cell sizes from the edge of the slime gland out to only a few cell lengths away before it starts to plateau, which suggests that most gland thread cell growth happens close to the epithelium. In full slime glands, gland thread cells were found much farther from the edge of the slime gland and were much larger on average compared with those in other refilling time points.  $n=5$  for each time point.

see, in our current histological analysis, approximately equal ratios of the areas occupied by thread cells and mucous cells at all refilling times; however, this was not the case. As for the vesicle to thread skein ratio, with the volume of thread cells and mucous cells increasing at the same rate, we would expect a steady increase in the mucous vesicle to thread skein ratio. This is a consequence of the number of vesicles increasing as mucous cells grow, but each gland thread cell only produces one thread skein even as it increases massively in volume. By some estimates, gland mucous cells contain between 8000 and 12,000 mucous vesicles (Leppi, 1968; Luchtel et al., 1991; Winegard, 2012). Based on the number of thread cells in our histological sections of full slime glands, we estimate that glands can contain between 6000 and 10,000 gland thread cells and likely similar numbers of gland mucous cells. The high mucous vesicle to thread skein ratio in full slime glands is likely the results of large gland mucous cells and gland thread cells being released, where each gland thread cell releases one large, long thread skein and each gland mucous cell releases thousands of mucous vesicles.

#### Origin of thread and mucous cells within the slime gland

Proliferating cells were found in the epithelial lining of the slime gland, but were not seen near the gland pore or in the interior of the gland (Fig. 6). PCNA-positive proliferating cells were sparse relative to the total number of cell nuclei seen in the epithelial lining of the gland, likely because PCNA is only expressed during the DNA synthesis phase (S phase) of the cell cycle and only a small proportion of the cells in the epithelial lining will give rise to the slime cells (Mathews et al., 1984; Prelich et al., 1987). These results

support the hypothesis that gland thread cells and gland mucous cells are produced from undifferentiated cells in the epithelial lining of the slime gland.

#### Changes in gland thread cell size and location within the slime gland

During refilling, a significant positive relationship was seen between the distance that the gland thread cell is from the edge of the slime gland and its cross-sectional area. Initially, there is a steep slope in the power curve fit to the data (data points between 0 and 40  $\mu\text{m}$ ), with the curve plateauing after the initial steep slope (data points between 40 and 2000  $\mu\text{m}$ ). This suggests that most growth happens close to the epithelial lining of the slime gland, which is reasonable as it minimizes the distance between growing cells and the capillaries that serve the slime gland (Lametschwandner et al., 1986). At 28 days post-sliming, we saw two peaks in frequency of gland thread cell size distribution in our histograms, one at 800  $\mu\text{m}^2$  and one at 2300  $\mu\text{m}^2$ . This likely indicates the growth of two distinct populations of gland thread cells during refilling, namely small gland and mucous cells close to the epithelium that were stimulated to grow when the gland was exhausted, and newly produced slime cells that arose via proliferation and differentiation within the epithelium.

Generally, as slime gland refilling proceeded, increased numbers of small gland thread cells were present near the edges of slime glands, while larger gland thread cells were found farther away from the edge as more cells were produced and grew below them. Because of the nature of sectioning an ellipsoidal cell such as a gland thread cell, some of the smaller cell cross-sections were likely

the result of cutting a larger cell near its edge, resulting in an underestimate of its maximum cross-sectional area. However, if our data on the smallest and largest gland thread cells are accurate, then the volume of the largest gland thread cells in a slime gland can be up to 1000 times larger than that of the smallest ones. For a given small cell, the appearance of the nucleus often made it possible to be confident that it had been sectioned close to its center. The smallest, and likely youngest, gland thread cells possess large, prominent nuclei (Winegard et al., 2014) and were usually found near the epithelium. This is consistent with our immunofluorescence results, which showed proliferative cells within the gland epithelium, often with small slime cells nearby. Our results are consistent with the hypothesis that gland thread cells and gland mucous cells originate from the undifferentiated cells lining the slime gland (Newby, 1946; Downing et al., 1981b; Spitzer et al., 1988).

### Model of gland refilling

Based on the histological observations and analysis presented here, we propose a new cellular model of slime gland refilling in hagfishes (Fig. 10). After the musculus decussatus contracts and squeezes exudate out of the gland, the gland is stimulated to begin the refilling process, which involves two main processes: growth of small, already differentiated slime cells near the gland epithelium, and the production of new cells. New cells destined to be gland thread cells and gland mucous cells are produced in the epithelial lining of the slime gland from undifferentiated cells in what we are coining the ‘proliferation zone’ of the slime gland. Slime cells are produced by differentiating from the undifferentiated cells in the epithelial lining of the slime gland. As these cells grow, they split the epithelial lining of the gland, with a thin layer of cells also being produced, wrapping around developing slime cells.

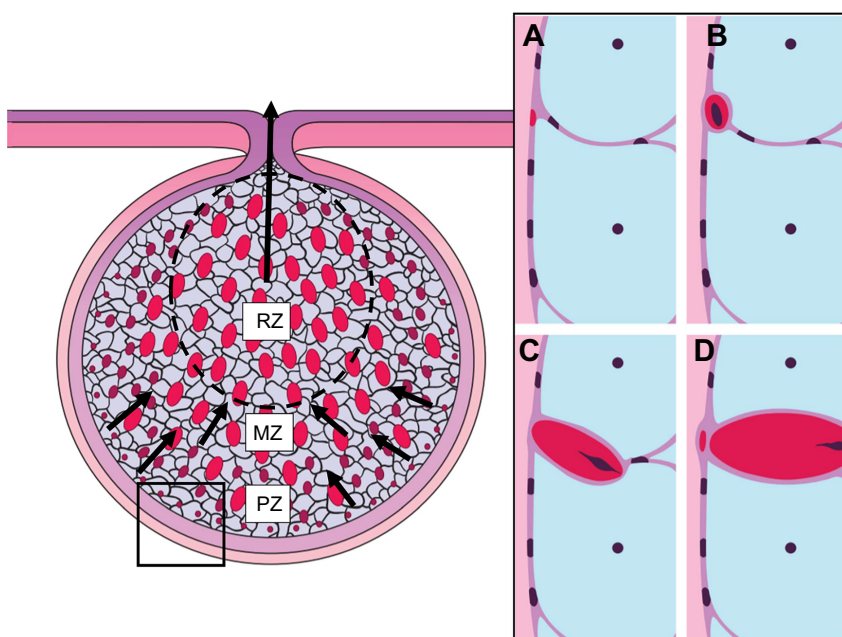
As cell proliferation continues, slime cells are pushed toward the center of the slime gland by subsequent cells being produced and growing below them. Slime cells continue to grow and mature as they pass through the maturation zone, which contains growing slime cells within various intermediate phases of development. Large slime cells can be found predominantly in the release zone, which we define as the area near the center of the slime gland that

contains the largest cells. When the musculus decussatus contracts, large slime cells in the release zone are ejected from the gland through the gland pore. As the slime gland refills, the musculus decussatus returns to its resting length, in preparation for ejection of the next bolus of slime exudate.

### Discrepancies in slime gland refilling time

Pacific hagfish slime gland refilling is a lengthy process, and likely represents a major energy investment for this species (Fudge et al., 2005). Schorno et al. (2018) found that refilling Pacific hagfish slime glands released equivalent amounts of exudate to full glands by 24 days post-sliming, indicating that they had likely refilled in this time. The present study found via histological cross-sections of refilling slime glands, that by 28 days post-sliming, glands were statistically equivalent in size to full slime glands. We should point out, however, that they were also not statistically different in size from the other refilling time points. This suggests that refilling was likely not complete after 28 days, as it was in the exudate study. This discrepancy in refilling rate may be due to differences in the experimental design between the two studies.

The hagfish in our previous study were each handled and anesthetized once before their exudate was collected (once to exhaust slime glands on left side of the animal) (Schorno et al., 2018). However, in the present study, to reduce the number animals used, each hagfish had multiple groups of slime glands emptied at several time points (Fig. 2A). Thus, each hagfish was handled and anesthetized five times before they were euthanized for tissue collection. Repeated handling and anesthesia of fishes can increase their stress levels, which in turn could have negative effects on their metabolism and recovery (Wendelaar Bonga, 1997; Vijayan and Moon, 1992; Wagner et al., 2003; Holloway et al., 2004). It is also possible that by stimulating glands in various regions of the hagfish body we created some discrepancies as well. The size of the slime glands may inherently differ significantly along the length of the body. If this were the case, then a gland that is much larger would appear to reach the same size or ‘refill’ much faster than other smaller glands along the body. However, the diversity of gland sizes among the hagfish body regions has not yet been investigated.



**Fig. 10. Schematic representation of Pacific hagfish slime gland refilling.** Slime cells (in this case a gland thread cell) are produced from small, undifferentiated cells among the epithelial cells in the epithelial lining of the slime gland in the proliferation zone (PZ; inset) of the slime gland. As cell proliferation continues, slime cells are pushed toward the center of the slime gland by subsequent cell divisions below them. Slime cells continue to grow as they pass through the maturation zone (MZ), which contains growing gland mucous cells (light purple) and gland thread cells (dark red) within various intermediate phases of development. Large gland mucous cells (light purple) and gland thread cells (red) are found predominantly in the release zone (RZ), the area closest to the center of the slime gland. When the slime gland is stimulated, large slime cells in the release zone are ejected from the slime gland through the gland pore via contraction of the gland musculature. (Inset) Slime cells are produced from the small, undifferentiated cells (red) found among the epithelial cells in the epithelial lining (purple) of the slime gland by growing out from within the epithelial lining of the gland. A–D represent the time series for production/development of the new gland thread cell as illustrated.

## Conclusions

This study expands our current understanding of how Pacific hagfish slime glands replenish their slime stores after they are exhausted. Our data are consistent with the finding that slime gland refilling is a surprisingly slow process, and that refilling of the glands is likely limited by the production of the complex, coiled protein-rich slime threads rather than the production of mucous. We found that changes in composition of the exudate during refilling correspond to changes in the slime cells within the glands, namely that increased numbers of gland thread cells within the gland early in refilling likely contribute to the lower mucin vesicle to thread skein ratio of the exudate. We also provide evidence that proliferative cells are found among the epithelial cells within the lining of the slime gland, and that these are likely the origin of new gland thread cells and gland mucous cells. Finally, we propose a model that outlines how refilling of the slime gland occurs, with most of their growth happening near the edge of the slime gland, and growing cells being pushed to the center of the gland via production and growth of subsequent cells below them.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: S.S., T.E.G., D.S.F.; Methodology: S.S.; Software: S.S.; Validation: S.S.; Formal analysis: S.S.; Investigation: S.S.; Data curation: S.S.; Writing - original draft: S.S., T.E.G., D.S.F.; Writing - review & editing: S.S., T.E.G., D.S.F.; Visualization: S.S., T.E.G., D.S.F.; Supervision: T.E.G., D.S.F.; Project administration: T.E.G., D.S.F.; Funding acquisition: T.E.G., D.S.F.

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