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RESEARCH ARTICLE

Unraveling inter-species differences in hagfish slime skein deployment

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ABSTRACT

Hagfishes defend themselves from fish predators by producing defensive slime consisting of mucous and thread components that interact synergistically with seawater to pose a suffocation risk to their attackers. Deployment of the slime occurs in a fraction of a second and involves hydration of mucous vesicles as well as unraveling of the coiled threads to their full length of ∼150 mm. Previous work showed that unraveling of coiled threads (or 'skeins') in Atlantic hagfish requires vigorous mixing with seawater as well as the presence of mucus, whereas skeins from Pacific hagfish tend to unravel spontaneously in seawater. Here, we explored the mechanisms that underlie these different unraveling modes, and focused on the molecules that make up the skein glue, a material that must be disrupted for unraveling to proceed. We found that Atlantic hagfish skeins are also held together with a protein glue, but compared with Pacific hagfish glue, it is less soluble in seawater. Using SDS-PAGE, we identified several soluble proteins and glycoproteins that are liberated from skeins under conditions that drive unraveling *in vitro*. Peptides generated by mass spectrometry of five of these proteins and glycoproteins mapped strongly to 14 sequences assembled from Pacific hagfish slime gland transcriptomes, with all but one of these sequences possessing homologs in the Atlantic hagfish. Two of these sequences encode unusual acidic proteins that we propose are the structural glycoproteins that make up the skein glue. These sequences have no known homologs in other species and are likely to be unique to hagfishes. Although the ecological significance of the two modes of skein unraveling described here are unknown, they may reflect differences in predation pressure, with selection for faster skein unraveling in the *Eptatretus* lineage leading to the evolution of a glue that is more soluble.

KEY WORDS: Adhesive, Glue, Mucus, Pacific hagfish, *Eptatretus stoutii*, Atlantic hagfish, *Myxine glutinosa*

INTRODUCTION

Hagfishes are benthic craniates that defend themselves from fish predators by secreting gill-clogging slime when they are attacked (Fudge et al., 2005; Lim et al., 2006; Zintzen et al., 2011). The slime originates within numerous epidermal slime glands that produce two main secretory cell types, gland mucus cells (GMCs) and gland thread cells (GTCs) (Downing et al., 1981a,b). GMCs produce large numbers of mucous vesicles and GTCs each produce a single intricately coiled slime thread that is estimated to be 150 mm long when laid end to end (Fudge et al., 2005). Contraction of the thin layer of muscle surrounding each gland serves to squeeze mature GMCs and GTCs through the gland pore into the surrounding seawater in a process known as holocrine secretion (Downing et al., 1981a,b). Passage through the narrow gland pore causes rupture of both GMCs and GTCs (Fernholm, 1981), and subsequent mixing with seawater leads to swelling of the mucous vesicles and unraveling of the coiled threads (also known as 'skeins') (Koch et al., 1991). The deployed mucus and threads interact with seawater to produce an ultra-dilute slime that catches on fish gills and clogs them (Lim et al., 2006), which typically leads to the predator aborting its attack (Zintzen et al., 2011).

Under natural conditions, the unraveling of slime threads from their coiled state to their full length occurs in a fraction of a second, but the process is not well understood (Lim et al., 2006; Zintzen et al., 2011). Newby (1946) suggested that Pacific hagfish (*Eptatretus stoutii*) slime threads are formed within GTCs 'under considerable pressure' and that release of this pressure via the osmotic rupture of the GTC cell membrane drives unraveling. Fernholm (1981) subsequently showed that GTCs from Pacific hagfish and Atlantic hagfish (*Myxine glutinosa*) lose their cell membranes as they pass through the slime gland duct, well before the skeins unravel, which ruled out Newby's osmotic rupture mechanism. Fernholm also reported observations of controlled skein unraveling under the microscope, and demonstrated that unraveling proceeds in both artificial seawater (ASW) and distilled water, but is slowed in double-strength artificial seawater (2×ASW) (Fernholm, 1981). Koch et al. (1991) showed that Pacific hagfish skeins can be stabilized in a 0.92 mol l^{-1} sodium citrate solution, and will unravel if this stabilizing solution is suitably diluted.

Winegard and Fudge (2010) investigated the requirements for unraveling in skeins from Atlantic hagfish, and found that they generally do not unravel spontaneously when exposed to seawater. Instead, *Myxine* skeins must be actively pulled apart by external forces, namely the hydrodynamic forces they experience during mixing with seawater. They also showed that the presence of mucus facilitates unraveling, most likely by attaching to the skeins and transducing turbulent mixing forces to them. More recently, Bernards et al. (2014) showed that skeins from Pacific hagfish unravel spontaneously in seawater, even in the absence of mucus and mixing forces. In Pacific hagfish, skein unraveling appears to be mediated by the presence of a seawater-soluble protein adhesive on and between loops of coiled thread (Fernholm, 1981; Bernards et al., 2014). After ejection from the slime gland, dissolution of the adhesive in seawater triggers unraveling by allowing strain energy stored within the threads to be released, causing loops of thread to spring off from the skein and adopt a more relaxed curvature than they had in the coiled state (Bernards et al., 2014).

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While much is known about the functional properties of hagfish slime and its thread and mucous components (Fudge et al., 2005; Lim et al., 2006; Zintzen et al., 2011; Winegard and Fudge, 2010; Herr et al., 2010, 2014; Ewoldt et al., 2011; Böni et al., 2016, 2017), little is known about the molecular underpinnings of this unusual adaptation that appears to be unique to hagfishes. Koch and colleagues reported the amino acid sequence of two heteropolymeric proteins of the intermediate filament family, named α and γ, which make up most of the proteins within the slime threads produced by GTCs (Koch et al., 1994, 1995). More recently, Herr et al. (2014) reported molecular and functional evidence for the presence of aquaporins and calcium-activated chloride channels in the membrane of hagfish slime mucous vesicles. Apart from these examples, little else is known about the molecular bases of hagfish slime production and deployment.

Although there are currently no hagfish genomes that have been sequenced, the advent of affordable transcriptomic methods has unlocked opportunities for exploring the genes and proteins involved in this trait. Because hagfish slime is produced within discrete glands that can be electrically stimulated to eject slime exudate and begin the refilling process, it is ideally suited to transcriptomic analysis, which provides detailed information about the genes that are expressed in a tissue of interest at a specific moment. Here, we used transcriptomic analysis coupled with SDS-PAGE and mass spectrometry to gain further insight into the molecules involved in a critical aspect of hagfish slime deployment – thread skein unraveling. We also carried out numerous *in vitro* functional assays to test the hypothesis that the differences in the behavior of Pacific and Atlantic hagfish skeins arise from differences in the solubility in seawater of the skein glue from these two species. Our results provide new insights into the molecular mechanisms involved in hagfish slime deployment and raise new questions about the origins of this evolutionary novelty.

MATERIALS AND METHODS

Experimental animals

Both species of hagfish were collected using baited traps at a depth of ∼100 m. Atlantic hagfish (*Myxine glutinosa* Linnaeus 1758) were collected 10 km southeast of Appledore Island, Maine, USA and Pacific hagfish [*Eptatretus stoutii* (Lockington 1878)] were collected in Barkley Sound near Bamfield, British Columbia, Canada. Both species were housed in 2000 liter tanks filled with chilled artificial seawater (34‰, 10°C) at the Hagen Aqualab at the University of Guelph, Guelph, Ontario, Canada. The hagfishes were fed a seafood medley once per month to satiety as per the University of Guelph Animal Care Protocol #2519.

Slime collection

Slime collection for protein analyses and functional assays was performed following procedures outlined in Bernards et al. (2014). Hagfish were placed in 3 liters of artificial seawater (ASW; 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 at 4°C for 30 min. Hagfish were placed on a chilled dissection tray lined with a seawater-wetted cloth with the slime glands on one of its sides facing up. The skin surrounding the glands was washed with ddH_2O, blotted dry and the glands were stimulated (80 Hz, 18 V) using a Grass SD9 Stimulator (Grass Instruments, Quincy, MA, USA) with a custom stimulation wand. Several glands were stimulated, each releasing a small pool of exudate, which was collected using a small spatula and placed either into mineral oil for whole slime observations, or 0.9 mol l^{-1} sodium citrate (Fisher Scientific, Ottawa, ON, Canada), 0.1 mol l^{-1} PIPES [piperazine-*N*,*N*′-bis(ethanesulfonic acid)] (Sigma-Aldrich; Oakville, ON, Canada) slime stabilization buffer (SB) (pH 7.4) for experiments requiring the separation of skeins and mucous vesicles. Regardless of the method of storage, slime was always used on the day that it was collected, as prolonged storage can affect slime viability (Böni et al., 2017).

SEM of skein and thread surface

Samples for scanning electron microscopy (SEM) were prepared according to the procedure outlined by Bernards et al. (2014); both Pacific and Atlantic hagfish slime samples were collected in SB and then underwent three washing cycles in SB to remove mucous vesicles. After the third SB wash, the samples were fixed in 3% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in phosphate-buffered saline and then dehydrated in an ethanol series (30%, 35%, 50%, 70%, 95%, 3×100%). Dehydrated samples were then critical point dried and sputter coated using an Emitech K550 Sputter Coater (Emitech Ltd, Ashford, Kent, England) for ∼2 nm Au/Pd coating (20 mA for 2 min). Samples were observed using a Hitachi S-570 scanning electron microscope (Hitachi High Technologies, Tokyo, Japan) and images were captured using the Quartz PCI Image Management System (v. 8.5). The amount of material on the skeins was scored by three volunteers, who were blinded to the various treatments, using a scale from 0–3, with 0 denoting no material, and 3 denoting abundant material on the skeins.

Unraveling assays

Unraveling assays ([NaCl]×temperature and trypsin) for Atlantic hagfish skeins were conducted following a modified procedure described for Pacific hagfish skeins by Bernards et al. (2014). For the [NaCl]×temperature trials, four samples of whole slime exudate from each of four hagfish were exposed to 11 different solutions varying in NaCl concentration, between 0.125 mol l^{-1} and 4 mol l^{-1} inclusively. This procedure was carried out at 5°C and at 35°C. To examine the effects of trypsin on unraveling of Atlantic hagfish skeins, four exudate samples from each of four hagfish were exposed to two treatments: 0.25 mol l^{-1} NaCl, and 0.25 mol l^{-1} NaCl+0.5 g l^{-1} trypsin, both at 35°C. To examine the effects of urea, four exudate samples from each of four hagfish from both species were exposed to five levels of urea (0, 0.5, 1.0, 1.5 and 2.0 mol l^{-1}) in a background of 5 mmol l^{-1} Tris-HCl (pH 8) and 0.25 mol l^{-1} NaCl. This concentration of NaCl was chosen because it is known to inhibit unraveling in Pacific hagfish skeins. SB was used as a negative control treatment. Unravelling was quantified from images of exudate exposed to the various treatments taken with a Nikon Eclipse 90i microscope using a 20× objective.

Slime thread and skein swelling assays

To further explore the mechanisms of unraveling in Atlantic hagfish skeins, we exposed them to ASW (34‰), 2×ASW (68‰) and ddH_2O in the absence of convective mixing using custom experimental chambers made from glass slides. On the slides, a hydrophobic barrier (3.5×2.5 cm) was drawn using a Liquid Blocker super PAP pen (Daido Sangyo Co., Ltd., Tokyo, Japan). The dimensions of this chamber allowed 0.5 ml of test solution to be deposited within the barrier walls with little surface curvature, thus preserving the imaging quality under the upright microscope without a cover glass, the placement of which would have created substantial shear forces. Electrical stimulation (as described above) was used to induce contraction of the muscle around a single slime gland, which resulted in expression of a small pool of exudate onto

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the skin. Exudate was collected with a micropipette from the skin of anesthetized hagfish and 0.2 µl was gently added to the experimental chamber that was pre-filled with one of the three test solutions. Observations of overall skein morphology and area were made using a 4× objective lens, with images acquired every minute after the addition of exudate for a total of 5 min. For measurements of thread diameter, a 40× objective with 2× optical zoom was used, with images captured at the same time intervals.

SDS-PAGE of ASW-soluble skein proteins

Pacific hagfish slime was collected in SB and underwent several SB washing cycles to remove mucous vesicles. After the final SB wash, the isolated skeins were exposed to one of two treatments: ASW (unraveling) or 4 mol l^{-1} NaCl (stabilizing). We used 4 mol l^{-1} NaCl instead of SB as the stabilizing treatment, because 4 mol l^{-1} NaCl stabilizes skeins (Bernards et al., 2014), but not all mucous vesicles, which we reasoned would make it easier to differentiate glue proteins from contaminating mucus proteins. Treated skein samples were centrifuged at 15,000 ***g*** for 10 s so that the threads formed a pellet at the bottom of the microfuge tube. The supernatant was removed, subjected to TCA/acetone precipitation, and the proteins were re-solubilized in Laemmli running buffer (10:1 2× SDS running buffer:β-mercaptoethanol). Samples were heated to 95°C and then loaded into 10% polyacrylamide gels, which were prepared and run according to the procedure described by Klaiman et al. (2011). Gels were either stained with Coomassie Brilliant Blue, or underwent periodic acid oxidation and Alcian Blue/silver staining for glycoproteins (Møller and Poulsen, 2009). Gels were scanned and analyzed by comparing relative band densities using Image Lab (v. 4.1) software (BioRad Laboratories).

Mass spectrometry of putative skein glue proteins

Four glycoprotein bands (210 kDa, 150 kDa, 130 kDa, 95 kDa) and one protein band (245 kDa) were cut out of the gels and prepared for mass spectrometry (MS) (i.e. reduced with DTT, alkylated, digested with trypsin and lyophilized) according to protocols described in Gundry et al. (2010). Mass spectrometry analysis was performed at the SickKids Proteomics, Analytics, Robotics and Chemical Biology Centre (SPARC BioCentre) in Toronto, Ontario, Canada using a Q-Exactive mass spectrometer according to protocols described in detail in Gundry et al. (2010).

RNA extractions and cDNA library construction

Total RNA was extracted from three replicates each of refilling (2 days post-sliming) and unstimulated slime glands from both species of hagfishes using a TRIzol® Plus RNA Purification Kit (Ambien; Life Technologies; 12183-555). Glands that had been exhausted of slime 2 days before they were collected were used to try and detect genes that are upregulated during the gland refilling process. Individual glands (~50–100 mg each) were manually homogenized using disposable homogenizing pestles in Eppendorf tubes containing 1 ml TRIzol™ reagent. RNA extractions followed a standard protocol provided with the purification kit. RNA quality and concentration were assayed using gel electrophoresis and spectrophotometry. RNA sequencing libraries were prepared using the NEB Next RNAseq library prep kit with multiplex oligos (New England Biolabs). Only one index primer was added to each sample per PCR reaction.

Illumina sequencing and sequence assembly

Sequencing was conducted on two lanes of paired end 150 sequencing using an Illumina HiSeq2500. Eight sequencing libraries were sequenced per lane. Sequencing and de-multiplexing was conducted at the Hubbard Center for Genome Studies at the University of New Hampshire. Following sequencing, raw datasets were error corrected using BFC (Li, 2015), filtered for low quality data and adaptor sequences using Trimmomatic (Bolger et al., 2014), and assembled using Trinity (Grabherr et al., 2011). For the Trinity assembly, only the two sequencing libraries from each refilling stage that had the largest amount of data (number of reads) were assembled. Following Trinity assembly, individual reads from each dataset were mapped back onto the assembly using Salmon (Patro et al., 2017) and imported into R using tximport (Soneson et al., 2015).

Screening of mass spectrometry data against transcriptomes

FASTA files of the two assembled hagfish species transcriptomes were loaded into PEAKS 8 software (Bioinformatics Solutions Inc.) and were used as a database against which the *de novo* assembled MS data were screened. The following modifications were considered within the search parameters: methionine oxidation, deamidation of asparagine and glutamine and carbamidomethylation of cysteine residues. The tolerance values used were 10 ppm for parent ions and 0.02 Da for fragment ions. Proteins were identified using a false discovery rate (FDR) cut-off of 1% with at least two unique peptides per protein. The top protein hits were identified as those with the highest $-10\log P$ values, percentage coverage, the number of peptides in the sequence and the number of unique peptides. The amino acid sequence of the top protein hit was then translated into a peptide sequence using *TransDecoder* (http://transdecoder.github.io/) and blasted against each of the hagfish species transcriptomes. Isoelectric point (pI) and molecular weight (mw) values were calculated for the

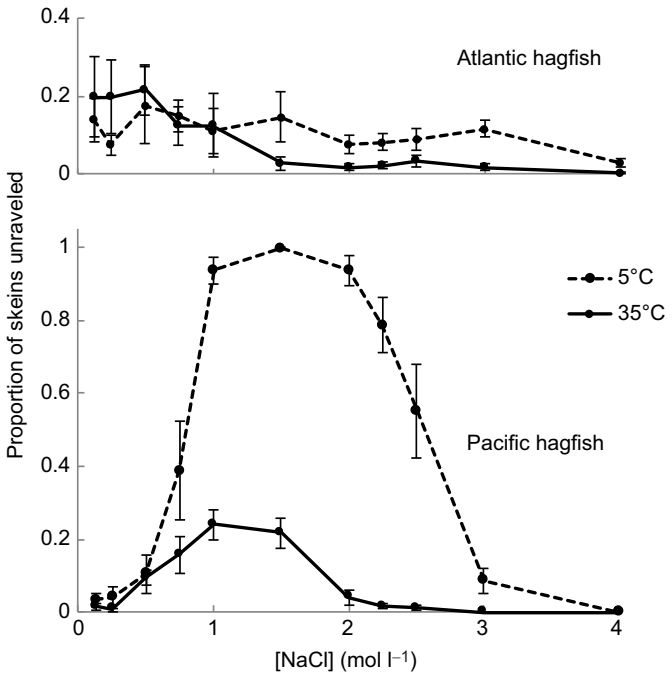

Fig. 1. Slime thread skein unraveling in various concentrations of NaCl in Atlantic and Pacific hagfish. In Atlantic hagfish, there was a statistically significant [NaCl] effect (P>0.05), but the magnitude of the effect was small. There were no significant temperature or [NaCl]×temperature interaction effects (P=0.40 and P=0.48 respectively) on Atlantic hagfish skein unraveling. Pacific hagfish data are reproduced for comparison from Bernards et al. (2014). Data are means±s.e.m.; n=4 (4 samples from each of 4 different hagfish).

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skein glue protein sequences using the ExPASy Bioinformatics Resource Portal (https://web.expasy.org/compute_pi/) (Gasteiger et al., 2005). Analysis of protein domain structure was carried out using the online tool PredictProtein (Yachdav et al., 2014). Raw sequence data are available under BioProject PRJNA497829 at the Sequence Read Archive.

Statistical analyses

All data were analyzed using SPSS v. 24.0 (SPSS Inc., Armonk, NY) with α=0.05. Relative band densities from SDS-PAGE gels were compared between unraveled and stabilized treatments using independent sample two-tailed *t*-tests. SEM surface material scores were compared between species and treatments (ASW versus SB) using a two-way ANOVA and *post hoc* Tukey's HSD testing. Main effects of temperature and [NaCl] on Atlantic hagfish skein unraveling efficiency, as well as interactive effects of temperature and [NaCl], were calculated using a two-way ANOVA. Proportion of skein unraveling was calculated for a given trial as the number of unraveled skeins divided by the total number of skeins observed in that trial. Effects of species and temperature, as well as interaction effects, on the proportions of pulled and spontaneous skein unraveling were compared using a two-way ANOVA with *post hoc* Tukey's HSD tests. Effects of trypsin over time, as well as interaction effects, on unraveling efficiency were calculated using a two-way ANOVA with post-hoc Tukey's HSD testing to compare the proportion of bundles unraveled between trypsin and control skeins at each time point. A two-way ANOVA with *post hoc* Tukey's HSD testing was conducted on the skein unravelling data to determine the effects of urea concentration and species on the proportion of skeins that unraveled during assays. Thread and skein dimensions from the swelling assays were normalized by dividing all measurements by the thread diameter or skein area at time 0. Effects of ASW, 2×ASW, and ddH_2O on Atlantic hagfish thread and skein dimensions over time were analyzed using repeated measures ANOVAs.

RESULTS

Temperature and NaCl concentration effects on Atlantic hagfish skeins

When exposed to a range of NaCl concentrations at 5°C and 35°C, only a small amount of unraveling occurred in Atlantic hagfish skeins, with the proportion of bundles that unraveled rarely exceeding 0.2 (Fig. 1). Temperature and [NaCl]×temperature interaction did not have significant effects on unraveling ($F_{1,0.703}$, P=0.405 and $F_{10,0.968}$, P=0.479, respectively). [NaCl] had a statistically significant effect on unraveling ($F_{10,2.393}$, P<0.05), with unraveling rates being slightly higher at lower NaCl concentrations (Fig. 1).

Spontaneous versus pulled unraveling

From the salt and temperature trials, we observed two distinct unraveling patterns in Pacific and Atlantic hagfish samples, which we refer to as 'pulled' and 'spontaneous' (Fig. 2). In pulled unraveling, the skeins appeared as if a length of thread has been pulled from the apical tip, with little disruption to the rest of the skein. In spontaneous unraveling, there was an energetic springing of successive coiled loops from the skein, resulting in a disorganized pile of thread loops. The proportion of unraveled skeins that exhibited spontaneous unraveling was significantly higher in Pacific hagfish samples compared with Atlantic hagfish samples ($F_{1,13.035}$, P<0.05), but temperature and the interaction of temperature×species did not have significant effects on spontaneous unraveling ($F_{1,1.260}$, P=0.269 and $F_{1,0.021}$, P=0.885, respectively). Conversely, pulled unraveling was significantly higher in Atlantic hagfish samples compared with Pacific hagfish samples ($F_{1,13.035}$, P<0.05), but temperature and the interaction of temperature×species did not have a significant effect on the proportion of skeins that exhibited pulled unraveling ($F_{1,1.260}$, P=0.269 and $F_{1,0.021}$, P=0.885, respectively) (Fig. 2).

SEM of skein and thread surfaces

Skeins were examined for evidence of surface material using SEM after exposure to ASW and a citrate-based SB that suppresses unraveling. Species alone had no significant effect on the amount of adhesive present on skeins after treatment (F(1, 0.760), P=0.386), however, treatment and the interaction of species/treatment both had significant effects on SEM surface scores ($F_{1,37.240}$, P<0.0001 and $F_{1,19}$, P<0.0001, respectively). There was no significant difference in the amount of adhesive present on SB-treated skeins between Atlantic hagfish and Pacific hagfish (P=0.074), or between ASW- and SB-treated Atlantic hagfish skeins (P=0.608). There was significantly less adhesive on ASW-treated Pacific hagfish skeins compared with SB- and ASW-treated Atlantic hagfish, and SB-treated Pacific hagfish skeins. (P<0.001) (Fig. 3).

Trypsin effects on Atlantic hagfish skein unraveling

The protease trypsin can trigger spontaneous unraveling of Pacific hagfish skeins under conditions under which they normally do not unravel (low salt and high temperature) (Bernards et al., 2014). It has also been shown to trigger unraveling of skeins that have been freeze

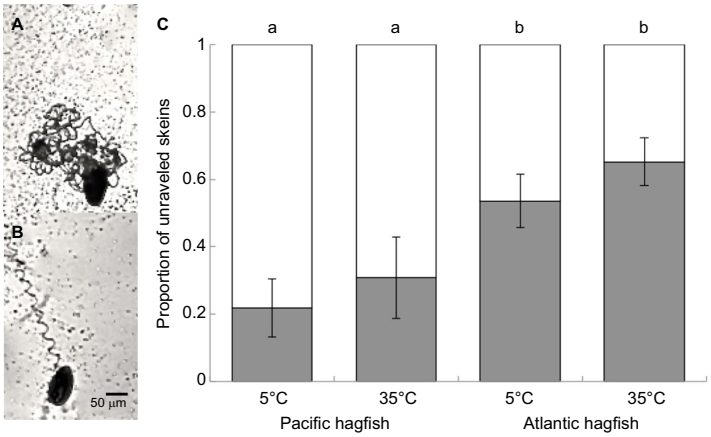

Fig. 2. Comparison of spontaneous and pulled unraveling of hagfish skeins. Micrographs showing examples of (A) spontaneous and (B) pulled unraveling of skeins. Scale bar is for both A and B. (C) Proportion of the total number of unraveled skeins that exhibited spontaneous or pulled unraveling. Treatments not sharing a lowercase letter are significantly different in their proportions of skeins that were unraveled by pulling (P<0.0001). Data are means±s.e.m. for n=11 trials, which included analysis of 3176 unraveled skeins.

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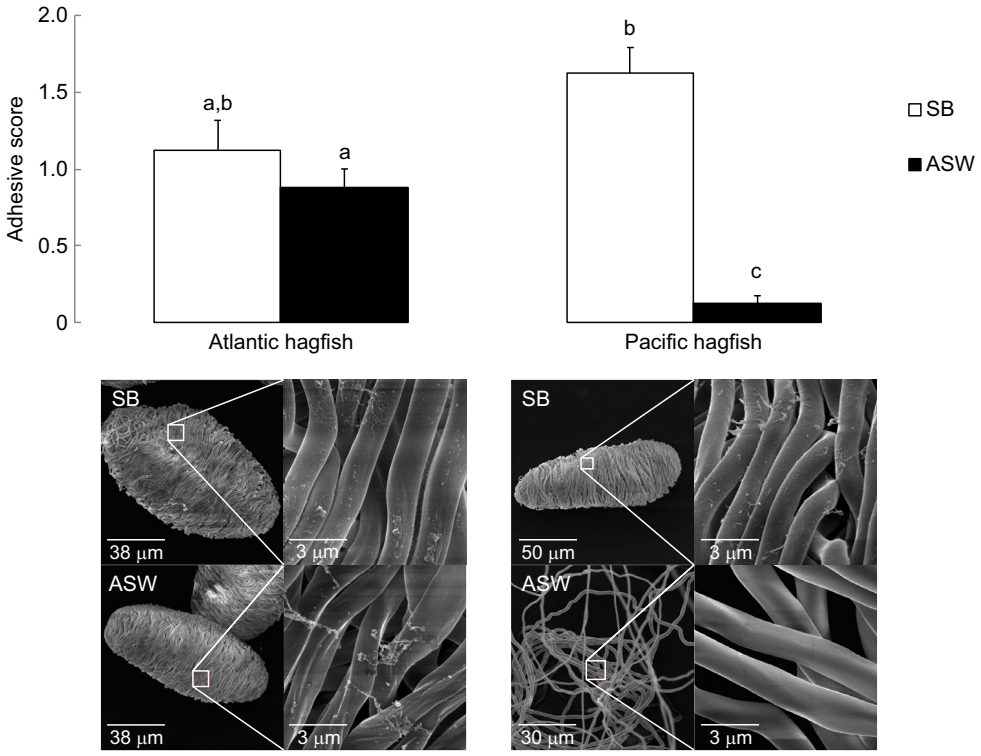

Fig. 3. Amount of surface material on and between the thread loops of Atlantic hagfish and Pacific hagfish skeins in stabilization buffer (SB) and artificial seawater (ASW). SEM images of Atlantic hagfish (left) and Pacific hagfish skeins (right) show that the material remains intact on both species in SB, and on Atlantic hagfish skeins in ASW, but is lost from Pacific hagfish skeins in ASW. Treatments not sharing a letter are significantly different (P<0.0001). Data are means±s.e.m. for n=20 images/treatment.

dried or stored for extended periods in SB (Böni et al., 2017). We reasoned that if the lack of spontaneous unraveling in Atlantic hagfish is due to the presence of skein glue that is insoluble in seawater, then we should be able to trigger unraveling in Atlantic hagfish skeins by exposing them to trypsin. We found that trypsin can indeed drive spontaneous unraveling in Atlantic hagfish, even under conditions that inhibit unraveling in Pacific hagfish. Treatment with trypsin, time and the interaction of time×treatment all had significant effects on the proportion of skeins that unraveled ($F_{1,2.915}$, P<0.0001, $F_{5,0.104}$, P<0.0001 and $F_{5,0.095}$, P<0.0001, respectively). The proportion of skeins that unraveled in 0.25 mol l^{-1} NaCl at 35°C did not change over the course of 5 min, remaining at ∼0.2 (P=1.00). However, after 5 min exposure to trypsin, almost 90% of skeins had begun to unravel spontaneously. The difference in the proportion of bundles that unraveled between trypsin and control treatments was statistically significant from 2 min of exposure onward (P<0.05 at 2 min, P<0.0001 at 5 min) (Fig. 4).

Urea effects on Atlantic hagfish skein unraveling

To test whether unraveling can be regulated by the solubility of skein glue, we exposed skeins from both species to solutions with varying urea concentrations. In a background of low salt (0.25 mol l^{-1} NaCl), which is known to inhibit spontaneous unraveling in Pacific hagfish skeins, urea had strong and consistent effects on unravelling in skeins from both species (Fig. 5). A two-way ANOVA revealed a strong main effect of urea concentration ($F_{4,60.692}$, P<0.001), but no significant effect of species ($F_{1,0.001}$, P=0.979) on the proportion of skeins unraveling. Tukey's HSD *post hoc* testing revealed that the proportion of skeins unraveled increased significantly from 0 to 0.5 mol l^{-1} urea, and peaked at a urea concentration of 1.0 mol l^{-1} (P<0.001). Unravelling decreased dramatically from 1.0 to 1.5 mol l^{-1} and was absent at 2.0 mol l^{-1} urea. Exposure to 1.5 mol l^{-1} and 2.0 mol l^{-1} urea solutions caused a dramatic increase in the transparency of the skeins, which was likely caused by denaturation of the thread proteins.

Thread and skein swelling in Atlantic hagfish

We induced thread swelling in Atlantic hagfish skeins by exposing them to ddH_2O (Fudge and Gosline, 2004). We also exposed them to ASW and 2×ASW. For each treatment, we measured thread diameter as well as skein area every minute for 5 min. When using a repeated-measures ANOVA with a Greenhouse–Geisser correction, the mean change in thread diameter significantly increased for the ddH_2O treatment ($F_{1.457,9.691}$, P<0.05), but did not significantly change with the ASW or 2×ASW treatments ($F_{2.966,1.866}$, P=0.167 and $F_{(3.779,1.119}$, P=0.359 respectively). Similarly, skein area did not significantly change when treated with ASW or 2×ASW ($F_{1.511,0.883}$), P=0.423 and $F_{1.182,2.902}$, P=0.139 respectively), but

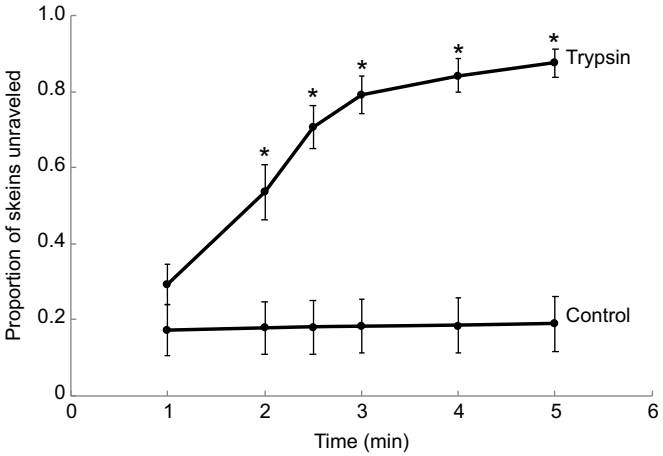

Fig. 4. Effect of trypsin on Atlantic hagfish slime thread skein unraveling in 0.25 mol l^{-1} NaCl at 35°C. Data are means±s.e.m. n=4 (4 samples from each of 4 different hagfish).*P<0.01, statistically significant difference between the proportion of bundles unraveled at a given time between trypsin and control treatments.

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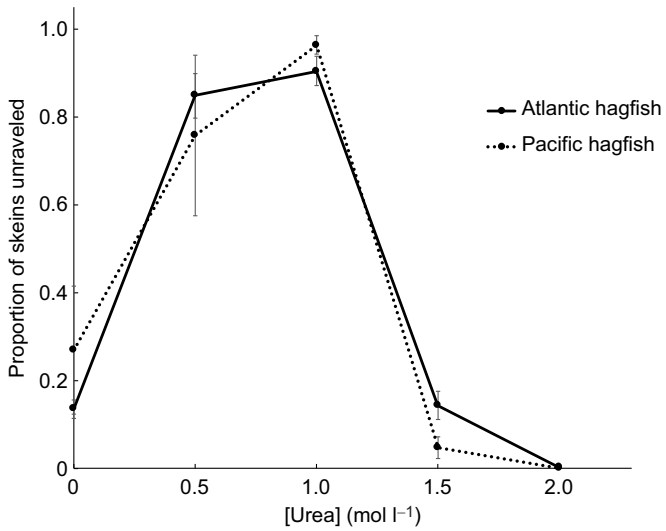

Fig. 5. Effect of urea on slime thread skein unraveling in Atlantic and Pacific hagfish slime. Unravelling was quantified in a background buffer chosen for its ability to suppress unravelling (0.25 mol l^{-1} NaCl and 5 mmol l^{-1} Tris-HCl, pH 8), with varying concentrations of urea. A two-way ANOVA revealed a strong effect of urea (P<0.001) and no effect of species (P=0.979). Data are means±s.e.m.; n=5.

significantly increased when treated with ddH_2O ($F_{1.212,9.067}$), P<0.05) (Figs 6 and 7).

SDS-PAGE of Pacific hagfish proteins

SDS-PAGE and Coomassie Brilliant Blue staining of ASW-soluble proteins from Pacific hagfish skeins revealed the presence of nine protein bands of molecular mass 18, 23, 35, 45, 52, 63, 75, 210 and 245 kDa (Fig. 8A,B). The 75 kDa protein band may have been contamination from the mucous portion of the slime (mucus-only gel not shown) (Spitzer et al., 1984). This protein band appeared in both unraveled and stabilized treatments and was therefore used as the reference band for relative band density analysis. The 63 kDa protein band was most likely one of the intermediate filament proteins (γ) that make up the slime thread (Spitzer et al., 1984; Koch et al., 1995). The only bands that showed a significant difference in relative band intensity between treatments were the 245 kDa (d.f.=4, t=3.970, P<0.05) and 210 kDa (d.f.=3, t=111.527, P<0.001) bands. There was no significant difference in relative band densities between stabilized (4 mol l^{-1} NaCl) and unraveled (ASW) treatments for the 52 kDa (d.f.=4, t=0.592, P=0.586), 45 kDa (d.f.=4, t=0.474, P=0.660), 35 kDa (d.f.=4, t=−0.605, P=0.578), 23 kDa (d.f.=4, t=−0.040, P=0.970) and 18 kDa (d.f.=4, t=0.283, P=0.791) bands. PAS Alcian Blue/silver staining of the protein gels revealed the presence of four large glycoproteins of approximate molecular size 95, 130, 150 and 250 kDa in the supernatant of isolated skeins exposed to ASW, none of which were present in the supernatant of skeins exposed to 4 mol l^{-1} NaCl (Fig. 8C).

Mass spectrometry and transcriptomics

The five protein bands analyzed using mass spectrometry generated hundreds of peptides, which mapped to numerous protein sequences translated from the Pacific hagfish transcriptome, which contained 54,406 assembled sequences. Fourteen protein sequences from the translated transcriptome had $-10\log P$ values higher than 100 (i.e. false detection P-values of 10^{-10}) and greater than 10 unique peptides mapping to each, with the strongest sequence hits having dozens of unique peptides mapping to them. All 14 of these sequences had high scoring sequence similarity in the Atlantic hagfish transcriptome, with the percentage of identical amino acid matches ranging from 39% to 97% (Table 1). Each band analyzed with mass spectrometry identified multiple proteins, with many of the top hits detected in multiple bands. For this reason, we have not attempted to assign molecular masses to the top protein hits other than to provide the predicted molecular mass of the protein sequence inferred from the Pacific and Atlantic hagfish transcriptomes (Table 2).

DISCUSSION

Two modes of unraveling

Previous work pointed to differences in the unraveling behavior of skeins from Atlantic hagfish and Pacific hagfish, with the former requiring the presence of mucus and vigorous hydrodynamic mixing forces and the latter being capable of spontaneous unraveling in seawater (Winegard and Fudge, 2010; Bernards et al., 2014). Our results are consistent with these conclusions.

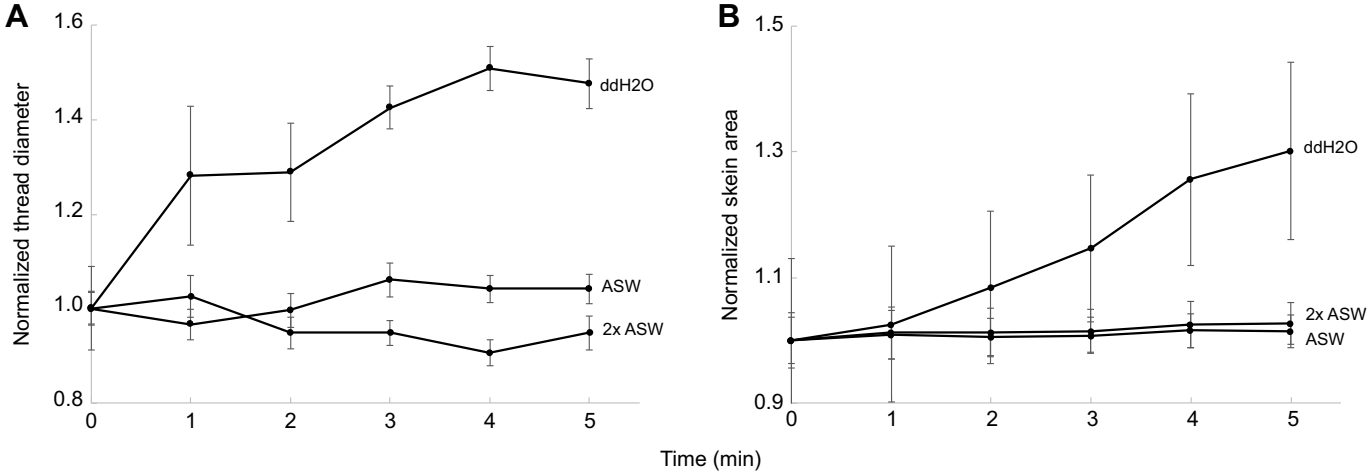

Fig. 6. Ionic effects over time on slime thread diameter and skein area in Atlantic hagfish thread skeins. Effect of 2×ASW on (A) thread diameter and (B) skein area. There was no significant change in thread diameter in ASW or 2×ASW (P=0.167, P=0.359 respectively), but ddH_2O caused significant swelling (P<0.05). Skein area remained constant in both ASW and 2×ASW (P=0.423, P=0.139 respectively), but ddH_2O led to a significant increase in skein area (P<0.05). Data are means±s.e.m.; n=8 for thread diameter, n=6 for skein area.

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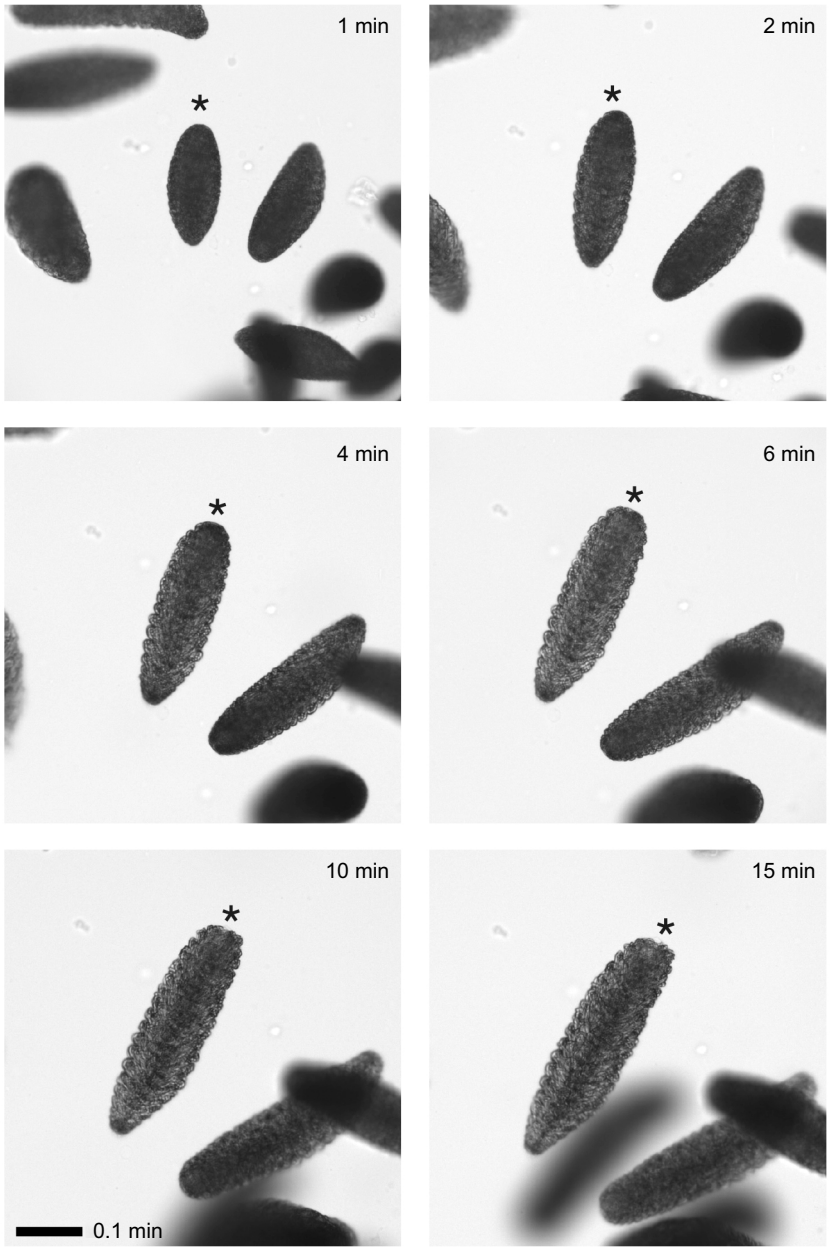

Fig. 7. Response of Atlantic hagfish skeins to ddH_2O exposure. While ddH_2O led to significant swelling of threads and skeins, some skeins resisted unraveling even after substantial amounts of swelling. Asterisk indicates the same skein in each panel.

While nearly all Pacific hagfish skeins exhibited some unraveling at intermediate salt levels (1–2 mol l^{-1} NaCl) in cold water, only a small fraction of Atlantic hagfish skeins unraveled under the same conditions (Fig. 1). In addition, of the skeins that did unravel during these trials, we observed substantial differences in the mode of unraveling, with most of the Atlantic hagfish skeins that unraveled exhibiting 'pulled' unraveling, and most Pacific hagfish skeins exhibiting 'spontaneous' unraveling. The pulled versus spontaneous distinction is a general trend, and not a black-and-white distinction, with some spontaneous unraveling in Atlantic hagfish skeins, as well as some pulled unraveling in Pacific hagfish skeins (Fig. 2). Furthermore, under natural conditions, deployment of thread skeins almost certainly involves both processes, as it is difficult to imagine how spontaneous unraveling alone could account for the speed of natural slime deployment (~100 ms) and the fact that slime threads that are fully unraveled and straightened are over 1000-times longer than intact skeins.

We tested the hypothesis that the reluctance of Atlantic hagfish skeins to unravel spontaneously in seawater arises from the presence of skein glue that is less soluble in seawater than its counterpart in Pacific hagfish. This idea is supported by our SEM results, which revealed that rinsing of Atlantic hagfish skeins in ASW did not significantly decrease the amount of surface material, whereas Pacific hagfish skeins appeared smooth and clean after seawater rinsing (Fig. 3). Our SEM results are also consistent with recent results reported by Böni et al. (2017), who used SEM to show the presence of putative glue filaments bridging adjacent loops of thread in Atlantic hagfish skeins that had been rinsed in MilliQ water. The skein glue solubility hypothesis is also supported by the fact that Atlantic hagfish skeins can swell considerably in ddH_2O without unraveling (Fig. 7). The swelling of the skeins in this case is likely to be due to swelling of the slime thread itself (Fig. 6A), with unraveling being prevented by insoluble, and possibly elastic, skein glue. In contrast, Pacific hagfish skeins unravel spontaneously in ddH_2O (data not

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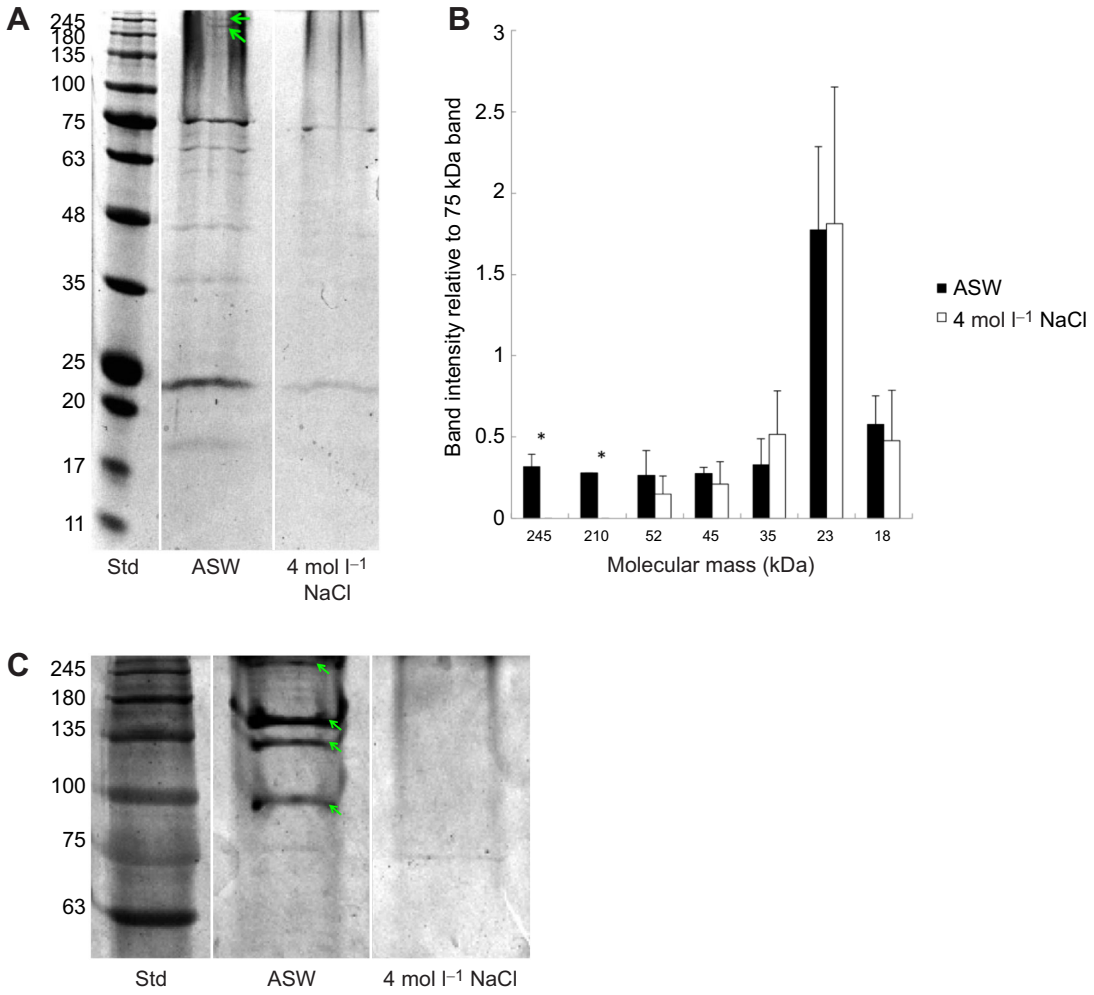

Fig. 8. SDS-PAGE of proteins and glycoproteins solubilized from Pacific hagfish thread skeins in ASW and 4 mol l^{-1} NaCl. (A) SDS-PAGE gel stained with Coomassie Brilliant Blue. Arrows indicate proteins that were significantly (*P<0.05) more soluble in solutions that cause skein unraveling (ASW) compared with solutions that inhibit unraveling (4 mol l^{-1} NaCl). (B) Densitometry analysis of gel in A. Band intensities were standardized against a 75 kDa band that may be a protein from the mucous fraction of the slime (Spitzer et al., 1984). Data are means±s.e.m.; n=3. (C) SDS-PAGE with PAS Alcian Blue/silver stain for glycoproteins solubilized from Pacific hagfish thread skeins in ASW and 4 mol l^{-1} NaCl. Arrows indicate the four most abundant glycoproteins, which were present in the supernatant of ASW-treated skeins, but absent in the supernatant of 4 mol l^{-1} NaCl-treated skeins; n=3. Molecular size standards (in kDa) are shown in first lanes of A and C (Std).

shown), probably due to the combined effect of thread swelling and the more soluble nature of the skein glue. We should note that in a recent publication, Böni et al. (2018) report substantial amounts of spontaneous unraveling of Atlantic hagfish skeins in ddH_2O. It is unclear why we observed mainly skein elongation in ddH_2O, although it may have something to do with minor differences in protocol used in the two studies. For example, Böni et al. (2018) used exudate stored in mineral oil, whereas we used fresh exudate that was used immediately after it was expressed from a hagfish.

Urea effects

We showed that urea can trigger spontaneous unraveling in skeins from both species of hagfish. The skein glue solubility hypothesis predicts that Pacific hagfish skeins should begin to unravel at lower urea concentrations than Atlantic hagfish skeins, but we did not see evidence of this; both kinds of skeins showed a very similar urea response. This may be because we conducted these trials in inhibitory, low salt (0.25 mol l^{-1} NaCl) conditions (Bernards et al., 2014), which were necessary for detecting whether urea was able to promote unraveling. It is possible that the differences in skein glue solubility that exist in seawater simply do not exist in these typically inhibitory low salt conditions, hence the similar effects of urea observed here. We also found it surprising that higher concentrations of urea (1.5 and 2.0 mol l^{-1}) exhibited far less unraveling than intermediate levels (0.5 and 1.0 mol l^{-1}). If urea drives unraveling via solubilizing skein glue proteins, then higher concentrations should be even better at solubilizing and more effective at promoting unraveling. One possible explanation is that high concentrations of urea denature the slime thread proteins, which leads to a dissipation of the strain energy that is normally released when the skein glue is disrupted. The fact that the skeins become transparent at these high urea concentrations is consistent with this hypothesis.

Urea is considered to be a chaotrope, which means that it tends to have denaturing and solubilizing effects on proteins (Breslow and Guo, 1990). Kosmotropes have the opposite effects on proteins, i.e. they tend to stabilize them and reduce their solubility. We find it intriguing that the fluid component of hagfish slime exudate contains high concentrations of two known kosmotropes, TMAO (101 mmol l^{-1}) and betaine (218 mmol l^{-1}) (Herr et al., 2010). Herr

Table 1. Pacific hagfish slime gland transcripts identified from mass spectrometry of proteins and glycoproteins liberated during skein unraveling and their Atlantic hagfish homologs

Pacific hagfish transcript	Pacific hagfish MS top hit	RefSeq top hit	RefSeq E-value	Atlantic hagfish transcript	% Identical matches	Alignment length	Atl E-value
Pac_DN49767	31419	Calpain-9	0	Atl_DN24799	85.3	647	0
Pac_DN53409	31417	No similarity	–	Atl_DN21632	60.1	686	0
Pac_DN55920	31421	Heat shock cognate 71 kDa protein	0	Atl_DN30735	96.9	613	0
Pac_DN52657	31426	PRY1-like	5e–64	–	–	–	–
Pac_DN58107	31425	No similarity	–	Atl_DN17663	69.7	343	4e–13
Pac_DN55215	31428	Galectin-4-like	1e–12	Atl_DN20319	52.0	333	3e–102
Pac_DN53909	31420	Periplakin	8e–16	Atl_DN33742	74.1	617	3e–116
Pac_DN56448	31431	Envoplakin	5e–48	Atl_DN33742	91.5	188	0
Pac_DN54810	31427	Hsp90	0	Atl_DN30250	97.3	445	2e–113
Pac_DN54049	31439	Transitional ER ATPase	0	Atl_DN17126	96.9	809	0
Pac_DN44409	31436	Periplakin-like	3e–33	Atl_DN33742	78.6	201	0
Pac_DN41322	31448	Envoplakin-like	2e–11	Atl_DN33742	69.8	338	7e–107
Pac_DN52950	31466	Arachidonate 15-lipoxygenase-like	8e–47	Atl_DN33952	39.6	187	2e–138
Pac_DN54141	31455	Hsp70	3e–107	Atl_DN23110	90.2	368	6e–38

The 14 sequences included here had >10 unique peptides mapping to them and $-10\log P$ values >100. Atl E-value is the E-value of the top hit when the Pacific hagfish transcript is BLASTed against the Atlantic hagfish transcriptome.

et al. (2010) tested the hypothesis that TMAO and betaine play a role in stabilizing mucous vesicles within GMCs, but found no evidence that they can. The results of the present study raise the possibility that TMAO and betaine act to stabilize the skein glue and prevent premature unraveling within GTCs.

Ecological implications

We have shown that skeins from two species of hagfish slime exhibit distinct unraveling behavior in seawater, and these differences appear to be mediated by differences in the solubility of skein glue proteins, which bridge neighboring thread loops in the slime gland and mediate the release of strain energy stored within the coiled thread. These results raise the question of why these differences exist and whether they are the result of divergent selective pressures acting on the *Myxine* and *Eptatretus* lineages. With their less-soluble skein glue, it seems reasonable that Atlantic hagfish skeins should take longer than Pacific hagfish skeins to unravel after being ejected from the slime glands, all other conditions being equal. This, however, is a prediction that remains to be tested. If *Myxine* skeins with insoluble skein glue represent the ancestral condition, then perhaps the differences we describe here arose via selective pressures for faster skein deployment in the *Eptatretus* lineage. One possibility is that the appearance of *Eptatretus* corresponded to a shift from a more obligate burrowing lifestyle in the ancestral population to a more epibenthic lifestyle, which exposed them to more predators. An increase in predation risk may have led to selection for faster slime deployment, which is likely limited by the speed of skein unraveling. Increases in the speed of unraveling may have come about by selection for skein glue that is less soluble in seawater. Future experiments should explore the consequences of the differences in skein glue properties described here on the deployment of whole slime exudate.

Identity of putative skein glue proteins

Using SDS-PAGE, we identified several proteins and glycoproteins that appear in skein rinsate under conditions that drive skein unraveling. Mass spectrometry of one protein and four of these glycoproteins generated numerous peptides that were then screened against a Pacific hagfish slime gland transcriptome, yielding 14 strong protein hits (Table 1).

Twelve of the protein sequences resemble known proteins in the RefSeq database, but two sequences (Pac_DN53409, Pac_DN58107) share homology with no known proteins. We propose that these sequences code for the structural proteins that make up the skein glue. Both sequences are rich in acidic amino acids and have low predicted pI values of 4.31 and 4.10, respectively. Homologous sequences in the Atlantic hagfish transcriptome (Atl_DN21632 and Atl_DN17663) also have low predicted pI values of 4.27 and 4.17, respectively (Table 2). Analysis of these sequences using PredictProtein (Yachdav

Table 2. Predicted isoelectric point (pI) and molecular mass of Pacific hagfish transcripts and their Atlantic hagfish homologs described in Table 1

Pacific hagfish transcript	pI	Molecular mass (kDa)	Atlantic hagfish transcript	pI	Molecular mass (kDa)
Pac_DN49767	8.12	74,132.7	Atl_DN24799	8.94	84,044.0
Pac_DN53409	4.31	69,319.0	Atl_DN21632	4.27	74,131.0
Pac_DN55920	5.57	71,280.5	Atl_DN30735	5.46	71,075.3
Pac_DN52657	5.28	28,975.3	–	–	–
Pac_DN58107	4.10	51,574.4	Atl_DN17663	4.17	37,731.0
Pac_DN55215	4.98	37,934.3	Atl_DN20319	4.93	37,531.7
Pac_DN53909	4.70	69,450.7	Atl_DN33742	5.03	188,538.7
Pac_DN56448	10.20	21,218.4	Atl_DN33742	5.03	188,538.7
Pac_DN54810	4.96	83,869.9	Atl_DN30250	5.00	83,349.4
Pac_DN54049	5.15	89,406.8	Atl_DN17126	5.09	89,892.3
Pac_DN44409	5.29	23,826.8	Atl_DN33742	5.03	188,538.7
Pac_DN41322	4.94	38,288.5	Atl_DN33742	5.03	188,538.7
Pac_DN52950	5.16	21,349.4	Atl_DN33952	5.24	71,829.2
Pac_DN54141	5.15	45,650.2	Atl_DN23110	5.60	96,499.0

et al., 2014) reveal a striking lack of domain structure, with most of the sequences scored as 'exposed' and very few scored as 'buried'. These attributes, along with their remarkably low pI values, suggest that these proteins are highly negatively charged at neutral pH and thus likely possess a high degree of conformational freedom; an attribute that is shared by proteins that make up elastomeric materials such as resilin and elastin (Tatham and Shewry, 2000). The ability of Atlantic hagfish skeins to resist unraveling in ddH_2O, even when the slime thread and the entire skein swells (Figs 6 and 7), is consistent with the possibility that strands of skein glue are elastomeric and thus able to accommodate large tensile strains before they fail.

Transcript Pac_DN49767 most closely resembles the protein calpain-9, which is a calcium-dependent cysteine protease expressed in the mucosa of the GI tract (Suzuki et al., 1996). We have shown in a previous paper (Bernards et al., 2014) and in the current study that the protease trypsin can drive unraveling of hagfish thread skeins *in vitro* under conditions where they normally do not unravel. Our finding that a calpain homolog may be present in the skein glue suggests that proteolysis may in fact be an integral component of skein glue dispersal during deployment in seawater. The use of a calcium-dependent protease is sensible given the extremely low calcium concentration within cells and the relatively high concentration of calcium in seawater. Further experiments on the effects of calcium and calpain inhibitors on unraveling will be needed to test whether calpain is in fact involved in skein unraveling.

Four sequences (Pac_DN53909, Pac_DN56448, Pac_DN44409 and Pac_DN41322) resemble the proteins periplakin and envoplakin, which are expressed in keratinocytes and are known to bind to the cytoskeletal elements known as intermediate filaments (Ruhrberg et al., 1997). Hagfish slime threads are almost exclusively composed of intermediate filament proteins (Spitzer et al., 1984), and it is not difficult to imagine how a protein that binds these cytoskeletal elements might be involved in a protein glue whose function it is to bind to the coiled slime thread and prevent it from unraveling in the cell. Alternatively, it is also possible that periplakin- and envoplakin-like proteins are involved in bundling of intermediate filaments within the slime thread. If the latter is the only function of envoplakin and periplakin in the skeins, however, it is difficult to imagine how these proteins would be solubilized by ASW.

We should note that the four sequences from the plakin family of proteins appear in different transcripts in the Pacific hagfish transcriptome, but as one very large transcript in the Atlantic hagfish transcriptome (Table 2). There are a few possible explanations for this observation. One is that there are errors in the Illumina sequence assemblies, with either a very large Pacific hagfish sequence being erroneously broken up into four sequences, or four Atlantic hagfish sequences being erroneously assembled into one very large sequence. It is also possible that the assemblies are accurate, and reflect real differences between the Pacific and Atlantic genes. Hennebert et al. (2014) showed that a protein involved in sea star adhesion is translated from a single large transcript, but then cleaved into four smaller proteins that are held together by disulfide bonds. Again, access to hagfish genomes will help us to resolve these questions.

One of the 14 proteins we identified (Pac_DN54810) most closely resembles the galectins, which are a large family of proteins that are also known as S-type lectins. Galectins bind specifically to glycan moieties containing galactose and its derivatives (Barondes et al., 1994). In the context of hagfish slime, one can imagine that a protein with the ability to bind glycans might be involved in forging cross-links between the slime threads and the mucous fraction, which consists of glycoproteins (Salo et al., 1983). Moreover, functional studies have demonstrated an affinity between the thread and mucous components (Koch et al., 1991; Winegard and Fudge, 2010). Galectins have been shown to be important in adhesion in several different groups, including echinoderms (Hennebert et al., 2014), cnidarians (Rodrigues et al., 2016) and mollusks (Smith et al., 2017) It is not clear why galectins would be present specifically in the skein glue, especially if the skein glue is designed to dissolve away from the skein surface (at least in Pacific hagfish). More work will be required to pin down the location and function of galectins in hagfish slime.

While our SDS-PAGE experiments were designed to isolate ASW-soluble skein glue proteins, we should caution that cytoplasmic proteins not involved in the skein glue may have been liberated during skein unraveling in ASW. Thread skeins within GTCs are tightly coiled structures (Fernholm, 1981; Winegard et al., 2014), and it is therefore possible that substantial amounts of soluble cytoplasmic proteins exist in the spaces between coiled loops. If this is the case, then the unraveling of skeins by ASW should liberate not only the soluble glue proteins, but also proteins trapped in these small, but potentially numerous pockets of cytoplasm. Some of the proteins we have identified have known functions that may be related to skein manufacture or unraveling. Others have no obvious connection to skein function, and we propose that they are cytoplasmic proteins liberated by the loosening of the skeins during unraveling. These proteins include: a PRY1-like protein, heat shock cognate 71 kDa protein, Hsp90, Hsp70 and arachidonate 15-lipoxygenase-like protein (Table 1).

Conclusions and future directions

Our results demonstrate that thread skeins from Pacific hagfish and Atlantic hagfish slime exhibit distinctly differently behaviors in seawater, with the former generally unraveling spontaneously, and the latter generally requiring external forces to overcome the seawater-insoluble skein glue. The ability of the chaotrope urea to drive skein unraveling in both Pacific hagfish and Atlantic hagfish skeins is consistent with the hypothesis that skein glue solubility is a major determinant of its function, with differences in solubility in seawater probably accounting for the differences in unraveling behavior observed here. Our results also point to the possibility that the high concentrations of the kosmotropes betaine and TMAO in hagfish slime exudate may be involved in skein glue stabilization and the prevention of premature skein unraveling within the slime glands. Mass spectrometry combined with transcriptomics has uncovered several leads in our efforts to identify and characterize the skein glue proteins, and raise the possibility that calcium-activated proteolysis may be involved in skein deployment. The publication of a hagfish genome will allow us to probe the proteins we have identified in greater detail, especially the two acidic proteins that we propose make up the skein glue and may be unique to the hagfish lineage.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.A.B., S.S., I.O., D.C.P., D.S.F.; Methodology: M.A.B., S.S., E.M., T.M.W., I.O., D.C.P., D.S.F.; Software: D.C.P.; Validation: M.A.B., S.S., D.C.P., D.S.F.; Formal analysis: M.A.B., S.S., T.M.W., I.O., D.C.P., D.S.F.; Investigation: M.A.B., S.S., E.M., T.M.W., I.O., D.C.P., D.S.F.; Resources: D.C.P., D.S.F.; Data curation: S.S., I.O., D.C.P., D.S.F.; Writing - original draft: M.A.B., D.S.F.; Writing - review & editing: M.A.B., S.S., D.C.P., D.S.F.; Visualization: M.A.B., S.S., E.M., T.M.W., I.O., D.C.P., D.S.F.; Supervision: D.C.P., D.S.F.; Project administration: M.A.B., D.C.P., D.S.F.; Funding acquisition: D.C.P., D.S.F.

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Data availability

Raw sequence data are available at the Sequence Read Archive under BioProject PRJNA497829.

References

Barondes, S. H., Cooper, D. N., Gitt, M. A. and Leffler, H. (1994). Galectins: structure and function of a large family of animal lectins. *J. Biol. Chem.* **269**, 20807-20810.

Bernards, M. A., , Jr, Oke, I., Heyland, A. and Fudge, D. S. (2014). Spontaneous unraveling of hagfish slime thread skeins is mediated by a seawater-soluble protein adhesive. *J. Exp. Biol.* **217**, 1263-1268.

Bolger, A. M., Lohse, M. and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* **30**, 2114-2120.

Böni, L., Fischer, P., Böcker, L., Kuster, S. and Rühs, P. A. (2016). Hagfish slime and mucin flow properties and their implications for defense. *Sci. Rep.* **6**, 30371.

Böni, L. J., Zurflüh, R., Widmer, M., Fischer, P., Windhab, E. J., Rühs, P. A. and Kuster, S. (2017). Hagfish slime exudate stabilization and its effect on slime formation and functionality. *Biol. Open* **6**, 1115-1122.

Böni, L. J., Zurflüh, R., Baumgartner, M. E., Windhab, E. J., Fischer, P., Kuster, S. and Rühs, P. A. (2018). Effect of ionic strength and seawater cations on hagfish slime formation. *Sci. Rep.* **8**, 9867.

Breslow, R. and Guo, T. (1990). Surface tension measurements show that chaotropic salting-in denaturants are not just water-structure breakers. *Proc. Natl. Acad. Sci. USA* **87**, 167-169.

Downing, S. W., Spitzer, R. H., Salo, W. L., Downing, J. S., Saidel, L. J. and Koch, E. A. (1981a). Threads in the hagfish slime gland thread cells: organization, biochemical features, and length. *Science* **212**, 326-328.

Downing, S. W., Salo, W. L., Spitzer, R. H. and Koch, E. A. (1981b). The hagfish slime gland: a model system for studying the biology of mucus. *Science* **214**, 1143-1145.

Ewoldt, R. H., Winegard, T. M. and Fudge, D. S. (2011). Non-linear viscoelasticity of hagfish slime. *Int. J. Non-Linear Mech.* **46**, 627-636.

Fernholm, B. (1981). Thread cells from the slime glands of hagfish (*Myxinidae*). *Acta Zool.* **62**, 137-145.

Fudge, D. S. and Gosline, J. M. (2004). Molecular design of the α-keratin composite: insights from a matrix-free model, hagfish slime threads. *Proc. R. Soc. B* **271**, 291-299.

Fudge, D. S., Levy, N., Chiu, S. and Gosline, J. M. (2005). Composition, morphology and mechanics of hagfish slime. *J. Exp. Biol.* **208**, 4613-4625.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Willkins, M. R., Appel, R. D. and Bairoach, A. (2005). Protein identification and analysis tools on the ExPASy server. In *The Proteomics Protocols Handbook* (ed. J. M. Walker), pp. 571-607. New York: Humana Press.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q. et al. (2011). Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nat. Biotechnol.* **29**, 644-652.

Gundry, R. L., White, M. Y., Murray, C. I., Kane, L. A., Fu, Q., Stanley, B. A. and Van Eyk, J. E. (2010). Preparation of proteins and peptides for mass spectrometry analysis in a bottom-up proteomics workflow. *Curr. Protoc. Mol. Biol.* **90**, 1-23.

Hennebert, E., Wattiez, R., Demeuldre, M., Ladurner, P., Hwang, D. S., Waite, J. H. and Flammang, P. (2014). Sea star tenacity mediated by a protein that fragments, then aggregates. *Proc. Natl. Acad. Sci. USA* **111**, 6317-6322.

Herr, J. E., Winegard, T. M., O'Donnell, M. J., Yancey, P. H. and Fudge, D. S. (2010). Stabilization and swelling of hagfish slime mucin vesicles. *J. Exp. Biol.* **213**, 1092-1099.

Herr, J. E., Clifford, A. M., Goss, G. G. and Fudge, D. S. (2014). Defensive slime formation in Pacific hagfish requires Ca2+- and aquaporin-mediated swelling of released mucin vesicles. *J. Exp. Biol.* **217**, 2288-2296.

Klaiman, J. M., Fenna, A. J., Shiels, H. A., Macri, J. and Gillis, T. E. (2011). Cardiac remodeling in fish: strategies to maintain heart function during temperature change. *PLoS ONE* **6**, e24464.

Koch, E. A., Spitzer, R. H., Pithawalla, R. B. and Downing, S. W. (1991). Keratin-like components of gland thread cells modulate the properties of mucus from hagfish (*Eptatretus stouti*). *Cell Tissue Res.* **264**, 79-86.

Koch, E. A., Spitzer, R. H., Pithawalla, R. B. and Parry, D. A. (1994). An unusual intermediate filament subunit from the cytoskeletal biopolymer released extracellularly into seawater by the primitive hagfish (Eptatretus stouti). *J. Cell Sci.* **107**, 3133-3144.

Koch, E. A., Spitzer, R. H., Pithawalla, R. B. and Castillos, F. A. (1995). Hagfish biopolymer: a type-I type-II homologue of epidermal keratin intermediate filaments. *Int. J. Biol. Macromol.* **17**, 283-292.

Li, H. (2015). BFC: correcting Illumina sequencing errors. *Bioinformatics* **31**, 2885-2887.

Lim, J., Fudge, D. S., Levy, N. and Gosline, J. M. (2006). Hagfish slime ecomechanics: testing the gill-clogging hypothesis. *J. Exp. Biol.* **209**, 702-710.

Møller, H. J. and Poulsen, J. H. (2009). Staining of glycoproteins/proteoglycans on SDS-gels. In *The Protein Protocols Handbook: Third Edition* (ed. J. M. Walker), pp. 569-574. New York: Humana Press.

Newby, W. W. (1946). The slime glands and thread cells of the hagfish, *Polistrotrema stouti*. *J. Morphol.* **78**, 397-409.

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* **14**, 417-419.

Rodrigues, M., Ostermann, T., Kremeser, L., Lindner, H., Beisel, C., Berezikov, E., Hobmayer, B. and Ladurner, P. (2016). Profiling of adhesive-related genes in the freshwater cnidarian *Hydra magnipapillata* by transcriptomics and proteomics. *Biofouling* **32**, 1115-1129.

Ruhrberg, C., Hajibagheri, M. A. N., Parry, D. A. D. and Watt, F. M. (1997). Periplakin, a novel component of cornified envelopes and desmosomes that belongs to the plakin family and forms complexes with envoplakin. *J. Cell Biol.* **139**, 1835-1849.

Salo, W. L., Downing, S. W., Lidinsky, W. A., Gallagher, W. H., Spitzer, R. H. and Koch, E. A. (1983). Fractionation of hagfish slime gland secretions: partial characterization of the mucous vesicle fraction. *Prep. Biochem.* **13**, 103-135.

Smith, A. M., Papaleo, C., Reid, C. W. and Bliss, J. M. (2017). RNA-Seq reveals a central role for lectin, C1q and von Willebrand factor A domains in the defensive glue of a terrestrial slug. *Biofouling* **33**, 741-754.

Soneson, C., Love, M. I. and Robinson, M. D. (2015). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research* **4**.

Spitzer, R. H., Downing, S. W., Koch, E. A., Salo, W. L. and Saidel, L. J. (1984). Hagfish slime gland thread cells. II. Isolation and characterization of intermediate filament components associated with the thread. *J. Cell. Biol.* **98**, 670-677.

Suzuki, K., Sorimachi, H., Yoshizawa, T., Kinbara, K. and Ishiura, S. (1996). Calpain: novel family members, activation, and physiologic function". *Biol. Chem.* **376**, 523-529.

Tatham, A. S. and Shewry, P. R. (2000). Elastomeric proteins: biological roles, structures and mechanisms. *Trends Biochem. Sci.* **25**, 567-571.

Winegard, T. M. and Fudge, D. S. (2010). Deployment of hagfish slime thread skeins requires the transmission of mixing forces via mucin strands. *J. Exp. Biol.* **213**, 1235-1240.

Winegard, T., Herr, L., Mena, C., Lee, B., Dinov, I., Bird, D. and Fudge, D. (2014). Coiling and maturation of a high-performance fibre in hagfish slime gland thread cells. *Nature Comm.* **5**, 3534.

Yachdav, G., Kloppmann, E., Kajan, L., Hecht, M., Goldberg, T., Hamp, T., Hönigschmid, P., Schafferhans, A., Roos, M., Bernhofer, M. et al. (2014). PredictProtein—an open resource for online prediction of protein structural and functional features. *Nucleic Acids Res.* **42**, W337-W343.

Zintzen, V., Roberts, C. D., Anderson, M. J., Stewart, A. L., Struthers, C. D. and Harvey, E. S. (2011). Hagfish predatory behaviour and slime defence mechanism. *Sci. Rep.* **1**, 131.