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Emptying and Refilling of Slime Glands in Atlantic (*Myxine glutinosa*) and Pacific (*Eptatretus stoutii*) Hagfishes

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SUMMARY

Hagfishes are known for their unique defensive slime, which they use to ward off gill breathing predators. While much is known about the slime cells (gland thread cells and gland mucous cells), little is known about how long slime gland refilling takes, or how slime composition changes with refilling or repeated stimulation of the same gland. Slime glands can be individually electro-stimulated to release slime, and this technique was used to measure slime gland refilling times for Atlantic and Pacific hagfish. The amount of exudate produced, the composition of exudate, and the morphometrics of slime cells were analyzed during refilling, and as a function of stimulation number when full glands were stimulated in rapid succession. Complete refilling of slime glands for both species took three to four weeks, with Pacific hagfish achieving faster absolute rates exudate recovery than Atlantics. We found significant changes in composition of exudate and morphometrics of slime cells from Pacific hagfish during refilling. Over successive stimulations of full Pacific glands, multiple boluses of exudate were released, with exudate composition, but not thread cell morphometrics, changing significantly. Finally, histological examination of slime glands revealed slime cells retained in glands after exhaustion. Discrepancies in volume of cells released that can be explained by contraction of striated muscle alone suggests other mechanisms may be involved in the exudate ejection. Our results provide a first look at the process and timing of slime gland refilling in hagfishes, and raise new questions about how refilling is achieved at the cellular level.

INTRODUCTION

Hagfishes (Myxini) are known for their unique defensive slime (Downing et al., 1981a; Newby, 1946), which is an effective defense against attacks by gill-breathing predators (Lim et al., 2006; Zintzen et al. 2011). The slime is produced by many slime glands that occur in a line down both sides of the hagfish. The slime glands are connected to the skin surface by a short duct, and are surrounded by striated muscle and a connective tissue capsule (Downing et al. 1981b; Blackstad, 1963; Koch et al., 1991). The slime glands contain two main secretory cells: gland thread cells and gland mucous cells. The gland thread cells each contain a single, elaborately coiled protein-rich thread formation, known as a skein, which is precisely bundled and organized into stacked thread loops within the cell (Winegard et al. 2014; Downing et al., 1984; Spitzer et al., 1984). The gland mucous cells are filled with hundreds of tiny mucus-containing vesicles (Luchtel et al., 1991; Salo et al., 1983). When the musculature around the slime glands contracts, it initiates a rapid holocrine secretion event; the two gland components are forced out through the narrow gland pore, and in the process, their plasma membranes are sheared off (Downing et al., 1981a, b). In seawater, the thread and mucus components interact to yield an ultra-dilute mucous gel (Downing et al., 1981b; Spitzer et al., 1987; Fudge et al., 2005; Ewoldt et al., 2011).

The mass of slime exudate from all the glands of a Pacific hagfish (*Eptatretus stoutii*) has been estimated to be 3-4% of body mass (Fudge et al., 2005), and therefore represents a significant energetic commitment. In spite of the large amounts of pre-exudate stored in the slime glands, hagfish appear to be economical with their use of the slime in two important ways. First, the slime is remarkably dilute, with mucus concentrations three orders of magnitude lower than other mucous secretions such as gastric mucus (Fudge et al., 2005). Second, hagfishes conserve slime by only releasing exudate from glands in the vicinity of an attack, and not as a whole body response (Lim et al., 2006; Zintzen et al., 2011). The large number of slime glands possessed by hagfishes (~79 pairs in Pacific hagfish and ~97 pairs in Atlantic) and their ability to release exudate locally likely reduce the chances that a hagfish will be left without slime to defend itself in subsequent attacks (Fernholm, 1998). Vulnerability to predators will also be affected by how many times a single gland can release exudate before it is empty, and how quickly depleted slime glands can recharge, which were both major foci of this study.

While the time scale for slime gland recovery after a sliming event has not been documented in any species of hagfish, Lametschwandtner et al. (1986) speculated about the relative rates of recovery in Pacific hagfish and Atlantic hagfish (*Myxine glutinosa*) based on differences in the vascular anatomy of the slime glands in these two species. In Atlantic hagfish, capillaries form a cage around the slime gland, whereas in Pacific hagfish, capillary loops also descend into the gland interior, which may increase the rate of nutrient delivery to the slime glands and decrease the recovery time in this species (Lametschwandtner et al. 1986).

In this study, we aimed to answer several fundamental questions about the refilling of slime glands in hagfishes. Using a population of captive Atlantic and Pacific hagfishes, we measured the time it takes for slime glands to refill after they have been emptied of slime exudate. We also investigated the possibility that a single slime gland can eject multiple boluses of exudate, and if so, whether the composition of the exudate changes with successive ejections. In addition, we examined the effects of recovery time after a sliming event on the composition of exudate in a gland, the relative proportions of threads and mucus in the exudate, as well as the morphometrics of the thread skeins.

METHODS

Experimental animals

Pacific hagfish (*Eptatretus stoutii*) were collected from Bamfield Marine Station in Bamfield, BC, Canada, and Atlantic hagfish (*Myxine glutinosa*) were collected from Passomoquody Bay, New Brunswick, Canada. All hagfishes utilized in this study were adults, but the age and sex of each individual was unknown. Both species were housed together in a 2000 litre Environmentally Controlled Aquatic Recirculating System filled with chilled artificial seawater (34 ppm, 10°C) at the Hagen Aqualab at the University of Guelph, Guelph, ON, Canada. Hagfish were isolated in floating bins within the tank for a minimum of one month (30 days) prior to our experiments to make sure their slime glands were completely full. Hagfish were fed squid to satiety once per month. All housing and feeding conditions were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #2519).

Hagfish anesthesia and slime collection

Hagfish were anesthetized by placing them in 3 L of artificial saltwater (Coralife, Energy Savers Unlimited, Inc., Carson, CA, USA) with 3 mL of a clove oil (Sigma-Aldrich, Oakville, ON, Canada) anesthetic solution (1:9 clove oil to 95% ethanol) and left until they ceased to respond to touch. Once fully anesthetized, the hagfish were removed from the artificial saltwater, rinsed with deionized water, patted dry using Kim Wipes (Kimberly-Clark Corporation, Irving, TX, USA), and placed on a dissection tray. The slime glands of the hagfish were induced to secrete slime exudate via mild electrostimulation (60Hz, 18V) using a Grass SD9 electric stimulator (Grass Instruments, Quincy, MA, USA). Expressed exudate was collected from the skin using a scoopula and either gently stirred into a 0.9 M sodium citrate (Fisher Scientific, Ottawa, ON, Canada), 0.1 M PIPES [piperazine-*N,N'*-bis(ethanesulfonic acid)] (Sigma-Aldrich, Oakville, ON, Canada) stabilization buffer (pH 6.7) or collected onto a pre-weighed paper towel (Fudge et al., 2003). If at any point during its handling a hagfish produced slime, it was excluded from the trials.

Successive stimulation trials

Samples from successive stimulations of the same glands were collected and analyzed to test whether the composition of the exudate changes as more and more exudate is expressed from the glands. Pacific hagfish (n=20) with full glands were anesthetized and had ten glands posterior to the gill pouches stimulated. A single stimulation was defined as touching the electro-stimulator wand to each slime gland only long enough for exudate to be released. The exudate produced from the first five glands during each stimulation was pooled and collected into individual microfuge tubes (Fisher Scientific, Ottawa, ON, Canada) containing 1.5 mL of stabilization buffer to be used for later analysis (one tube per stimulation). The final five glands' exudate was collected onto pre-weighed paper towels to determine the mass of exudate produced. Exudate from each stimulation was collected in this manner until the glands were exhausted. Time between stimulations ranged from 15-30 seconds depending on the time needed to collect the previous stimulation's sample. Microfuge tubes containing samples of exudate from each stimulation were gently mixed by inversion, and 20 µl subsamples were removed and analyzed as described above.

Slime gland regeneration trials

To determine how long it takes for hagfish slime glands to re-fill, a series of exudate collection trials were conducted using one hundred individuals of similar mass from each species (Pacific 51.54±2.53 g; Atlantic 42.14±1.23g), with ten individuals used for each of the ten time intervals examined. For each recovery time treatment, all glands on the left side of each specimen were exhausted completely (no longer producing exudate) at time zero using repeated electrostimulation, while keeping the glands on the right side unstimulated and serving as an internal control. Experimental hagfish were kept separate from others by placing them in a smaller floating container in the tank. Hagfish were isolated for various time intervals (4 days, 8 days, etc.) and removed from the tank at desired days post sliming to have their slime exudate collected. Each individual had one refilling timepoint (left side of hagfish) and full glands (right side of hagfish) from which slime was collected. After recording hagfish body mass, slime from each side of the animal was collected separately onto pre-weighed paper towels to determine the mass of slime produced by the refilling side and the full side of the animal.

Animal euthanasia and gland dissection

Exhausted and unstimulated (‘full’) glands were harvested from five Pacific hagfish for histological analysis. Hagfish were anesthetized then euthanized by severing the notochord and dorsal nerve cord directly using a large pair of scissors. Hagfish were dissected according to a protocol developed by Winegard (2012), which allows the slime glands to be separated from the myotomal muscle in which they are embedded while still attached to the skin at the gland pore. Dissected slime glands were placed in fixative for later histological analysis.

Fixation, embedding and staining of hagfish tissues

Harvested gland tissue was fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) solutions in preparation for paraffin embedding. Paraformaldehyde containing fixatives were prepared in stabilization buffer (0.9 M sodium citrate, 0.1 M PIPES; pH 6.7) to reduce swelling of the mucous vesicles within the gland. Fixed tissues were processed for paraffin embedding in the Ontario Veterinary College Veterinary Histology Unit using a routine overnight protocol. Hagfish slime gland diameter was measured with calipers, trimmed and sagittally sectioned (5 μm thick sections) to the approximate center of the gland using a rotary

microtome. Sections were placed on frosted glass microscope slides (Fisher Scientific, Ottawa, ON, Canada) for histological staining. Whole glands were stained with hematoxylin and eosin (HandE; Fisher Scientific, Ottawa, ON, Canada), and slides were covered with cover glass (22x50 mm; Fisher Scientific, Ottawa, ON, Canada) then sealed with Cytoseal XYL (Richard Allen Scientific, San Diego, USA). The Cytoseal dried overnight before visualization.

Brightfield imaging of exudate and histology samples

Slides with exudate and histology samples 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) driven by NIS Elements AR software (Nikon Instruments, Inc., Melville, NY, USA). For each stimulation subsample, three images of the exudate were taken for analysis (3 replicates per sample). For histological sections, multiple tile images of gland sections taken at 10x magnification were stitched together using the 'scan image' function in NIS elements. Exudate sample images were analyzed using ImageJ software (Abramoff et al., 2004). Exudate sample images were analyzed for number of thread skeins present (number of thread skeins/area of view), thread cell diameter along the short axis (d1) and long axis (d2) (µm), and mucous vesicle concentration (number of vesicles/area of view) (Fig. 1). The long and short axis measurements of thread skeins were used to calculate thread skein volume ($4/3*\pi*(d_1/2)^2*d_2/2$), where each thread skein was treated as an ellipsoid. Histology samples were analysed using NIS Elements AR software.

Statistical analysis

All statistical analyses were run using SPSS v. 23.0 (SPSS Inc., Armonk, NY, USA) with an $\alpha=0.05$. Non-positive values (0 or negative values) were excluded from the analyses. Outliers identified by SPSS software were also excluded from analyses and figures. For the successive stimulations of full glands trials, non-linear regression analyses were performed on the percent of exudate released with each stimulation data, as well as the data for the mucous vesicle to thread skein ratio released with each stimulation. A one-way ANOVA test with post-hoc LSD was conducted on the thread skein morphometrics (short axis d1, long axis d2, volume) data to test for differences in thread skein size between stimulations. Due to the nested design of the study, replicate number (3 replicates per subsample) and individual number (n=20 for successive stimulations trial) were used as break variables for statistical analysis of successive stimulation

exudate composition samples. A two-way ANOVA with post-hoc Tukey's HSD and LSD tests was conducted on gland refilling data for both species to test for the effects of days post sliming and species on the proportion of refilling. A two-way ANOVA with post-hoc Tukey's HSD and LSD tests was also conducted on the absolute slime gland refilling timeline data (total slime from full glands/number of slime glands; total slime from full glands/mass of the hagfish) between the two species. A one-way ANOVA with post-hoc LSD testing was used to analyze whether the morphometrics of skeins in collected exudate significantly differed as a function of days post sliming. Non-linear regression analysis was performed on the mucous vesicle to thread skein ratio data as a function of days post sliming.

RESULTS

Successive stimulations of 'full' Pacific hagfish slime glands

The percent total exudate released significantly decreased with successive stimulations of the full glands, with more than half of the gland contents being released during the first and second stimulation (42.1±3.2%, 23.7±1.5%, respectively) (Fig. 2A). A logarithmic curve was fit to the percent of total exudate released per stimulation data ($R^2 = 0.731$; $f=359.419$; $p<0.001$). Non-linear regression analysis revealed that stimulation number had a significant effect on the percent of total exudate released ($df = 2, 132$; $f=429.378$; $p<0.0001$).

Thread skein dimensions were remarkably consistent over successive stimulations of full glands (Fig. 2B). Full slime glands, on average, released larger sized thread skeins (d1 = 72.5±0.4 µm, d2 = 158.8±0.8 µm, volume = $4.6x10^3 \pm 5.6x10^3$ µm^3) over successive stimulations. Thread skein short axis length (d1) ($df = 7, 125$; $f=0.448$; $p=0.870$), long axis length (d2) ($df = 7, 125$; $f=0.779$; $p=0.606$) and volume ($df = 7, 125$; $f=0.318$; $p=0.945$) were found to not significantly differ among stimulations. The mucous vesicle to thread skein ratio in the exudate decreased significantly with successive stimulations of the full Pacific hagfish slime glands (Fig. 2C). The mucous vesicle to thread skein ratio was highest in the initial stimulation of the slime gland, and consistently decreased with each successive stimulation. An inverse curve was fit to the mucous vesicle to thread skein ratio data ($R^2 = 0.552$; $f=143.982$; $p<0.001$). Non-linear regression analysis revealed that stimulation number had a significant effect on this ratio ($df = 2, 117$; $f=167.716$; $p<0.0001$).

Histological analysis of exhausted and full glands from Pacific hagfish

H&E stained histological sections from Pacific hagfish slime glands that were stimulated until exhaustion revealed that these glands were not devoid of slime cells (Fig. 3A). Full glands on average had nearly 3.5 times the cross-sectional area of exhausted glands (full glands = 7.83±1.10 mm^2, exhausted glands = 2.15±0.25 mm^2), and contained almost triple the number of thread cells (full glands = 680±91, exhausted glands = 246±31) (Fig 3B). Many of the exhausted gland sections seemed to have fewer gland thread cells in the centre of the gland compared to full glands, with more gland thread cells found near the periphery of the gland near the gland capsule. The circumference of exhausted glands was about half that of corresponding full glands ($C_{exhausted}/C_{full}$ = 0.53±0.06).

Timeline for slime gland refilling in Pacific and Atlantic hagfishes

We found that slime gland refilling is a process that takes multiple weeks in both Pacific and Atlantic hagfishes (Fig. 4). Refilling slime glands for both species were found to release equivalent masses of exudate compared to full slime glands by 24-28 days post sliming indicating that they had refilled by this timepoint [Tukey post hoc testing; Pacific hagfish: 24, 28 and 32 days post sliming ($p=0.829$, $p=0.953$, and $p=1.000$ respectively); Atlantic hagfish: 28 and 32 days post sliming ($p=0.995$, $p=1.000$ respectively)]. A two-way ANOVA revealed that days post sliming had a significant effect on proportion of refilling ($df=8$; $f=129.784$; $p<0.0001$), but revealed no significant effect of species ($df=1$; $f=3.464$; $p=0.065$) and no significant interaction effect of species and days post sliming on proportion of refilling ($df = 8$; $f=1.745$; $p=0.092$).

Exudate mass measurements from full slime glands also allowed us to compare the absolute amount of stored exudate in Pacific and Atlantic hagfishes. By dividing the total exudate mass collected from one side of a hagfish by the number of slime glands (79 for Pacific hagfish, 97 for Atlantic hagfish), we calculated the average amount of exudate obtained from each gland. For Pacific hagfish, the average was 0.0053 ± 0.0034 g and for Atlantic hagfish, the average was 0.0028 ± 0.0012 g, a difference that was statistically different (t-test, $df = 198$, $t = 6.811$, $p < 0.001$). If the same data are normalized for body size (and not the number of slime glands), the values are 8.3 ± 4.6 g exudate/kg for and 6.5 ± 2.0 g exudate/kg for Pacific and Atlantic hagfish, respectively, which is also a significant difference (t-test: $df = 198$, $t = 3.557$, $p < 0.001$).

Pacific hagfish refilling exudate composition analysis

Pacific hagfish slime glands in the later stages of refilling (21, 28 days post sliming) and those that were full required significantly more stimulations to exhaust them compared to slime glands in the earlier stages of refilling (7, 14 days post sliming) (one-way ANOVA: df=4; f=18.023; $p<0.0001$). Slime glands in the earlier stages of refilling (14, 21 days post sliming) also released significantly smaller thread skeins on average compared to those in the later stages of refilling and those that were full (Post-hoc LSD testing: $p<0.05$) (Fig. 5A). Thread skein short axis length (d1) (df = 4, 35; f=4.881; $p<0.01$), long axis length (d2) (df = 4, 35; f=4.536; $p<0.01$) and volume (df = 4, 34; f=4.723; $p<0.01$) all differed significantly over the course of Pacific hagfish slime gland refilling. The mucous vesicle to thread skein ratio significantly increased with refilling of the Pacific hagfish slime gland, but was significantly reduced at 14 and 21 days post sliming (Post-hoc LSD testing: $p<0.0001$) compared to the proportion released from full glands (Fig. 5B). A quadratic curve was fit to the mucous vesicle to thread skein ratio in refilling exudate data (R^2 = 0.378; f=10.627; $p<0.001$). Non-linear regression analysis revealed that days post sliming had a significant effect on this ratio (df = 3, 35; f=68.803; $p<0.0001$).

DISCUSSION

Timeline for slime gland refilling in Pacific and Atlantic hagfishes

This study provides the first ever timeline of slime gland refilling in hagfishes. The refilling process takes several weeks after the slime glands have been exhausted, with Pacific hagfish glands refilling marginally faster than Atlantic hagfish glands. It is important to keep in mind that the data shown in Fig. 4 are normalized against the mass of exudate obtained from the previously unstimulated side of the body, and therefore provide no information about the absolute rate of exudate production. Absolute measurements of exudate mass from full glands of both species reveal that Pacific hagfish glands contain almost double the mass of pre-exudate as Atlantic hagfish glands. Thus, while the glands in both species refill in approximately the same amount of time, the absolute rate of exudate production is about twice as fast in Pacific hagfish, a result that is consistent with the more elaborate vascular anatomy in this species (Lametschwandtner et al., 1986). This increased absolute rate of exudate production in Pacific hagfish may reflect more intense predation pressures on this species. Furthermore, the lower metabolic rate and cardiac

function of the Pacific hagfish means that they expend an even larger fraction of their total energy budget to refill a given slime gland (Munz and Morris, 1965; Hansen and Sidell, 1983; Steffenson et al., 1984; Forster et al., 1991).

Implications of long gland refilling times

While there is no striking difference in relative refilling rates between both species, the time required for complete refilling of the glands is surprising, taking more than three weeks in the two species. Such a long recovery time raises the question of how hagfishes avoid being depleted of slime, which would make them vulnerable to attacks by predators. One possibility is that each slime gland contains enough exudate to participate in several defensive sliming events. Our successive stimulation data demonstrate that it is at least physiologically possible for hagfishes to release only a fraction of their slime gland contents at a time. However, it is also possible that our results are an artefact of the electrical stimulation protocol we used, and that the release of slime in the wild is an all-or-nothing response. In this case, a subsequent attack by a predator in the same area would indeed lead to a diminished sliming defense response.

It is also not clear why the slime glands take so long to refill. One factor may be the low metabolic rates of hagfishes, which are the lowest of any craniate (Forster, 1990). It is also possible that the elaborate thread skeins produced by gland thread cells simply take a long time to manufacture and are a limiting step in the refilling process. While the mechanism of skein production in gland thread cells is unknown, the leading hypothesis suggests that the 150-mm thread elongates from a single growing end (Fudge and Schorno, 2016). If this is the case, then the rate of thread elongation must be on average about 6 mm per day, or about 60 times the length of a single gland thread cell, assuming a 24-day maturation time. While it is known that the thread undergoes both lengthening and thickening during its development, the relative rates and timing of these processes are unknown (Winegard et al. 2014). Given that most mucus-producing epithelia continuously produce mucus, including those in the hagfish epidermis, it is difficult to imagine that mucus production is the limiting factor in the refilling of the slime glands.

Regulation of slime gland exudate refilling

It is not clear whether ejection of exudate from the slime gland initiates refilling, or whether production of slime cells is a continuous process similar to the production and turnover of

gametes in seminiferous tubules (Clermont and Perey, 1957; Klein et al., 2010). We propose that slime cell production is initiated after exudate is ejected, with division, differentiation, maturation, and growth of slime cells continuing until the gland is full. However, full slime glands are known to contain small gland thread cells near the gland epithelium, which at first glance may imply a continuous production of new cells (Newby, 1946; Downing et al., 1981 a; Downing et al., 1984). Another interpretation is that these small gland thread cells are arrested in their development and resume growing and maturing after exudate is ejected and refilling commences. Keeping numerous small thread cells near the epithelium may reduce the time to refilling compared to a process that relies completely on the production of all new cells. Further examination of slime glands at several stages of refilling, including staining for apoptotic and proliferative markers, should allow us to answer these remaining questions about the cellular mechanisms of slime gland refilling.

Exhausted Pacific hagfish slime glands contain slime cells

As seen in histological cross-sections, exhausted Pacific hagfish slime glands were on average about one third the area of 'full' glands, and exhausted glands contained fewer gland thread cells on average. However, exhausted glands were not devoid of gland thread cells and gland mucous cells, which raises the questions of how and why some cells are ejected from the glands and others are retained. One way to approach this question is to consider the mechanics of the thin layer of striated muscle (i.e. the *musculus decussatus*) that surrounds the slime gland capsule. When these muscle fibers contract, slime cells are squeezed out through the narrow gland pore and rupture in the process, releasing their thread and mucous secretory products. Striated muscle can generally contract about 10% of its total length (Rassier et al., 2003; Peterson et al., 2004; Herzog et al., 2008). If we assume the slime gland is a sphere with radius r, circumference C, maximal cross-sectional area A, and volume V, then a contraction of 10% the muscle around the gland will reduce the circumference to 0.9C, the radius to 0.9r, the area to 0.81A, and the volume to 0.73V. However, histological analysis of empty glands reveals that the latter have values of 0.53r, 0.53C, 0.28A, and 0.15V compared to their corresponding full glands. These numbers change the pertinent question from "how do the slime glands retain the cells they do?" to "how do the glands eject as many cells as they do?" given the limitations of striated muscle shortening. One possible mechanism may be that the muscle fibers in the capsule are connected in series to elastic elements that stretch as the gland is refilled, even after the muscle fibers have stretched to their limit. In this

scenario, contraction of the muscle fibers causes some shortening, but more importantly, increases pressure within the gland above the threshold pressure needed for pre-exudate to begin to flow through the narrow gland pore. Once resistance at the gland pore is overcome, exudate is ejected out of the gland primarily by the relaxation of previously stretched series elastic elements. Another possibility is that contraction of adjacent myotomal muscle assists in squeezing more exudate out of the glands than could be achieved by contraction of the *musculus decussatus* alone. Further work is needed to the test the viability of these hypotheses.

The fact that not all mucous and thread cells are ejected from slime glands stimulated to exhaustion raises the question of which cells get ejected and which remain. Intercellular adhesion forces between cells may be relevant here, with immature cells being strongly attached to their neighbors and retained, and mature cells exhibiting lower adhesion, and thus a greater likelihood of getting squeezed out the gland pore. A network of putative nurse cells (gland interstitial cells) may also be involved in the retention of immature cells in the gland (Fudge et al., 2015). Regardless of the mechanism, it seems sensible that the gland can preferentially eject mature cells and retain immature ones, as deviating from this pattern would be a waste of resources and likely would reduce the slime's predator-repelling efficacy.

Thread skein morphometrics in Pacific hagfish exudate during emptying and refilling

Compositional analysis of Pacific hagfish slime exudate revealed that thread skein size in full slime glands was conserved from the first stimulation to the last. This is interesting, given that histological sections show small gland thread cells in full glands near the gland epithelium (Fig. 3). The discussions of muscle mechanics and cell adhesion above are the simplest explanations for these observations, i.e. small cells are not released from full glands because the *musculus decussatus* can only contract so much, and the smaller gland thread cells near the gland epithelium are well adhered. The result is a preferential release of mature gland thread cells closer to the center of the gland. The conservation of thread skein size over multiple stimulations from full glands is also interesting because glands that are in the process of refilling release thread skeins that are substantially smaller than those released from full glands, but release lower mucous vesicle to thread skein ratios at these timepoints. This is consistent with the findings of Spitzer et al. (1988), who showed that in recently slimed glands, smaller thread skeins represented a higher percent of the total number of thread skeins present within a dissected slime gland. A proximate explanation of this pattern is that gland mucous cells regenerate and grow faster in depleted glands than gland

thread cells, which makes sense given the complexity of thread production in gland thread cells. Thus, contraction of the *musculus decussatus* results in ejection of the most mature thread cells, which in the latter case, are not fully mature, and smaller than those ejected from full glands. While ejection of smaller thread skeins likely has consequences for the function of the slime, such effects have not yet been investigated. It has been previously suggested that the threads provide a wide array of properties to the slime, including imparting cohesiveness, preventing mucin wash-out allowing for better clogging, and providing anchoring points for the mucins within the slime (Fudge et al., 2005; Lim et al., 2006; Böni et al., 2016). It is possible that ejection of smaller thread skeins results in a reduced clogging ability of the slime, however, ejection of sub-optimal slime is undoubtedly preferable during an attack to releasing no slime at all.

Mucous vesicle to thread skein ratio in Pacific hagfish exudate during emptying and refilling

We found a stark decrease in the mucous vesicle to skein ratio over successive stimulations of the full Pacific hagfish slime glands to exhaustion. This pattern may arise via gland mucous cells being more abundant in areas of the gland interior that are close to the gland pore and they are squeezed out first. The functional significance of changing the mucous vesicle to thread skein ratio is not entirely clear, although Koch et al. (1991) demonstrated that manipulating this ratio *in vitro* using sodium citrate stabilized slime exudate can result in predictable changes in slime cohesion. This ratio also varied as a function of the refilling time. At 14 and 21 days post sliming, the mucous vesicle to thread skein ratio was significantly less than all other time points in the refilling cycle. This pattern may simply be the result of a spike in the number of thread skeins that are mature enough to be ejected but not yet fully mature. Alternatively, slime glands may have evolved an adaptive mechanism to compensate for releasing smaller thread skeins during early refilling. Releasing smaller cells in larger quantities may make up for the shorter length of the thread skeins being released. Perhaps it is not the mucous vesicle to thread skein ratio that is important, but rather the vesicle to thread length ratio that is more critical. Because the relationship between thread length and skein size is not known, we are currently unable to evaluate whether this ratio is conserved.

Conclusions

This study provides detailed information about hagfish slime exudate and how it varies as a function of several factors, including recovery time after the slime glands are depleted, species, and, in experiments where the glands are stimulated in rapid succession to release exudate, stimulation number. Our data demonstrate that slime glands from Atlantic and Pacific hagfishes take three to four weeks to refill completely, with Pacific hagfish achieving faster rates of absolute exudate recovery. We found that individual Pacific hagfish slime glands can release multiple boluses of exudate, with the mass of each successive bolus decreasing exponentially. In full Pacific hagfish glands, thread skein morphometrics were conserved from the first bolus of exudate expressed to the last, although the mucous vesicle to the thread skein ratio declines as more exudate is expressed. We also found that exudate composition and thread cell morphometrics shift during Pacific hagfish gland refilling, with a general trend toward larger skeins being expressed as gland recovery time increases. Finally, histological analysis of full and exhausted glands from Pacific hagfish revealed that a larger volume of cells is expressed than can be explained by the contraction of striated muscle fibers in the *musculus decussatus*, suggesting that other mechanisms may be involved in the ejection of holocrine secretion products from the slime glands.

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COMPETING INTERESTS

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this study.

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Figures

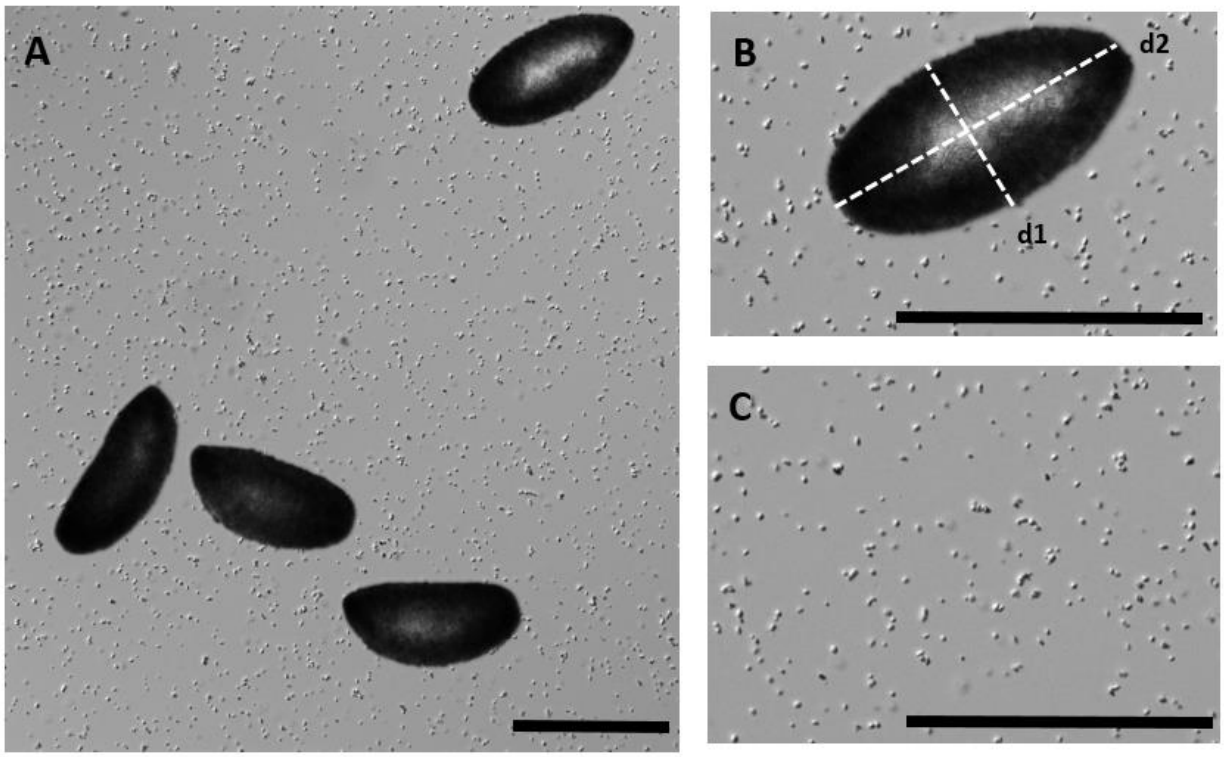

Figure 1. Brightfield image illustrating the composition of stabilized, diluted Pacific hagfish slime gland exudate collected via electrical stimulation of a slime gland. A) Exudate sample collected into 1 ml of S.B. during a successive stimulation trial; B) Thread skein morphometric measurements recorded from slime samples (d1 = short axis, d2 = long axis). Thread skein concentrations (number of thread skeins/area of view, μm^2) was also recorded; C) Mucous vesicle concentrations were recorded by counting the number of vesicles per area of view (number of mucous vesicles/area of view, μm^2). Scale bar = 250 µm.

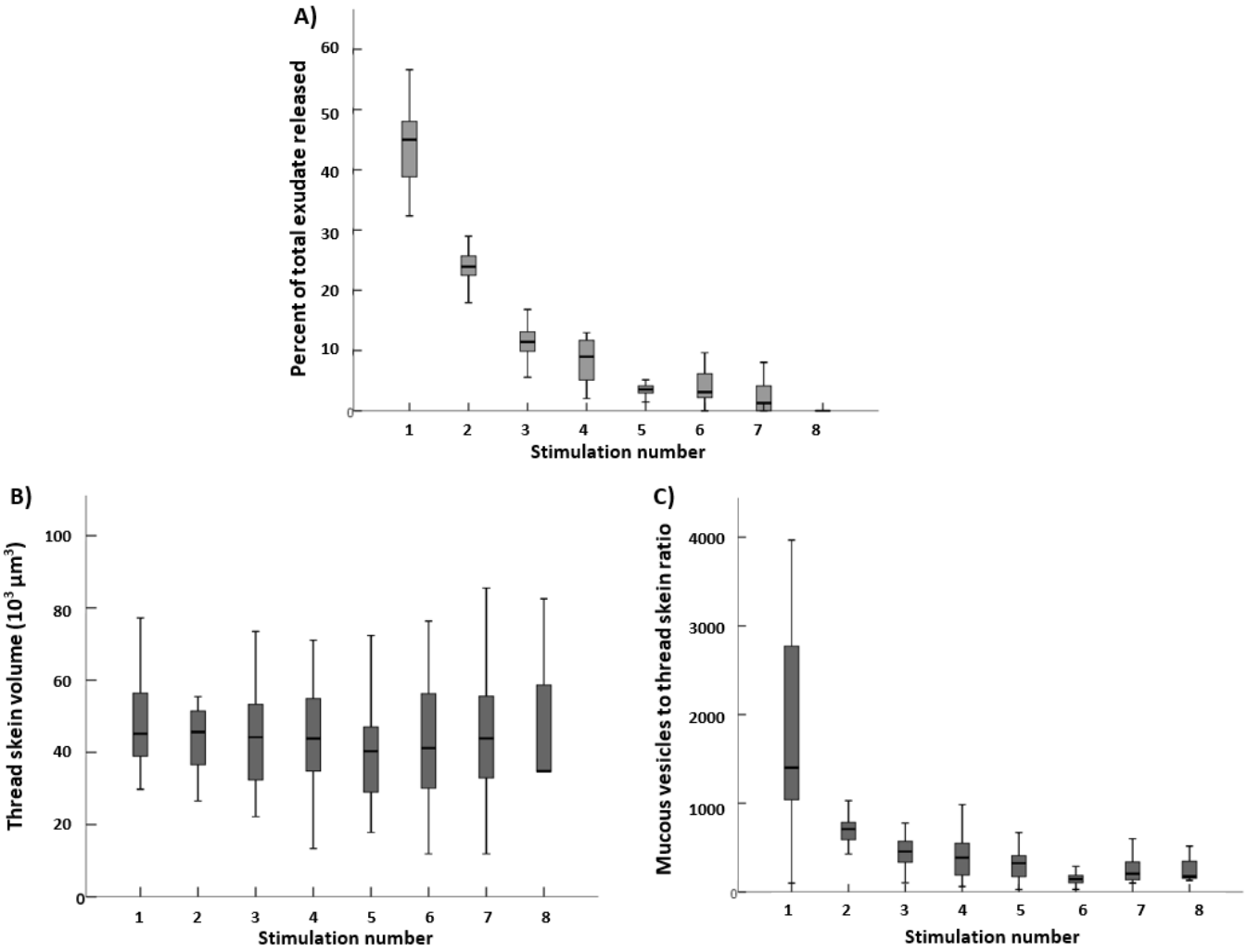

Figure 2. Emptying of Pacific hagfish slime glands. A) Mass of exudate released decreased significantly over successive stimulations of the slime gland, requiring up to eight electro-stimulations for exhaustion in some individuals and over half of the gland contents released in the first two stimulations (Non-linear regression: df = 2, 132; f=429.388; p<0.0001); B) Thread skein volume was conserved from the first stimulation of the slime gland to the last (one-way ANOVA: df = 7, 125; f=0.32; p=0.95); C) Mucous vesicle to thread skein ratio in exudate significantly decreased with successive stimulations of the slime gland (Non-linear regression: df = 2, 117; f=167.72; p<0.0001) (n=20 for each stimulation).

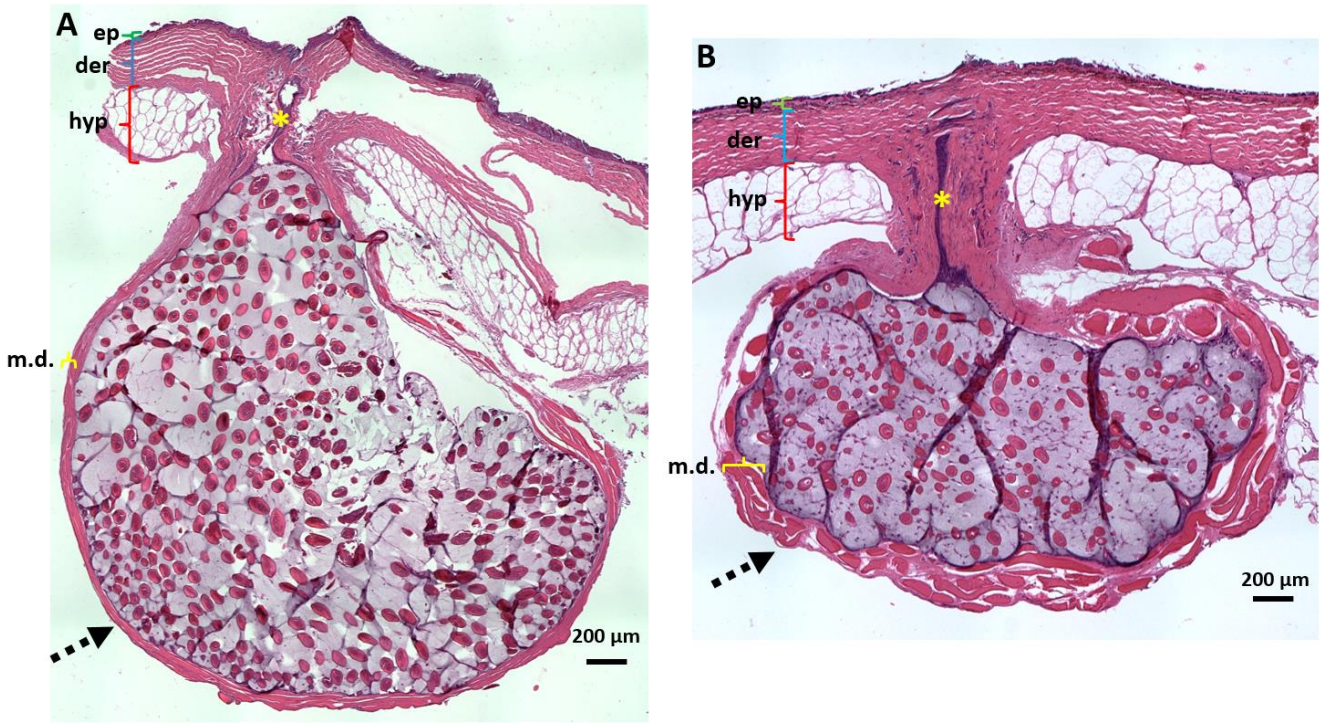

Figure 3. Cross-sections through H&E stained slime glands of Pacific hagfish comparing slime distribution in 'exhausted' and 'full' slime glands. Sagittal cross-sections through slime glands illustrating the gland pore (*), and gland capsule (dashed arrow). Epidermis (ep), dermis (der) and hypodermis (hyp) are continuous with the slime gland epithelium. The *musculus decussatus* (m.d.) surrounds the slime gland. Note the increase in thickness of the gland capsule wall/muscle layer in the empty glands compared to the full glands. Gland thread cells stain pink within the gland, while gland mucous cells stain purple. A) 'full' slime gland, harvested without stimulation of the gland; B) exhausted slime gland, harvested immediately after stimulation of the gland until exhaustion. The exhausted gland still retains slime cells but the gland itself is much smaller and possesses distinct creases.

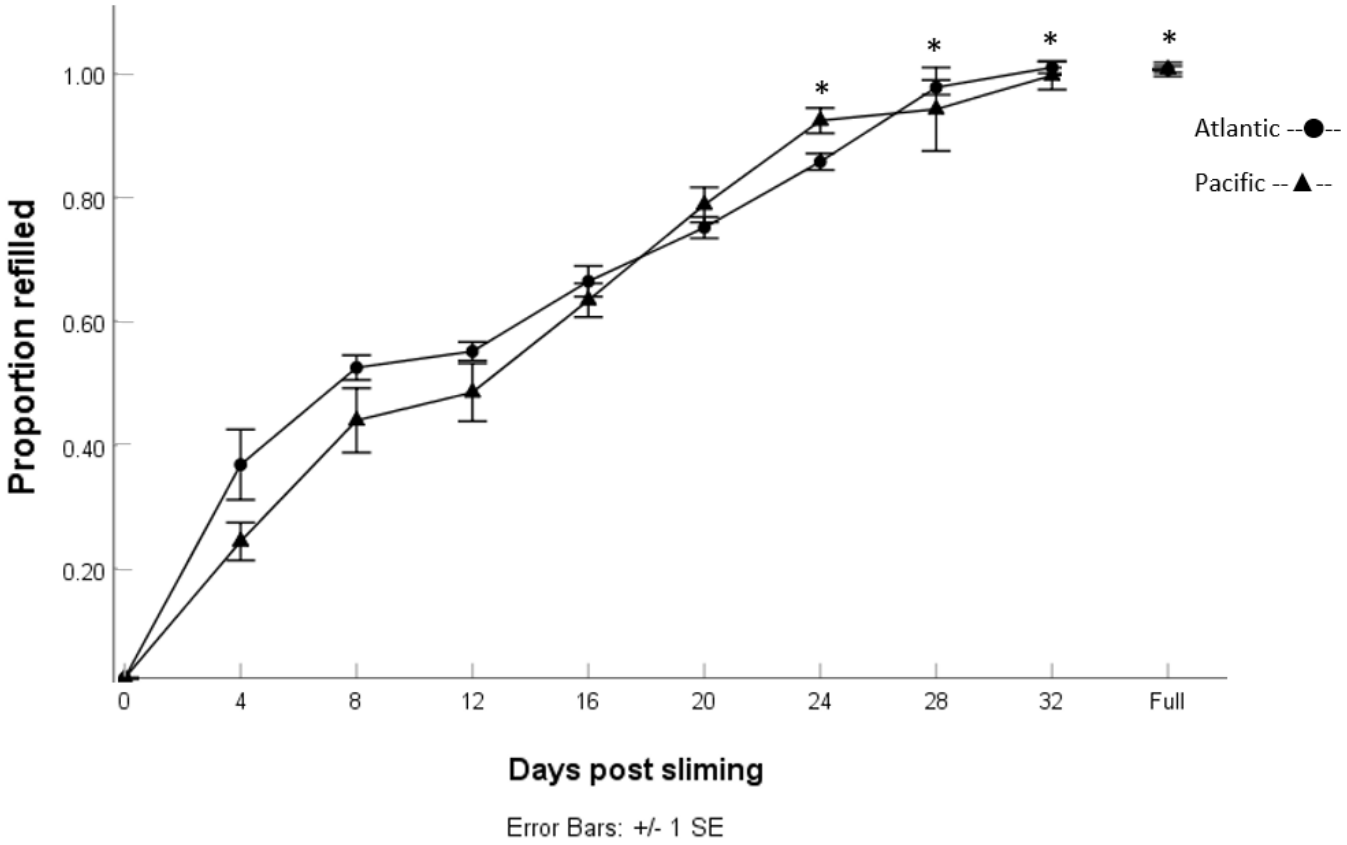

Figure 4. Timeline for slime gland refilling in Atlantic (--▫--) and Pacific (--▲--) hagfishes. Slime gland regeneration trials were conducted by collecting slime from the refilling (left) side of the animal and comparing it to the mass of slime collected from the full (right) side of the animal (proportion = mass of refilling/mass of full exudate). Slime glands were refilled when the proportion of refilling was not significantly different from full glands. Slime glands of Pacific hagfish were found to be refilled by 24 days post sliming ($p>0.05$) while slime glands of Atlantic hagfish were refilled after 28 days post sliming ($p>0.05$). Error bars are ±s.e.m, n=10 for each timepoint, asterisks denote statistically equivalent proportions of exudate produced compared to full glands.

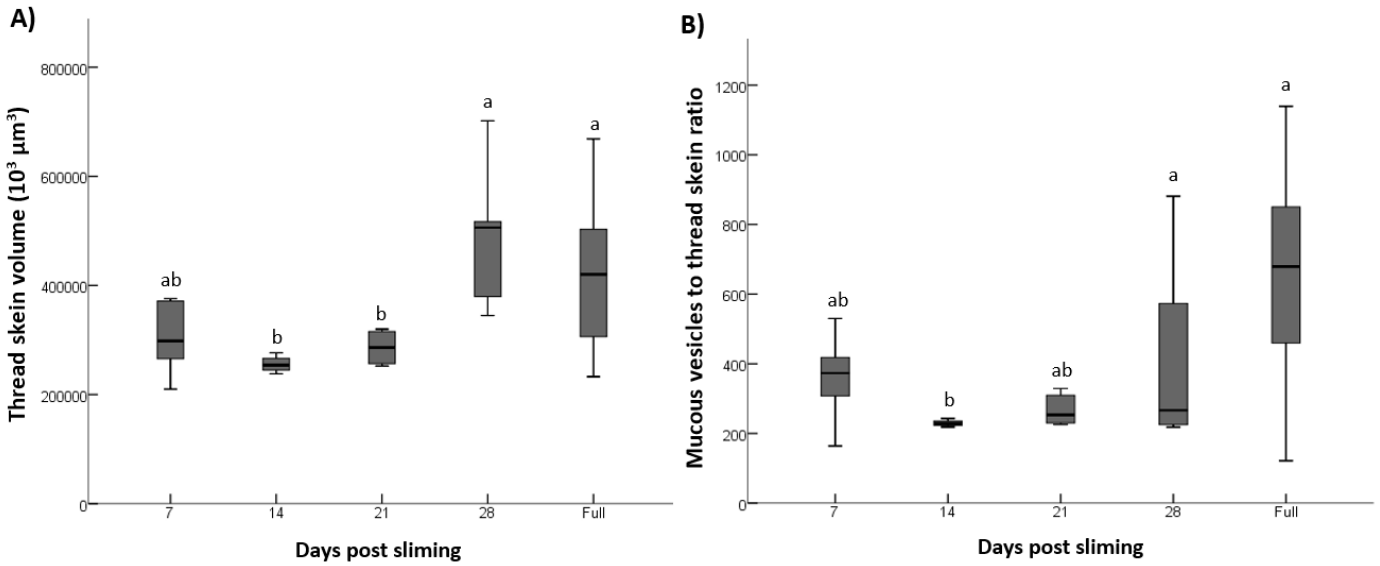

Figure 5. Properties of exudate from refilling Pacific hagfish slime glands. A) Thread skeins in the exudate differed considerably during refilling, with slime glands at 14 and 21 days post sliming releasing significantly smaller thread skeins on average (Post-hoc LSD testing: $p<0.05$); B) Mucous vesicle to thread skein ratio in the exudate varied significantly over refilling time (Non-linear regression: df = 3, 35; f=68.803; $p<0.0001$), with slime glands at 14 and 21 days post sliming releasing exudate with a lower mucous vesicle to thread skein ratio (Post-hoc LSD testing: $p<0.0001$) (n=5 for each timepoint, lowercase letters denote significant difference between timepoints within parameters).