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RESEARCH ARTICLE

High concentrations of trimethylamines in slime glands inhibit skein unraveling in Pacific hagfish

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ABSTRACT

Hagfish defend themselves from fish predators by producing large volumes of gill-clogging slime when they are attacked. The slime consists of seawater and two major components that are ejected from the slime glands: mucus and threads. The threads are produced within specialized cells and packaged into intricately coiled bundles called skeins. Skeins are kept from unraveling via a protein adhesive that dissolves when the skeins are ejected from the slime glands. Previous work revealed that hagfish slime glands have high concentrations of methylamines including trimethylamine *N*-oxide (TMAO), trimethylglycine (betaine) and dimethylglycine (DMG); however, the function of these compounds in the slime glands is unknown. We hypothesized that methylamines have stabilizing effects on the skeins that prevent premature unraveling in the gland. To test this hypothesis, we quantified the effect of methylamines on skein unraveling in Pacific hagfish and found that TMAO and betaine have inhibitory effects on skein unraveling *in vitro*. Furthermore, we found that TMAO is a more effective inhibitor of unraveling than betaine, but the presence of TMAO synergistically boosts the inhibitory action of betaine. Glycine and DMG were far less effective inhibitors of unraveling at natural concentrations. Our results support the hypothesis that high levels of trimethylamines in the slime glands may act to hold the coiled thread skeins together within gland thread cells, and they may do so by stabilizing adhesive proteins. These results advance our knowledge of skein stabilization and deployment and provide yet another example of trimethylamines functioning to stabilize proteins in a marine organism.

KEY WORDS: TMAO, Betaine, Hagfish, Methylamines, Mucus

INTRODUCTION

Hagfishes are deep-sea animals that are able to rapidly produce large amounts of slime when they are attacked (Ferry, 1941; Newby, 1946; Fudge et al., 2005). The slime is highly effective at thwarting attacks by fish predators because of its ability to lodge onto the gills and clog them (Lim et al., 2006; Zintzen et al., 2011). The slime is produced within numerous ventrolateral glands, each of which contains two major types of cells: gland mucous cells (GMCs) and gland thread cells (GTCs) (Downing et al., 1981a,b). Each individual GMC is able to produce hundreds of mucin vesicles, and each GTC produces a single ∼150 mm long intermediate filament-rich thread (Koch et al., 1994, 1995) that is tightly coiled into an ellipsoidal structure called a thread skein. When a hagfish is attacked, a thin layer of muscle in each slime gland in the vicinity of the bite contracts, ejecting mature GMCs and GTCs through a narrow gland pore. Ejection strips the cells of their plasma membranes (Fernholm, 1981) and allows their contents (mucous vesicles and skeins) to interact with seawater and each other to produce the gill-clogging slime.

In recent years, researchers have focused on understanding the process by which, in a fraction of a second after ejection from the gland, thousands of ellipsoidal thread skeins (Schorno et al., 2018a, b) unravel into slime threads that are approximately 1000 times longer than the skeins themselves (Koch et al., 1991; Winegard and Fudge, 2010; Winegard et al., 2014; Bernards et al., 2014, 2018; Böni et al., 2016; 2017; Chaudhary et al., 2019). The current model of skein unraveling posits that a proteinaceous glue is involved in both skein stabilization in the gland and the regulation of skein deployment in seawater. The glue appears to hold back strain energy contained in the coiled thread, such that when it is disrupted or dissolved, unraveling proceeds via relaxation of the thread (Bernards et al., 2014). Recent work has shown that in seawater, Atlantic hagfish (*Myxine glutinosa*) skeins are more resistant to unraveling than Pacific hagfish (*Eptatretus stoutii*) skeins, which may be a result of a less soluble skein glue in the former (Bernards et al., 2018). The ecological and/or evolutionary significance of this difference is not yet known. One of the key tests of the skein glue hypothesis involved exposing skeins to the protease trypsin under conditions that normally inhibit skein unraveling (e.g. high temperature and low salt). This experiment showed that trypsin can drive spontaneous unraveling under inhibitory conditions in both *M. glutinosa* and *E. stoutii* skeins (Bernards et al., 2014; 2018). These results indicate that the coiled thread contains strain energy, and that the skein glue is made of protein, a result that is corroborated by recent mass spectrometry analysis (Bernards et al., 2018). Furthermore, urea, which has known chaotropic (i.e. solubilizing) effects on proteins, also has the ability at high concentrations to drive skein unraveling under inhibitory conditions (Bernards et al., 2018). This result suggests that the difference between skein stabilization within GTCs in the slime gland and skein unraveling in seawater may be the result of differences in the chemical composition of GTC cytoplasm and seawater, with skein glue being generally insoluble in the former and soluble in the latter. This line of reasoning raises the question of what compounds in the GTC cytoplasm might be present to stabilize the skein glue and prevent premature unraveling in the slime gland.

While we do not know the detailed chemical composition of GTC cytoplasm, we do know the composition of the fluid component of exudate that is expressed from slime glands. This fluid can be obtained by centrifuging exudate, which results in a layer of thread

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skeins on the bottom, a layer of mucous vesicles in the middle, and a layer of clear fluid on top. Herr et al. (2010) analyzed this 'supernatant' fluid and found that it contains an ionic profile similar to that of the cytoplasm of hagfish cells. However, the fluid also contains high concentrations of methylamines, with the trimethylamines trimethylglycine (betaine) and trimethylamine *N*-oxide (TMAO) occurring in the highest concentrations (Table 1). Herr et al. (2010) tested the hypothesis that these compounds are involved in the stabilization of mucous vesicles in slime glands and found no evidence that they can prevent vesicle rupture, even at very high concentrations. In the present study, we tested the hypothesis that the methylamines present in hagfish slime glands exert a stabilizing effects on skeins, which keeps them in a coiled state and prevents premature unraveling. Betaine and TMAO are both known to counteract the denaturing effects of urea in fishes and mammals (Yancey and Somero, 1980; Garcia-Perez and Burg, 1991), and we reasoned that they might have similar effects on proteins involved in keeping skeins in a condensed state within hagfish slime glands. This hypothesis predicts that increasing the concentration of these compounds should inhibit skein unraveling, which we tested with a series of *in vitro* unraveling assays.

MATERIALS AND METHODS

Experimental animals

Slime exudate was collected from Pacific hagfish, *E. stoutii* (Lockington 1878), housed in a 1000 l tank filled with chilled artificial seawater (ASW; 34‰, 8°C) at Chapman University, CA, USA. Hagfish were fed a variety of fish, squid, polychaetes and beef to satiety. Although hagfishes do not fall under the jurisdiction of the Chapman University Institutional Animal Care and Use Committee (IACUC), all animal protocols used for this research were based on guidelines of the Canadian Council on Animal Care (https://www.ccac.ca/en/standards/guidelines/), which does require animal use protocols for hagfishes.

Exudate collection

Hagfish were anesthetized by immersion in 3 l of ASW containing 5250 µl of anesthetic solution (1:9 clove oil to 95% ethanol) and left until the hagfish failed to respond to touch stimuli. Anesthetized hagfish were placed on a chilled dissection tray and a region on the right side of the caudal end was blotted until dry. Slime glands were individually induced to release exudate using a stimulator wand connected to a Grass S48 stimulator (60 Hz, 1 ms duration, 18 V). A Teflon spatula was used to collect the pool of exudate that was secreted on to the skin after stimulation and transferred into mineral oil. Exudate was stored on ice or in a refrigerator (4°C) until use up to 48 h after collection.

Table 1. Concentration of organic methylamines in the supernatant of fresh Pacific and Atlantic hagfish slime exudate

	Concentration (mmol l^{-1})	
Compound	Pacific hagfish	Atlantic hagfish
TMAO	79.1±25.3	101.3±4.8
Glucose	2.4±2.3	1.2±0.2
m-Inositol	1.2±0.8	2.3±0.7
Taurine	1.3±0.3	2.1±0.4
Betaine	365.6±29.7	218±7
Glycine	1.8±0.6	79.9±7.5
β-Alanine	3.5±1.6	2.3±0.7
Creatine	0.4±0.1	15.0±1.4
DMG	N/A	68.6±6.0
Serine/alanine	15.1±7.4	N/A
Total	470.4±39.8	490±10

TMAO, trimethylamine *N*-oxide; DMG, dimethylglycine. Values for Atlantic hagfish are reproduced from Herr et al. (2010). Betaine values for Pacific hagfish might be an overestimate and DMG values an underestimate if the DMG peak merged with the betaine peak in these samples. Values are means±s.e.m., *N*=3.

Analysis of *E. stoutii* exudate supernatant

We centrifuged freshly obtained exudate from Pacific hagfish (as described above) at 9168 ***g*** for 5 min, which resulted in a clear layer of supernatant. Osmolality of the supernatant was measured from three 10 µl samples obtained from three hagfish using a Wescor Vapro 5600 Osmometer, calibrated using four osmolality standards (ranging from 100 to 2000 mOsm kg^{-1}) (R^2=0.9999). For organic methylamine analysis, supernatant samples were weighed to a precision of 0.0001 g, homogenized in 9:19 parts of cold 7% perchloric acid in a glass dounce, and stored overnight at 4°C to precipitate proteins. The samples were then centrifuged at 15,000 ***g*** for 30 min at 4°C, and the supernatants were removed. These were then neutralized to pH 6.5–7.5 with 2 mol l^{-1} KOH, and lipids and particulates were removed using a solid-phase C18 cartridge (Sep-Pak; Waters Inc., Milford, MA, USA) capped with a 0.22 µm filter. TMAO contents were determined according to Wekell and Barnett (1991) as modified by Kelly and Yancey (1999). Briefly, 20–50 µl of supernatant from each homogenate (either before or after neutralization) was added to 100 µl of reagent mixture with iron, which reduces TMAO to trimethylamine (TMA). The TMA was then extracted in toluene and reacted with a picric acid–toluene reagent. Spectrophotometry was used to quantify the concentration of the TMA picrate products, which were compared to TMAO/TMA standards treated identically. TMAO content of the original samples was calculated based on all dilutions made in the processing.

Other organic solutes (sugars, methylamines and uncharged free amino acids) were identified and quantified by high performance liquid chromatography (HPLC) as described by Wolff et al. (1989). In brief, 50 µl of each neutralized, filtered homogenate was pumped (Perkin Elmer 200 MicroPump) through a Sugar-Pak I column (Waters, Inc.) that separates solutes by size and polarity. Compounds were then detected by their refractive indices (Bio-Rad 1755 detector) and the peaks integrated with Powerchrom (eDaq Ltd) on a microcomputer (Windows 7). Standards of known methylamines were used regularly to determine the HPLC retention time of each solute and its refractive index.

Unraveling assays

The effects of various slime gland components [TMAO, betaine, DMG, glycine] on skein unraveling were assessed *in vitro* using a custom protocol based on Bernards et al. (2014). To detect possible inhibitory effects, all test solutions contained 1 mol l^{-1} NaCl, which is known to promote spontaneous unraveling of *E. stoutii* skeins (Bernards et al., 2014). The TMAO, betaine, DMG and glycine concentrations used were 100, 200, 300, 400 and 500 mmol l^{-1} and 1000 mmol l^{-1}. We also conducted trials with solutions of TMAO and betaine in a 1:2 molar ratio for total concentrations of 100, 200, 300, 400 and 500 mmol l^{-1} and 1000 mmol l^{-1}. Unraveling trials were also conducted using solutions containing a mixture of four solutes (glycine, DMG, TMAO, betaine) combined in the molar ratio found in Atlantic hagfish supernatant (1:1:1.5:2) for total methylamine concentrations of 450 and 900 mmol l^{-1}.

For the unraveling assays, small (35 mm) glass-bottom Petri dishes (Azzota Corporation) were chilled to 5°C using an incubation stage (Pecon GmbH) attached to a chiller (RC Control, IKA). The dishes were chilled prior to visualization of the skeins under the

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microscope. Exudate from 10 different hagfish was exposed to six different concentrations (100, 200, 300, 400, 500, 1000 mmol l^{-1}) of each of the various osmolytes of interest. A 1 µl drop of exudate was placed towards the center of the chilled glass-bottom Petri dish and, immediately after, a 0.5 ml of the test solution was pipetted across the drop of slime. A Zeiss Imager.M2 microscope was used to capture images with a 5× objective lens and an Axiocam 506 mono camera. After 30 s, 6–8 non-overlapping fields of view of the image were captured, allowing us to assess the degree of unraveling for 13,586 skeins across all treatments. Zen Pro software (v2.3) was used to count the total number of skeins, and skeins were counted as unraveled if any loose thread was visible. Counts of intact and unraveled skeins were performed by at least two individuals for each image and percentage unraveling for each trial was calculated as the number of unraveled skeins divided by the total number and multiplied by 100%.

Statistical analyses

All figures represent means±s.e.m., unless otherwise stated. Raw data for percentage unraveled skeins under the various unraveling assays were pooled for subsequent tests of statistical significance. Multifactor ANOVA was performed to reveal the effects of methylamine type, concentration and the interaction between the two. Shapiro–Wilk normality tests revealed that our data were not normally distributed. Therefore, we performed non-parametric Kruskal–Wallis ANOVA to evaluate the effects of concentration on skein unraveling within each methylamine type. To reveal the effects of concentration on the inhibition of skein unraveling, we performed non-parametric Mann–Whitney–Wilcoxon tests. Multifactor ANOVA was performed to reveal significant effects of methylamine type, concentration and the interaction of these two factors on skein unraveling in betaine, TMAO and TMAO plus betaine (1:2) (Fig. 4). Dunn *post hoc* tests were conducted to examine pairwise differences for all multifactor ANOVA. We used RStudio v1.1.456 to analyze data, and considered comparisons with $P \leq 0.05$ as significant for all tests.

RESULTS

Pacific hagfish supernatant osmolality and composition

The mean (±s.e.m.) osmolality of supernatant samples from three Pacific hagfish was 1078.3±44.1 mOsm kg^{-1}, or approximately isoosomotic with seawater. Analysis of organic osmolyte concentration revealed betaine and TMAO to be the most abundant compounds by far, with average values of 366 and 79 mmol l^{-1}, respectively (Table 1).

Methylamine effects on skein unraveling

We performed unraveling assays in the presence of osmolytes found in the supernatant of hagfish exudate to measure their effects on skein unraveling. Fig. 1 shows representative images of tests with TMAO and betaine, where the presence of 100 mmol l^{-1} TMAO, 100 mmol l^{-1} betaine or a combination of TMAO plus betaine in a ratio of 1:2 (33 mmol l^{-1} TMAO+67 mmol l^{-1} betaine) allowed skeins to unravel, while 1000 mmol l^{-1} TMAO, 1000 mmol l^{-1} betaine or a combination of TMAO plus betaine in a ratio of 1:2 (333 mmol l^{-1} TMAO+667 mmol l^{-1} betaine) inhibited unraveling. Unraveling as a function of solute and concentration is shown in Fig. 2: solute type (d.f.=4, F=189.44, $P<2.2\times10^{-16}$) and concentration (d.f.=6, F=87.13, $P<2.2\times10^{-16}$) had significant effects on skein unraveling, as well as the interaction between the type and concentration (d.f.=17, F=30.04, $P<2.2\times10^{-16}$) (multi-factor two-way ANOVA). TMAO, betaine and DMG all had

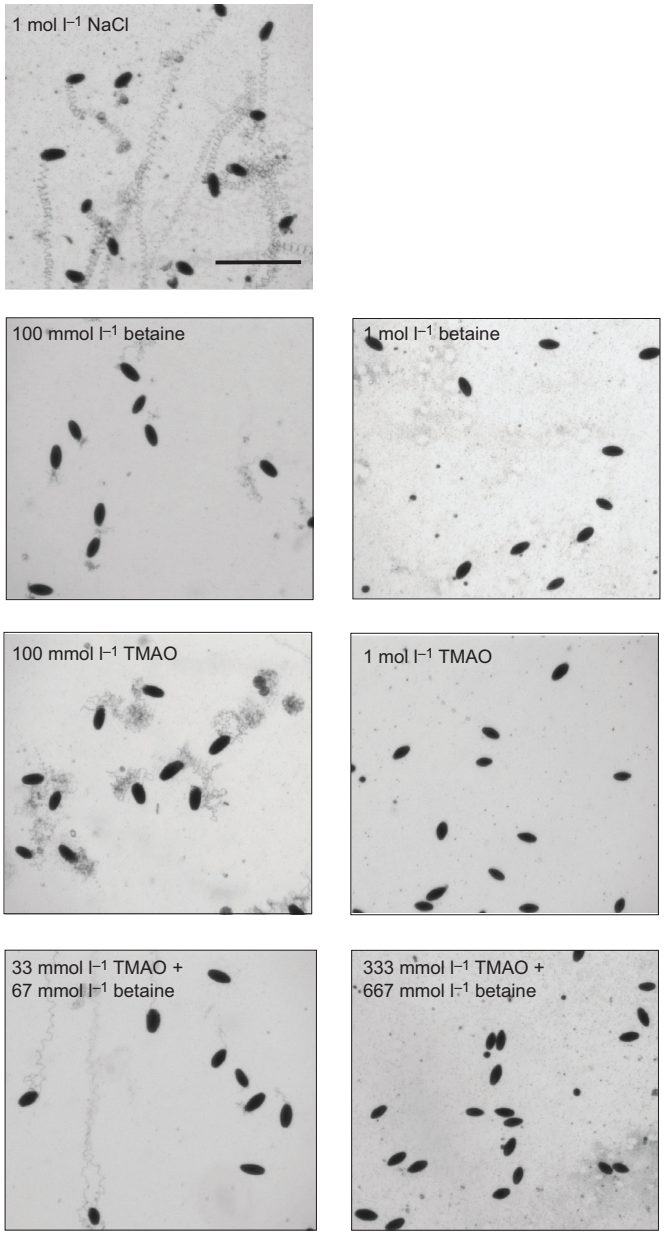

Fig. 1. Representative images from skein unraveling trials. Skeins were exposed to various trimethylamine concentrations at 5°C in a background of 1 mol l^{-1} NaCl, which is known to promote unraveling. Note that high levels of TMAO and betaine, as well as a combination of the two in a 1:2 ratio (333 mmol l^{-1} TMAO plus 667 mmol l^{-1} betaine) resulted in substantial inhibition of skein unraveling compared with NaCl controls, but lower concentrations were less effective.

substantial inhibitory effects on skein unraveling at high concentrations (1000 mmol l^{-1}), with only TMAO showing an ability to suppress the majority of unraveling at concentrations lower than 400 mmol l^{-1}. Glycine was unable to inhibit skein unraveling even at 1000 mmol l^{-1} (Fig. 2).

We analyzed the combination of three methylamines (DMG, TMAO and betaine) plus glycine in a ratio 1:1:1.5:2 with an approximate total methylamine concentration found in the supernatant of Pacific hagfish of 450 mmol l^{-1} and twice that concentration (950 mmol l^{-1}), which mimics the total osmolality observed in slime exudate as well as hagfish tissues and blood. We found that the 450 mmol l^{-1} solution had a substantial inhibitory

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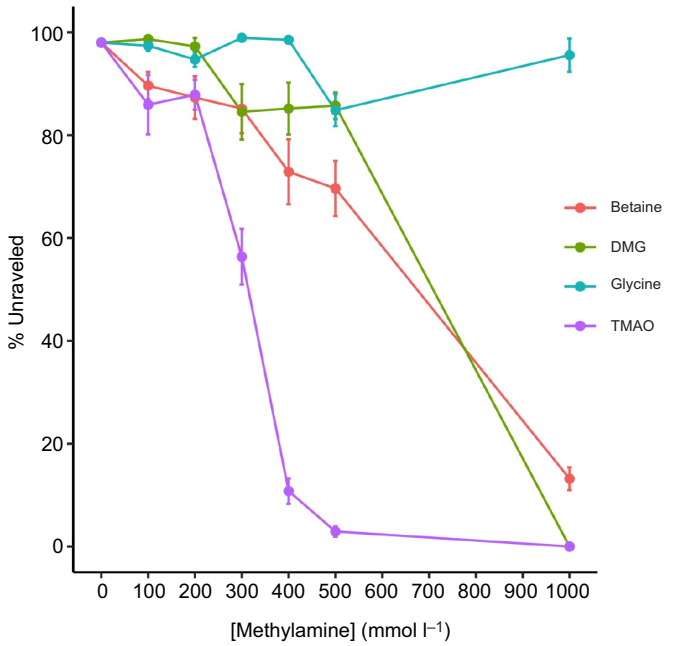

Fig. 2. The effect of methylamines and glycine on skein unraveling. Skeins were observed at 100–1000 mmol l^{-1} concentrations of methylamines at 5°C in a background of 1 mol l^{-1} NaCl, to promote skein unraveling. Data points and error bars represent mean±s.e.m. percentage unraveling. A multi-factor ANOVA revealed significant effects of methylamine type (d.f.=3, F=256.31, P<2.2×10^{-16}) and concentration (d.f.=5, F=147.74, P<2.2×10^{-16}) as well as the interaction between the two (d.f.=15, F=31.26, P<2.2×10^{-16}). Non-parametric Kruskal–Wallis ANOVA revealed significant effects of concentration on skein unraveling within each methylamine type (betaine: d.f.=4, P<5.34×10^{-9}; DMG: d.f.=4, P<2.23×10^{-16}; glycine: d.f.=5, P<2.97×10^{-5}; TMAO: d.f.=4, P<2.2×10^{-16}; TMAO plus betaine: d.f.=4, P<3.93×10^{-5}).

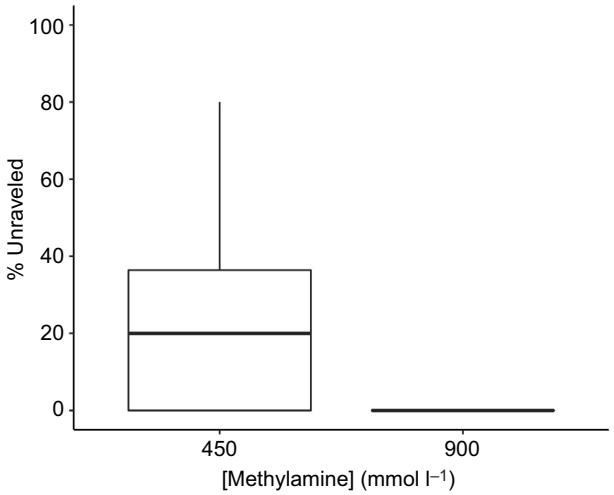

Fig. 3. The effect of 450 and 900 mmol l^{-1} methylamines (DMG, TMAO, betaine) and glycine on skein unraveling. Methylamines were prepared in the molar ratio found in Atlantic hagfish exudate (1:1:1.5:2) in a background of 1 mol l^{-1} NaCl, to promote skein unraveling. Both treatments exhibited substantial inhibition, with the higher concentration causing complete inhibition of skein unraveling (Mann–Whitney–Wilcoxon test, n=3, W=288, P=3.30×10^{-5}).

effect on unraveling, with only about one-fifth of skeins exhibiting unraveling. Doubling the concentration to 900 mmol l^{-1} resulted in complete inhibition of unraveling (Fig. 3; Mann–Whitney–Wilcoxon test, W=288, P=3.30×10^{-5}).

As TMAO and betaine are the dominant osmolytes in hagfish slime gland exudate, we conducted trials in which they were combined in their approximate natural molar ratio (1:2) to test whether their effects are additive or whether there are interactive effects between the two. The results demonstrate that the inhibitory effects of the two compounds are not simply additive, with combinations of the two exerting stronger inhibition than one would expect from each compound alone. Another way of expressing this is that although betaine was less effective at inhibiting unraveling at low concentrations than TMAO, solutions in which the two were combined were equally effective as the same concentration of TMAO (Fig. 4).

DISCUSSION

The main goal of this study was to determine the function of the high levels of methylamines that are present in the slime glands of hagfishes, and we tested the hypothesis that these compounds act to stabilize coiled thread skeins within gland thread cells. Our results demonstrate that of the four methylamines we tested, the trimethylamine TMAO is the most effective inhibitor of unraveling. Betaine was less effective at inhibiting unraveling on its own but exhibited a synergistic effect when it was combined with TMAO. DMG was only effective at the highest concentration tested, 1000 mmol l^{-1}, and glycine was generally ineffective at inhibiting unraveling over the entire concentration range tested. This pattern of stabilization, TMAO>betaine>DMG>glycine, is consistent with other studies showing that an increased degree of methylation increases stabilizing ability and that of the two trimethylamines, TMAO is typically stronger than betaine (Yancey, 2005, 2015; Street et al., 2006).

While TMAO inhibited unraveling in the vast majority of skeins at concentrations of 500 mmol l^{-1} and 1000 mmol l^{-1}, lower concentrations were not able to stabilize all the skeins. Indeed, at concentrations of TMAO corresponding to those we measured in the supernatant of Atlantic and Pacific hagfishes (101 mmol l^{-1} and 79 mmol l^{-1}, respectively), inhibition was far from complete, with most skeins unraveling. How then is it possible that TMAO can stabilize thread skeins in the slime glands? Although betaine is not a very effective stabilizer on its own, our results demonstrate that TMAO and betaine act synergistically to inhibit skein unraveling. The combined concentrations of these two compounds (319 mmol l^{-1} and 445 mmol l^{-1} in Atlantic and Pacific hagfishes, respectively) are quite high and at these concentrations, stabilization is far more effective, with unraveling being inhibited in the majority of skeins. Another thing to consider is that the concentration of organic osmolytes that we measured in the supernatant of centrifuged slime exudate does not necessarily correspond to the concentration of those compounds within the GTCs, where the thread skeins are produced and reside. If most of the TMAO and betaine in the supernatant originates in the GTCs, and the GTCs contribute only a small fraction of the volume of the supernatant, then it is likely that the concentrations of these compounds are considerably higher in the cytoplasm of these cells than in the supernatant.

Our *in vitro* unraveling results support the hypothesis that high levels of TMAO and betaine in the gland may function to prevent premature unraveling of thread skeins within gland thread cells. These results raise the question of how exactly these compounds influence skein unraveling and one possibility is that they reduce the solubility of adhesive proteins. TMAO is known to be an effective stabilizer of proteins and nucleic acids (Zou et al., 2002; Singh et al.,

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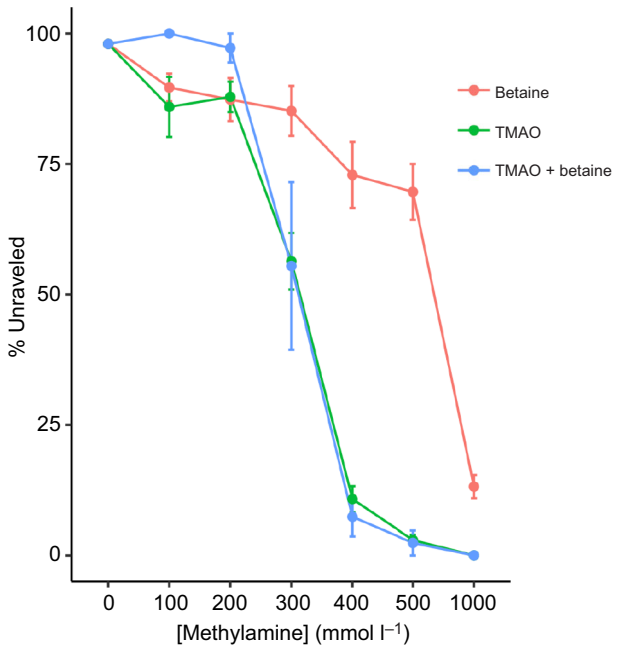

Fig. 4. Concentration effects of betaine, TMAO and the interaction of the two on skein unraveling. All solutions contained a background of 1 mol l^{-1} NaCl, to promote skein unraveling. Multi-factor ANOVA revealed that methylamine (d.f.=2, F=119.66, P<2.2×10^{-16}), concentration (d.f.=6, F=156.93, P<2.2×10^{-16}) and the interaction of these two factors (d.f.=12, F=14.22, P<2.2×10^{-16}) significantly affect skein unraveling in betaine, TMAO and TMAO plus betaine (1:2).

2005), but the mechanism is not clear. One possibility is that it acts to exclude the hydration layer around proteins, which stabilizes hydrophobic interactions and makes them less soluble (Liao et al., 2017). In contrast, compounds such as urea are believed to bind to proteins and disrupt hydrophobic interactions, increasing their solubility and causing them to denature (Street et al., 2006; Yancey, 2005). The antagonistic effects of urea and TMAO are well described in elasmobranchs, which accumulate urea as an osmolyte and counter its effects on enzymes with TMAO (Yancey and Somero, 1980). Recently, Bernards et al. (2018) showed that urea can promote unraveling of hagfish thread skeins under typically inhibitory conditions (i.e. low salt and high temperature). These opposing effects of urea and TMAO on thread skein unraveling are consistent with the view that urea and TMAO have counteracting effects on protein structure and solubility (Yancey and Somero, 1980; Yancey, 2005), and further support the hypothesis that skein unraveling may be mediated by adhesive proteins. Regardless of the mechanism of its action, our results provide another example of TMAO being used by a marine animal to adjust the function of its proteins.

While the potent stabilizing effects of TMAO alone on thread skein unraveling is consistent with effects shown in other systems, the synergistic inhibitory effect of TMAO and betaine together is more difficult to explain. Betaine alone was a weak inhibitor of skein unraveling, but its potency increased greatly in the presence of TMAO, with effects of solutions containing both compounds at a ratio of 1:2 resembling those of TMAO alone. This is the first report of such a synergistic effect between TMAO and betaine, although synergistic enhancement of elasmobranch enzyme substrate binding was reported for a mixture of TMAO, sarcosine, betaine and β-alanine (Yancey and Somero, 1980). This warrants further investigation to see whether protein stabilization is a general effect that applies to all proteins or whether it only pertains to a few systems such as hagfish thread skein glue. In addition to its role as a counteracting solute in elasmobranch tissues and blood, TMAO is also known to increase in concentration with increasing depth in the tissues of several marine animals, including elasmobranchs, teleosts and several invertebrates (Kelly and Yancey, 1999; Yancey et al., 2014; Downing et al., 2018). The leading hypothesis to explain this trend is that very high hydrostatic pressure can have destabilizing effects on protein structure, and TMAO counteracts this effect and helps maintain protein function (Street et al., 2006; Liao et al., 2017). Hagfishes are a diverse group of animals whose 76 described species (Knapp et al., 2011) inhabit a massive depth range, from the relatively shallow inshore hagfish (*Eptatretus burgeri*), which has been caught at 10 m, to the black hagfish (*Eptatretus deani*), which can live at depths greater than 2700 m (Fernholm, 1998). It would be interesting to see whether hagfishes follow the trend of increasing TMAO levels with depth, in both their tissues and blood, as well as in their slime glands, where levels are already high (Yancey, 2015). The two species for which we have data on organic osmolyte concentration in the slime glands, *E. stoutii* and *M. glutinosa*, have similar depth ranges and therefore are not informative on this issue.

Our analysis of supernatant from Pacific hagfish slime gland exudate revealed an osmolality of 1078 mOsm kg^{-1}, which is consistent with the view that hagfishes are osmoconformers and thus possess serum and tissue osmolalities that are similar to that of seawater (McFarland and Munz, 1958; Bellamy and Jones, 1961). Analysis of the organic osmolyte composition was also generally consistent with the result obtained in Atlantic hagfish by Herr et al. (2010), who first reported the remarkably high concentration of methylamines in Atlantic hagfish slime glands, and also found betaine and TMAO to be the most abundant osmolytes. One of the biggest differences between the osmolyte composition of Atlantic and Pacific hagfishes was for betaine, which had a concentration of 218 mmol l^{-1} in the former and 366 mmol l^{-1} in the latter. Two other substantial differences were in levels of DMG and glycine, both of which were present in substantial amounts in the Atlantic hagfish (67 mmol l^{-1} and 80 mmol l^{-1}, respectively), but were absent or nearly absent in the Pacific hagfish. In spite of these differences, the total concentration of organic osmolytes in the two species differed by only 4% and they are statistically the same, which may suggest that the levels of various osmolytes in the gland are not regulated as tightly as the total osmolarity of organic solutes. Another possibility is that the volume fraction of gland thread cells and gland mucous cells differed substantially between the exudate samples from the two species and, as suggested above, osmolyte composition may be highly heterogeneous in the various fluid fractions that make up the exudate. It is also possible that a large HPLC peak for betaine in the Pacific hagfish samples overwhelmed smaller DMG and glycine peaks, resulting in an overestimate of betaine and proportionate underestimate of DMG and glycine.

Herr et al. (2010) tested the hypothesis that high levels of methylamines in slime glands are involved in the stabilization of mucous vesicles and found that neither TMAO nor betaine is able to inhibit the rupture of slime vesicles *in vitro*, even at high concentrations. Here, we tested an alternative hypothesis – that methylamines act to stabilize the skeins and prevent premature unraveling in the gland. Our results are consistent with this hypothesis. One possible mechanism for this effect is that dilution of methylamines during mixing of slime gland exudate with seawater results in an increase in the solubility of skein glue. This in turn promotes unraveling by allowing the release of stored strain energy in the thread skein (Bernards et al., 2014) and/or reducing

the force required to pull the thread from a coiled skein (Chaudhary et al., 2019).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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