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Comparison of the organic matrix found in intestinal $CaCO_3$ precipitates produced by several marine teleost species

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ABSTRACT

Marine bony fish poses the unique ability to hydrate from imbibed seawater. They accomplish this, in part, by the precipitation of inorganic carbonate mineral in their intestine, which lowers luminal osmotic pressure and allows for water uptake. It has recently been described that in the Gulf toadfish (*Opsanus beta*) this $Ca(Mg)CO_3$ precipitation occurs under the regulation of an organic matrix. To date no investigations have aimed to determine if this phenomenon applies more generally to marine fish. Here, intestinally derived precipitates were collected from gray snapper (*Lutjanus griseus*), white grunt (*Haemulon plumieri*), European flounder (*Platichthys flesus*), as well as Gulf toadfish, and their matrices were extracted. The ability of these matrices to regulate $CaCO_3$ production was determined using an *in vitro* calcification assay, which revealed that the matrix derived from each of the tested species increased precipitation at low concentrations, while inhibiting it at higher concentrations in full agreement with the earlier studies on toadfish. Matrix extracted from European flounder precipitates was then analyzed by mass spectrometry, leading to the identification of over 50 unique proteins. When the identities of these proteins were compared to previous investigation of toadfish precipitate matrix, nearly 35% were found to overlap between the flounder and toadfish analyses, suggesting conserved mechanisms of precipitation control. The effects of using different sodium hypochlorite (NaOCl) solutions during precipitate purification on the resulting organic matrix are also discussed.

1. Introduction

Marine bony fish live in a medium that has an approximately threefold higher salt content then their body fluids (Larsen et al., 2014). Having such a large osmotic gradient between the body fluids and the environment provides a host of challenges, mainly the passive loss of water to the environment and the gain of excess salt. The large respiratory surface required for adequate oxygen extraction exacerbates this problem as the gills, which are well suited for gas exchange, are problematic from an osmoregulatory standpoint as some diffusion of salt and water is unavoidable (Sardella and Brauner, 2007). To maintain proper salt and water balance, marine fish drink seawater and employ solute-coupled water movement to absorb water across the intestine (Grosell, 2013), while excess salt is excreted across the gills via mitochondrial rich cells (Marshall and Grosell, 2006). Solute-coupled water movement in the intestine is completed almost exclusively via the transport of monovalent ions, as the intestinal epithelium is relatively impermeable to divalent ions (mainly Ca^{2+}, Mg^{2+} and SO_4^{2-}). This leads to the concentration of these divalent ions in the intestinal fluid due to the removal of water, and it is the buildup of these ions that eventually inhibits water transport (Genz et al., 2011). To allow for further water absorption, HCO_3^- is transported into the intestinal lumen in exchange for Cl^- via the SLC26a6 anion exchanger, which reacts with free luminal Ca^{2+} (and to a lesser extent Mg^{2+}), to form $Ca(Mg)CO_3$ (Grosell, 2006; Grosell et al., 2009; Kurita et al., 2008; Wilson et al., 2002). This carbonate mineral is eventually excreted into the environment as a waste product, where it plays a substantial role in oceanic inorganic carbon cycling (Wilson et al., 2009; Woosley et al., 2012). Without this intestinal precipitation, which lowers luminal osmotic pressure by up to 100 mOsm/kg (Grosell et al., 2009), marine fish would be unable to take up sufficient water to rehydrate, and would therefore be unable to inhabit marine environments (Grosell, 2013).

Although all marine teleosts are believed to produce carbonate precipitates in their intestine, the morphology and composition of the mineral produced varies greatly both between species, as well as within the same species (Perry et al., 2011; Salter et al., 2012; Salter et al., 2014). Magnesium content is also highly variable, ranging from ~1% to over 50% (Heuer et al., 2016; Perry et al., 2011; Salter et al., 2012).

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Studies have also reported numerous different polymorphs and crystal structures in fish-derived precipitates, suggesting there is much variation in these parameters across taxa as well (Foran et al., 2013; Perry et al., 2011; Salter et al., 2017; Salter et al., 2012; Salter et al., 2014; Walsh et al., 1991). This variation in crystal structure and composition has major implications in regards to the fate of the produced precipitates (whether they dissolve in the water column or accumulate in the sediments), and is described in detail by Salter et al. (2017). Such variability in precipitate composition across species is somewhat surprising as the intestinal fluid chemistry appears to show little variation between species (Grosell et al., 2001; Marshall and Grosell, 2006; Wilson et al., 2002), suggesting that there is some other mechanism that affects carbonate morphology.

Recent studies have found that the carbonate precipitates produced in the marine teleost intestine are not entirely inorganic, but instead contain an at least partially proteinaceous organic matrix (Schauer and Grosell, 2017; Schauer et al., 2016). This matrix can modulate the rate of $CaCO_3$ precipitation *in vitro*, as well as modify the incorporation of magnesium into the mineral (Schauer et al., 2016). Effects of this matrix on crystal morphology have also been reported (Schauer and Grosell, 2017). However, to date, the intestinal precipitate organic matrix has only been studied in a single species, the Gulf toadfish (*Opsanus beta*). To determine if the organic matrix produced by toadfish is representative of those produced by other fish, and how the matrix varies between species, a comparative study looking at the function and composition of precipitate matrix produced by four different species was completed here. Precipitates were collected from three subtropical species including the white grunt (*Haemulon plumieri*), gray snapper (*Lutjanus griseus*), and Gulf toadfish (*Opsanus beta*), as well as one temperate species – the European flounder (*Platichthys flesus*). Organic matrices were then extracted from the precipitates and analyzed using an *in vitro* calcification assay to measure their ability to regulate precipitation. Matrix extracted from the flounder precipitates was also analyzed by mass spectrometry (MS) to identify its protein constituents.

2. Materials and methods

2.1. Experimental animals

Gulf toadfish (25–50 g) were obtained as bycatch from commercial bait shrimp fishermen operating out of Dinner Key, Miami, Florida. Fish were brought to the Rosenstiel School of Marine and Atmospheric Science at the University of Miami, ectoparasite treated, and held in 60 l glass aquaria on flow-through sand filtered seawater originating from Biscayne Bay (32–37 ppt, 21–26 °C). Fish were given a minimum of two weeks to acclimate to the lab prior to the onset of experiments and were fed squid to satiation once weekly unless stated otherwise. Prior to precipitate collection, fish were transferred to six, 30 l aquaria at a density of 6 fish per tank.

Gray snapper (80–220 g) and white grunt (50–120 g) were caught by hook and line angling off Key Biscayne, Florida (license number SAL-16-1448-SR). Fish were kept in a livewell with a continual flow of seawater until brought back to the University of Miami Experimental Hatchery, where they were treated for ectoparasites and transferred to 750 l holding tanks (2–4 fish per tank) on flow through, 1 μm filtered seawater from Biscayne Bay (32–36 ppt, 31–33 °C). Fish were allowed to acclimate for five days prior to the start of experimentation, and were fed a diet of chopped squid to satiation daily unless stated otherwise. All experimental protocols were completed in accordance with and approved by the University of Miami Animal Care and Use Committee (protocol no. 13–225).

European flounder (350–450 g) were caught by trawling in Oresund (56° 2′49″N, 12°37′34″E) (fishing permit 12-7410-000008) and were held in separate 160 L tanks in a 10 °C, flow-through filtered sea water system at the Marine Biological Section, University of Copenhagen, Elsinore, Denmark. The fish were fed three times a week with sliced herring unless stated otherwise. Housing and experimentation was done in accordance to the Animal Experiments Inspectorate's guidelines.

2.2. Precipitate collection and purification

Food was withheld from all fish for a period sufficient to ensure that the gastrointestinal tract was cleared prior to the collection of samples (one week for toadfish and flounder; 48 h for snapper and grunt). Excreted precipitates were collected from the tank bottom by filtering siphoned water through a fine (~500 μm) mesh and frozen at −80 °C until further processing.

Frozen precipitates were thawed, and precipitates originating from the same individual fish (or tank of fish) were combined, rinsed 2× with ultrapure water, and then bleached in a NaOCl solution containing approximately 5% available chlorine at 4 °C overnight. NaOCl solutions were either diluted from a solution containing 14.5% available chlorine (Alfa Aesar), or purchased as a dilute solution certified to have the correct (5%) available chlorine concentration (Ricca Chemical Company). Bleached precipitates were then collected by a 10 min, 3500 g spin and again rinsed 2× with ultrapure water to remove residual bleach. Inorganic mineral was then removed by incubation in a 0.5 M ethylenediaminetetraacetic acid (EDTA) solution containing 1 x HALT protease inhibitor cocktail (Thermo Scientific) for 3 h at 4 °C, leaving behind a solution which contained the organic matrix. EDTA was removed and matrix was transferred into tris buffered saline (TBS; 150 mM NaCl, 10 mM tris, pH 7.5) and concentrated to a volume of 200 μl via ultrafiltration using Amicon spin concentrators (EMD Millipore) with a 3 kDa molecular weight cutoff. Residual EDTA was removed by overnight dialysis (3.5 kDa molecular weight cutoff) into TBS at 4 °C. Protein concentration was determined using the bicinchoninic acid (BCA; Thermo Scientific) method per the manufacturer's instructions. Isolated matrix was immediately analyzed via an in vitro calcification assay (see below), or stored at −80 °C until further analysis.

2.3. In vitro calcification assay

The ability of the isolated matrix to modulate $CaCO_3$ precipitation was tested using a previously described *in vitro* calcification assay (Schauer and Grosell, 2017; Schauer et al., 2016). Briefly, isolated matrix in TBS was added at different concentrations to a 96-well microplate, along with two thymol blue containing solutions (one lacking HCO_3^- and one lacking Ca^{2+}) that when combined, produce a solution that mimics the intestinal fluid chemistry of teleosts. The pH of each well was then monitored via the presence of thymol blue in the solutions by measuring the absorbance at 594 nm for a period of 20 h. All calcification assays were completed at 26 ± 1 °C. The change in pH can be used as a proxy for $CaCO_3$ production, as an H^+ ion is released for every molecule of $CaCO_3$ that forms. From the pH data, two metrics (nucleation time and calcification rate) were calculated for each well. Nucleation time is a measure of the time required for precipitation to begin, where calcification rate is the maximum rate of precipitation throughout the experiment. More detail about how the nucleation time and calcification rate metrics were calculated can be found in Schauer et al. (2016).

Significant ($p < 0.05$) differences in nucleation time and calcification rate were determined via a repeated measures ANOVA for normally distributed data, or the non-parametric Quade test for datasets failing the normality assumption (Quade, 1979). Subsequent multiple comparisons were completed via the Holm-Sidak method for normally distributed data, or post-hoc Quade multiple comparisons using the PMCMR package for R (version 3.3.1) using a Benjamini-Hochberg multiple testing correction.

2.4. Bleach titration

Available chlorine concentrations in the NaOCl solutions used in the purification of the precipitates were determined via titration under acidic conditions with sodium thiosulfate ($Na_2S_2O_3$) in the presence of iodine. A 0.2 N sodium thiosulfate solution was first standardized against a solution of 0.1 N potassium dichromate in the presence of sulfuric acid, potassium iodide, and starch. This standardized solution was then used to titrate 4–7 g of the NaOCl solutions that were acidified with glacial acetic acid. The titration endpoint was visualized by the addition of KI and water-soluble starch.

2.5. Mass spectrometry analysis

All mass spectrometry analysis was completed at the Colorado State University Proteomics and Metabolomics Facility (Fort Collins, Colorado, USA). Organic matrix isolated from the European flounder intestinal precipitates was analyzed as described elsewhere (Schauer and Grosell, 2017). Briefly, 10 μg of isolated protein was trypsin digested and ~2 μg of digested peptides were loaded onto an online, reverse-phase C18 column and eluted via a 30-min linear gradient of increasing acetonitrile directly into an Orbitrap Velos Pro MS (Thermo Scientific) operating in data-dependent acquisition mode. Raw MS data was searched using Mascot (version 3.3; Matrix Science) against a database that included all nucleotide sequences (including expressed sequence tags [EST]) available for *P. flesus* on the NCBI database (downloaded April 15, 2017) that were translated into all six possible reading frames, as well as the list of common contaminating proteins from the common Repository of Adventitious Proteins (cRAP; http://www.thegpm.org/crap/). The final database consisted of ~120 k sequences and was reverse concatenated to allow for false discovery rate (FDR) calculation. Search data was loaded into Scaffold (version 4.7.3; Proteome Software) for data filtering and visualization. Maximum 1.0% protein and peptide FDR limits were imposed, and a minimum of two unique peptides was required for protein identification. Manual BLAST searching against the NCBI non-redundant database was used to determine likely identifications and functions. Isoelectric points of identified proteins were calculated using the isoelectric point calculator web service (Kozlowski, 2016).

3. Results

3.1. Calcification assays

Isolated matrix samples collected from all four species contained similar amounts of protein (data not shown), suggesting that all species contain an organic matrix. Analysis of the isolated matrices revealed a significant effect of matrix concentration on both nucleation time and calcification rate for all species (Fig. 1), with the exception of nucleation time in European flounder, which showed a strong trend ($p = 0.06$). No precipitation was observed throughout the entirety of the 20 h assay when high concentrations (0.025–0.25 μg/ml depending on the species) of matrix were present. The exact point at which precipitation was completely inhibited varied somewhat between replicates. Only a single sample from the gray snapper showed precipitation at concentrations $>2.5 \times 10^{-3}$ μg/ml, and only one sample from flounder showed precipitation at a concentration of 5×10^{-3} μg/ml. Additionally, one toadfish matrix sample completely inhibited calcification at 5×10^{-2} μg/ml, where the others only showed complete inhibition at concentrations ≥ 0.25 μg/ml.

The overall trends observed in both nucleation time and calcification rate were similar between the species examined (Fig. 2). Not surprisingly, at the lowest concentrations tested, both nucleation time and calcification rate were similar to the no protein controls. At moderate concentrations, the nucleation time tended to decrease, indicating a decrease in the time required for precipitation to begin. This trend reversed at higher concentrations, eventually leading to the complete cessation of precipitation during the 20 h experiment. Calcification rates showed the reverse of these trends, consistent with overall increases or decreases in $CaCO_3$ production, as low nucleation times and high calcification rates lead to increased precipitation, and vice-versa. Calcification rate for the white grunt and gray snapper showed an increase at moderate matrix concentrations, and a decrease at high concentrations. Interestingly, the increase in calcification rate seemed absent (or at least reduced) in the European flounder, but a bimodal effect was observed with the toadfish matrix. Also of note was an apparent discrepancy in the necessary concentration of matrix required to produce the aforementioned effects. In the flounder, snapper and grunt, similar concentrations of matrix elicited similar responses. However, in the toadfish, concentrations approximately one order of magnitude higher than the other species were required to see a comparable effect.

The observed effects from the toadfish matrix appeared less pronounced and occurred at substantially lower concentrations than previous investigations have reported (Schauer and Grosell, 2017; Schauer et al., 2016). Additionally, unlike in other investigations, mass spectrometry analysis of the matrix extracted from precipitates purified here proved unsuccessful for all of the species tested, with few or no proteins identified in the samples, and almost nothing being eluted from the reverse phase column during the expected portions of the acetonitrile gradient (data not shown). We therefore sought to determine what could be the source of these discrepancies.

3.2. Comparison of NaOCl solutions

For the aforementioned investigations, a NaOCl solution from a different supplier (Ricca Chemical Company; catalog number 7495.5) was used than for all previous investigations on toadfish precipitate matrix (Schauer and Grosell, 2017; Schauer et al., 2016). This new bleach solution, which contained 5% available chlorine, from Ricca Chemical was used directly as supplied, where previous investigations have diluted a stock solution from Alfa Aesar (catalog number 33369) which contained 14.5% available chlorine to approximately 5% available chlorine. All other materials and methods used here were identical to those employed in the previous investigations. It was therefore hypothesized that the NaOCl solutions may have been responsible for the observed changes in the calcification assay and mass spectrometry analyses.

To address this idea, a sample of precipitates collected from toadfish was spilt, and half were purified using the NaOCl from Ricca Chemical, and half using the diluted Alfa Aesar stock. Notably, precipitates purified using the Ricca Chemical bleach solution appeared lighter in color than those treated with the Alfa Aesar solution. Additionally, aggregates (1–2 mm) of smaller mineral crystals that were collected from the tank bottom could still be observed in the Alfa Aesar bleach treated samples, whereas mineral treated with the Ricca Chemical bleach solution were reduced almost entirely to a fine powder. The resulting matrix was analyzed using the *in vitro* calcification assay, and compared to previously reported results (Fig. S1). Matrix extracted from the precipitates purified using the Ricca Chemical bleach closely mimicked the results from the toadfish analysis completed for the previously discussed species comparison, where the Alfa Aesar purified precipitate matrix was more reminiscent of previously reported results (Schauer et al., 2016). However, inhibition of precipitation (increased nucleation time, decreased calcification rate) compared to the no protein controls was not observed in the Alfa Aesar purified matrix at the concentrations tested, but a decrease in promotion was observed at 5 μg/ml than at lower concentrations. Unfortunately, there was not sufficient sample to test higher concentrations to determine if precipitation could be inhibited with this sample.

To investigate potential differences between the different NaOCl solution, free available chlorine concentrations were determined via titration with $Na_2S_2O_3$. The available chlorine concentration was

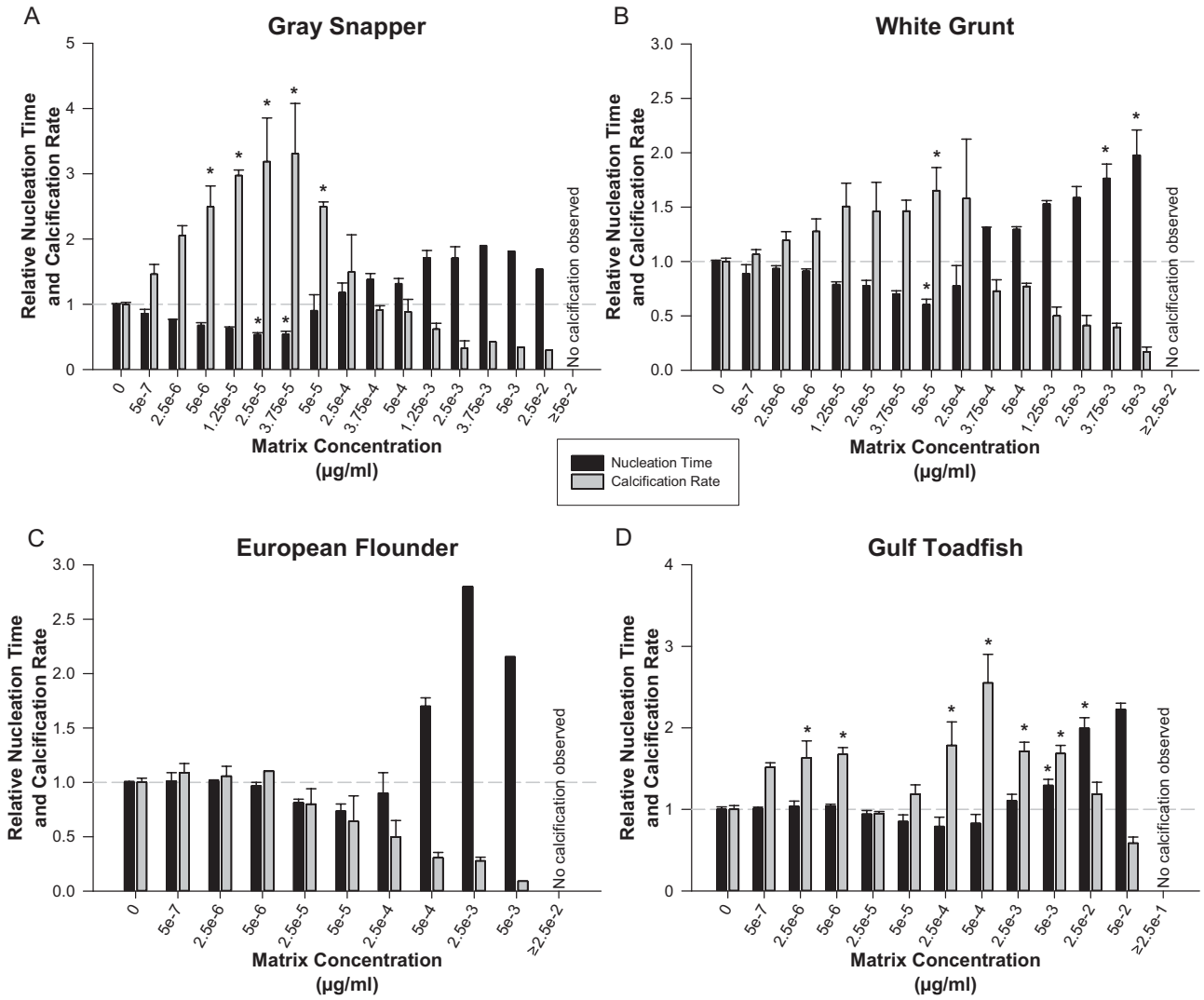

Fig. 1. Results from the *in vitro* calcification assay showing the effects of varying concentration of isolated precipitate organic matrix derived from four different species. Data from gray snapper (A), white grunt (B), European flounder (C), and Gulf toadfish (D) are shown. All precipitates were purified using the Ricca Chemical NaOCl solution. Nucleation time (black bars) and calcification rate (gray bars) reveal the effect of matrix on the time required for the start of precipitation and the max rate of precipitation, respectively. No substantial precipitation was observed throughout the 20 h assay for the highest concentrations of matrix, regardless of the species. Asterisks indicate values that significantly ($p < 0.05$) differ from the no protein control as determined via a Quade multiple comparison test. n = 3 biological replicates for gray snapper and white grunt, n = 2 for European flounder, and n = 6 for Gulf toadfish. Values are represented as mean ± SEM.

slightly higher in the Ricca bleach (4.856 ± 0.067% w/w; mean ± SD) than the diluted Alfa Aesar bleach (4.377 ± 0.155% w/w; Fig. S2A). To determine if this small change in available chlorine was sufficient to affect the precipitate purification, precipitates were collected from more Gulf toadfish, split into five individual samples, and then purified using five different dilutions of Alfa Aesar NaOCl, ranging from ~4 to 5% available chlorine (Fig. S2B). All other purification steps were identical. The matrix was then extracted and analyzed using the *in vitro* calcification assay. No obvious differences in nucleation time or calcification rate were observed between any of the matrices using the different bleach dilutions (Fig. S3).

3.3. Reanalysis of flounder matrix

Unfortunately, additional samples from the gray snapper and white grunt were not available, so the analysis of their matrix using the Alfa Aesar NaOCl was not possible. However, additional precipitates from two European flounder were obtained and were therefore purified using the Alfa Aesar bleach solution. A calcification assay was completed on matrix isolated from these samples as well (Fig. 3). This matrix significantly decreased nucleation time at moderate concentration ranges (5×10^{-3} µg/ml–0.25 µg/ml), but no significant changes in calcification rate were observed. The calcification rate data was somewhat variable, but an increasing trend in calcification rate at similar concentrations to where nucleation times were decreased was observed. Overall, the effects on $CaCO_3$ production of the flounder matrix from the precipitates purified using the two bleach solutions were similar. However, lower concentrations of the matrix purified from the Ricca Chemical precipitates was required to see effects similar to those observed from the Alfa Aesar purified samples, and the magnitude of those effects were lower. This corresponded well with what was observed in the comparison of toadfish precipitates purified using the two different NaOCl solutions (Fig. S1).

Additionally, mass spectrometry analysis was completed on these flounder matrix samples that were purified using the Alfa Aesar NaOCl solution, and 58 unique proteins from 70 unique loci (excluding contaminants from the cRAP database) were identified between the two replicates (Table S1). Intriguingly, six of these proteins (10%) contained known calcium binding domains (Table S1). When the identities of these proteins were compared to previously published investigations of

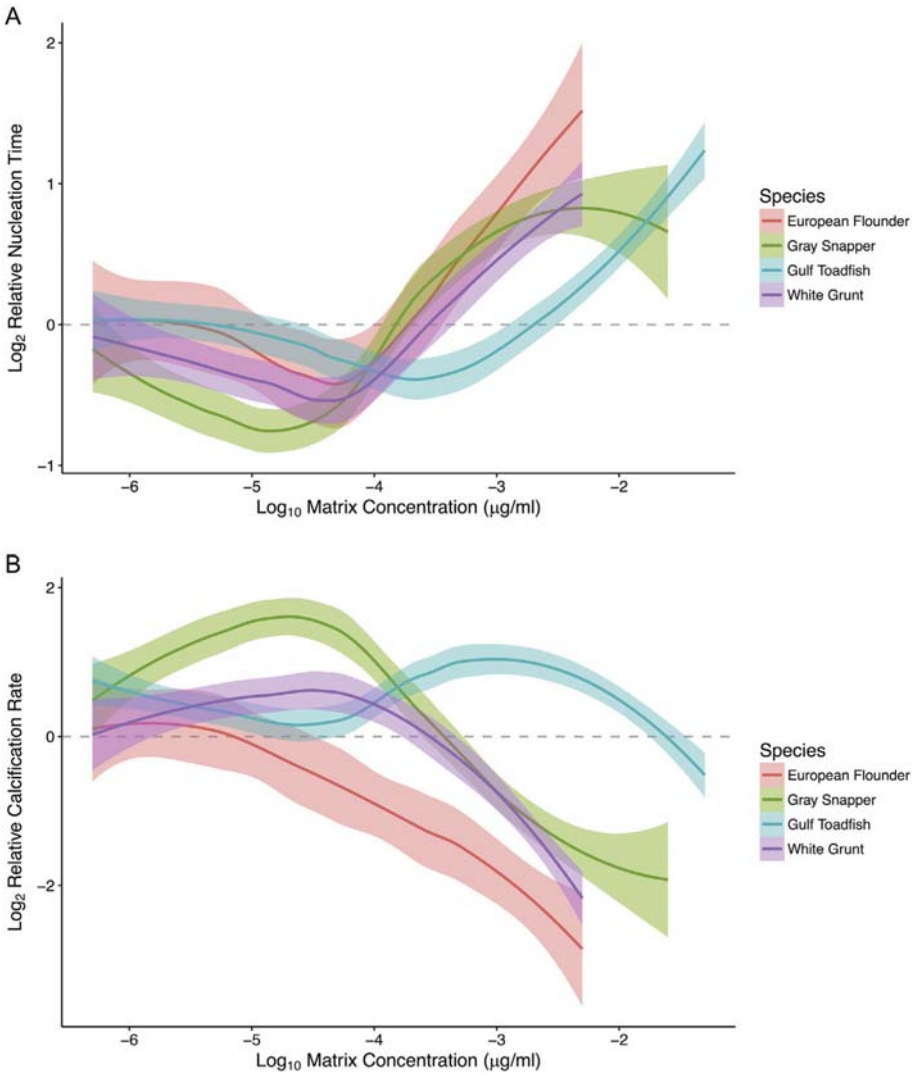

Fig. 2. Comparison of general trends in nucleation time and calcification rate between species. Curves fitted to the raw data and smoothed via the loess method are shown for both nucleation time (A) and calcification rate (B) as well as their 95% confidence intervals. Lines stop at the highest concentration at which calcification was observed throughout the 20 h experiment. Precipitate matrix used for this experiment was extracted from precipitates purified using the Ricca Chemical NaOCl solution.

toadfish matrix (Schauer and Grosell, 2017; Schauer et al., 2016), a substantial overlap was observed (Fig. 4). Most notably, eight proteins were common to all four datasets, including the flounder analysis. A list of all 221 proteins identified between the datasets illustrated in Fig. 4 can be found in Table S2.

4. Discussion

Results presented here illustrate that the presence of an at least partially proteinaceous matrix associated with intestinal $CaCO_3$ precipitates is not confined solely to Gulf toadfish. The presence of such a matrix in two additional tropical species (gray snapper and white grunt), as well as a temperate species (European flounder), suggests that the matrix is found across different temperature regimes, and different teleost taxa. However, it should be noted that previous investigations of the intestinal precipitates produced by these species has shown that they all produce high magnesium calcite, with ellipsoid morphology (Salter et al., 2012; Woosley et al., 2012). As the composition and morphology of the intestinally produced precipitates can vary significantly among species, it is possible that the matrix differs in species with dramatically different mineral compositions or morphologies. This would be an interesting point of consideration for future investigations.

In the species studies here, the matrix seems to serve similar functions by increasing $CaCO_3$ precipitation at low concentration, and inhibiting mineral formation at higher concentrations. The benefits of such a concentration dependent precipitation regulatory mechanism are intriguing, as has been discussed previously (Schauer et al., 2016). In theory, such regulatory activity could tightly control precipitation without the need for changes in gene transcription, protein translation, or protein modification. Assuming a constant amount of matrix protein is present in the intestinal lumen, when a fish drinks, the regulatory matrix proteins in the lumen would be diluted due to the influx of water causing an increase in precipitation and allowing for additional water absorption across the intestinal epithelium. Once much of the water has been absorbed, the proteins would become more concentrated, preventing further precipitation, which could otherwise lead to an intestinal blockage. Further investigations looking at the absolute quantitation of matrix proteins in the intestinal fluid could help to determine the feasibility of this proposed model.

Similar to toadfish matrix, the average isoelectric point (pI) of the 58 proteins identified in the European flounder matrix was only slightly acidic (6.6) (Schauer and Grosell, 2017). This is somewhat surprising as the presence of highly acidic proteins in the organic matrix is a hallmark of many biomineralization systems (Alvares, 2014; Marin and Luquet, 2007). However, it is important to note that the isoelectric

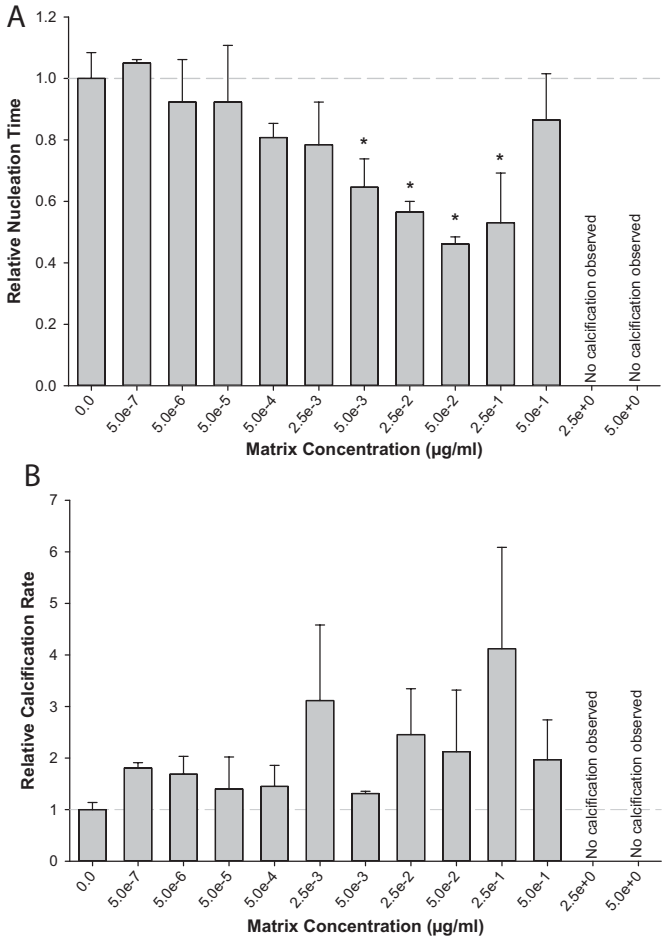

Fig. 3. Effects of the organic matrix isolated from European flounder precipitates purified with the Alfa Aesar NaOCl solution on *in vitro* CaCO3 production. Nucleation times (A) and calcification rates (B) relative to the no proteins controls are shown. Asterisks indicate values that significantly ($p < 0.05$) differ from no protein controls as determined by repeated measure ANOVA, and subsequent Holm-Sidak multiple comparisons.

points for each protein were calculated directly from the sequences in the database, and since the flounder and toadfish sequences were derived from a poorly curated nucleotide database, there were likely portions of the sequences that represent untranslated regions of the gene. Therefore, the calculated isoelectric points might not be indicative of the acidity of the native protein. This is further supported by the fact that the average pI of the peptides identified in the MS analysis of flounder matrix (~5.5) was lower than that of the whole proteins. Notably, the median pI for the identified peptides is quite acidic, at 4.4, suggesting that the mean value is influenced by a relatively small number of basic peptides. This is also of interest as basic proteins are known to be involved in biomineralization, although such proteins are less common than their acidic counterparts (Arivalagan et al., 2017).

Of the 58 proteins identified in the flounder matrix, 20 (34%) have previously been identified in the toadfish matrix (Fig. 4 and Table S2), suggesting that the protein constituents of the organic matrix are at least somewhat conserved across species. The proteins found in both the toadfish and flounder organic matrix are of particular interest, as their conservation across species suggests these proteins may play a role in the regulation of precipitation. Most intriguing are the eight proteins that were found in all studies of organic matrix completed to date (Fig. 4 inset). Of these eight proteins, seven (all but prothymosin alpha) have been identified in proteomic investigations of fish skin mucus (Cordero et al., 2015; Cordero et al., 2016; Jurado et al., 2015; Pérez-Sánchez et al., 2017; Rajan et al., 2013). *In vivo*, the intestinal carbonate precipitates are covered in a thick layer of mucus (Humbert et al., 1986; Schauer et al., 2016; Walsh et al., 1991), so it is possible that these proteins are therefore only present in association with the precipitates due to this mucus coating. However, should these proteins be purely contaminants, it would be expected that those proteins that are most abundant in the mucus (such as mucins) would be those that are most likely to end up in the precipitates, which was not the case. Further, the presence of these proteins in the mucus does not eliminate the possibility that they are involved in regulating precipitation. Indeed, it has been previously suggested that even abundant mucus proteins such as mucin-2 may be involved in inhibiting precipitation in toadfish (Schauer and Grosell, 2017), and other mucin-like proteins have been implicated in mollusk biomineralization (Marin et al., 2000). Therefore, the presence of these proteins in mucus should not lead to their exclusion from further consideration.

When taken together, the presence of calmodulin in all the proteomic investigations of teleost intestinal precipitate matrix completed to date, as well as this protein's well characterized ability to bind calcium, it seems likely that this protein is involved in regulating precipitation *in vivo*. Calmodulin, and a very similar protein referred to as calmodulin-like protein (CaLP), are both expected to play a role in mollusk biomineralization (Fang et al., 2008; Li et al., 2016; Yan et al., 2007). Additionally, the presence of calmodulin inhibitors was shown to reduce otolith growth (a $CaCO_3$ mineral produced in teleosts) in isolated rainbow trout sacculi (Mugiya, 1986). Most of the previous investigations of the role of calmodulin in biomineralization have looked at the involvement of this protein in the matrix or surrounding tissues, or has investigated the role of the protein in modulating the morphology of the resulting mineral when calmodulin is present in the precipitation medium. The direct effect of calmodulin on precipitation rates does not appear to have been studied to date, therefore it is difficult to know what role this protein may play in the teleost intestine. Future investigations analyzing the regulatory activity of purified calmodulin could address this question.

From all the evidence collected thus far on the regulation of teleost intestinal precipitation by an organic matrix, the protein that seems most likely to be playing a substantial role is prothymosin alpha. This protein was one of the eight proteins that was identified in all the matrix characterization investigations compared in Fig. 4, and is not known to be found in mucus, nor to be highly abundant in the intestinal fluid (Schauer et al., 2016), suggesting that it is enriched in the organic matrix by some other means. Further, this protein was shown to be upregulated in both the intestinal epithelium as well as the intestinal fluid (with the greatest increase in the fluid) during hypersalinity exposure, a condition known to increase intestinal $CaCO_3$ production (unpublished data). Prothymosin alpha is small (~13 kDa), highly acidic (pI = 3.6), and protein structure predictors suggest it is likely disordered (Yachdav et al., 2014). All these attributes are hallmarks of biomineralization related proteins (Marin and Luquet, 2007; Wojtas et al., 2012). The function of this protein remains poorly characterized, but it has been implicated in cell cycle control, cell survival, immune response, and embryonic development (Donizetti et al., 2008; Ioannou et al., 2012). The localization of the protein in humans varies by tissue, but is most commonly identified in the nucleus or cytoplasm (Kijogi et al., 2016). However, instances of extracellular secretion have been documented, albeit via a non-traditional, vesicle independent fashion (Matsunaga and Ueda, 2010). Notably, in teleosts there are two distinct isoforms of prothymosin alpha (ptmaa and ptmab), which likely arose from a gene duplication event early in the teleost lineage after their separation from tetrapods (Donizetti et al., 2008). The two genes differ in expression both temporally and spatially during zebrafish embryonic development (Donizetti et al., 2008), but further investigation of the two isoforms has yet to be completed. The protein identified in the toadfish and flounder precipitates appears to be derived from the ptmab gene, although the poor characterization of this gene in fish species makes it difficult to determine for certain. Taken together, it seems highly likely that this protein is involved in the regulation of carbonate

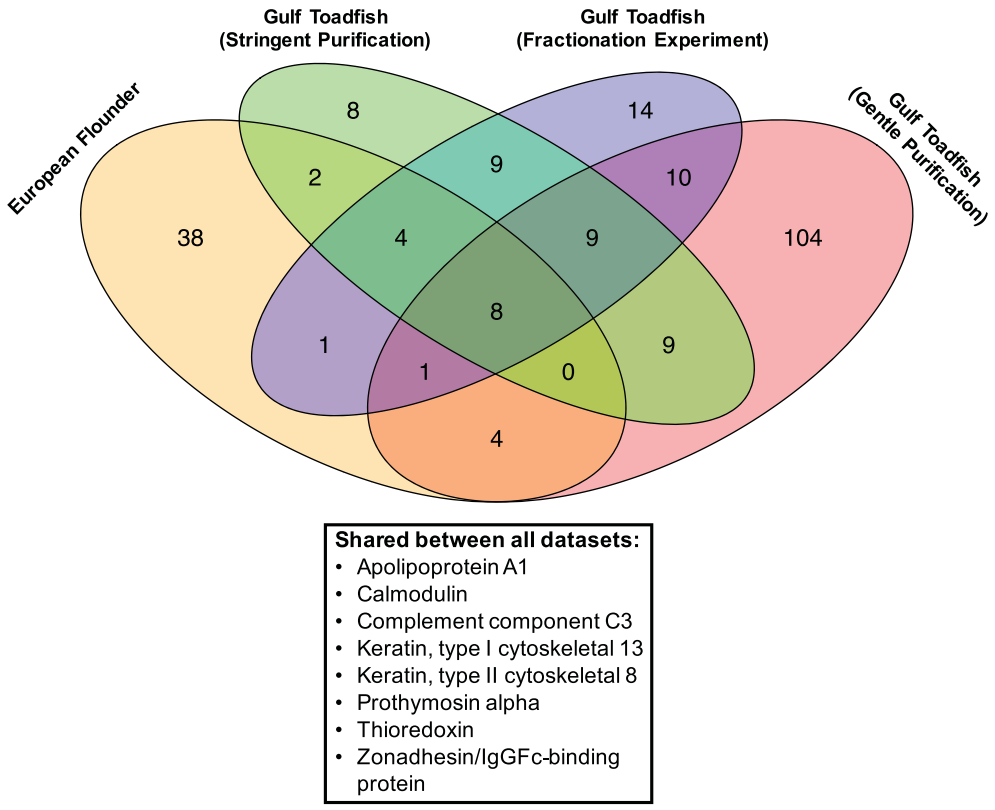

Fig. 4. Comparison of proteins identified in European flounder precipitate matrix and previous investigations of Gulf toadfish matrix. Likely protein identities were determined via BLAST searching against the NCBI non-redundant database and were manually compared between datasets. Inset shows proteins that are shared between all datasets. Toadfish data is derived from Schauer et al. (2016) and Schauer and Grosell (2017).

mineral production, and future investigations of this protein are certainly warranted.

Although prothymosin alpha and calmodulin are the most likely candidates to be involved in the regulation of intestinal precipitation, it is likely that a number of other proteins are involved in the process as well. Other potential candidates identified here include zonadhesin and calreticulin. Zonadhesin is a known protein in the 'biomineralization toolkit' and is believed to play a role in the binding coral calicoblastic cells to the forming skeleton, and may also bind coral acid-rich proteins (CARPs) that are involved in regulating skeleton production (Bhattacharya et al., 2016; Mass et al., 2016). Although the function this protein may play in teleost intestinal precipitation is unclear, it was one of the proteins shared between all the studies summarized in Fig. 4. Calreticulin is another calcium binding protein that has been implicated in calcium transport and biomineralization in corals and pearl oysters (Allemand et al., 2011; Shi et al., 2013), and is also found in the matrix vesicles responsible for the production of calcium phosphate for bone formation (Balcerzak et al., 2008; Cui et al., 2016). This protein was found in the flounder matrix as well as the toadfish matrix extracted from the more gently purified intestinal precipitates (Table S2), suggesting it too may be involved in the regulation of intestinal precipitation in fish. Further, this protein was found to be more abundant in the intestinal fluid of hypersalinity acclimated toadfish compared to those is seawater (unpublished data), providing further evidence in may be involved in regulating intestinal precipitation *in vivo*.

Unfortunately, differences in available chlorine concentrations failed to explain why the use of the two different NaOCl solutions used for the precipitate purifications had such drastic effects on the activity of the resulting matrix (Fig. S1). It is difficult to pinpoint what could be the source of the observed differences, as both solutions are marketed as containing only water and NaOCl, but it seems likely that at least one of the solutions was contaminated in some way. Regardless, the integrity of the matrix was clearly affected differently by the two solutions used. It seems likely that the Ricca Chemical solution was more potent than the Alfa Aesar, as evidenced by the lighter color of the resulting precipitates, as well as the inability to analyze the resulting matrix by MS, likely due to severe degradation of the proteins. Such degradation could also explain why only very low concentrations of matrix were necessary to affect the precipitation reaction. Extremely stringent purification would likely leave only the proteins most embedded in the $CaCO_3$ intact, removing any other contaminants. Assuming the proteins embedded deep in the precipitates are those primarily responsible for controlling the early stages of precipitation, the enrichment of these proteins compared to contaminants would mean that less total protein would be required to observe the same effects than a solution containing more proteins not involved in the biomineralization process. Alternatively, the differences in ability to modulate precipitation could be due directly to the degradation of the proteins themselves. Previous investigations of other biomineralization systems, as well as *in vitro* mineralization, have shown that small peptides can be strong regulators of $CaCO_3$ precipitation (Elhadj et al., 2006; Gunthorpe et al., 1990; Kim et al., 2006). It is therefore possible that the peptides derived from degradation of whole proteins were actually more potent regulators of calcification than the intact proteins. Future investigations could address these questions by assessing the precipitation regulatory ability of whole intestinal precipitate matrix, versus that which has been digested with a protease such as trypsin or pepsin.

In conclusion, the work completed here demonstrates that the presence of proteins in association with intestinally produced carbonate mineral precipitates is not unique to Gulf toadfish, but is also found in white grunt, gray snapper, and European flounder. The organic matrix across these species has similar effects on *in vitro* $CaCO_3$ production, with low concentrations promoting precipitation, and higher concentrations having an inhibitory effect. Mass spectrometry analysis of European flounder organic matrix was also completed, resulting in the identification of over 50 proteins in the matrix of precipitates produced by this species. The results from all previous mass spectrometry investigations of intestinal precipitate matrix are compared to the data collected here, providing further evidence as to which proteins may be

ultimately responsible for regulating $CaCO_3$ precipitation *in vivo*. Finally, a substantial influence on matrix calcification regulatory activity due to the NaOCl solution used during the purification of the precipitates is described, and it is shown that the effect is not due to available chlorine concentrations.

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