IDENTIFICATION AND REMOVAL OF ADHESIVE PROTEINS FROM GOLDFISH AND BAITFISH EGGS AND EGG MASSES

Reporting Period
January 1, 2014 – November 1, 2014

Funding Level 12 Months……………………………………………………..$45,000

Participants
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University of Arkansas at Pine Bluff..................Anita Kelly

PROJECT OBJECTIVES

Objective 1. Determine the protein composition of the egg matrix of goldfish and ballyhoo.

Objective 2. Identify the compounds that will de-stick goldfish and ballyhoo eggs from spawning substrate.

ANTICIPATED BENEFITS

This project is a continuation of SRAC funded research directed at identification of proteins associated with egg adhesion. Previous work from this collaborative team was unsuccessful in protein identification and it was believed that difficulties in protein extraction could be accomplished within a brief period of time. Once the glycoprotein compounds in the chorions have been identified, goldfish and ballyhoo eggs adhered to substrate would then be exposed to various compounds to assess if the eggs are removed from the substrate and still remain viable. Practical application of research results from this effort will directly help hatcheries in removing eggs from spawning substrate for more efficient egg incubation. It is our expectation that these results could be applied to other fish species.

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS

Objective 1. *Determine the protein composition of the egg matrix of goldfish and ballyhoo.*

Identification of contamination from gels

All progress within this reporting period and prior to the formal initiation of this project has been completed with a consistent supply of fertilized eggs of both ballyhoo and koi from collaborators at the University of Florida and University of Arkansas at Pine Bluff. In October of 2013 egg shell proteins extracted from Gulf killifish using a 3M urea buffer were different in molecular weight when compared to samples from the previous year. Gulf killifish eggs were used as an experimental substitute since koi and ballyhoo eggs would be available from collaborators in 2014. Figure 1A shows lane 1 as a molecular marker, lane 2 as the proteins from a 3M urea extraction, and lane 3 as proteins extracted from the previous year. When stained for glycoproteins the new samples did not indicate that any of the extracted proteins were

glycoproteins (Figure 1B, lane 2) in comparison to previously extracted samples (Figure 1B, lane 3). To proceed, we further investigated the potential that the previous or current protein samples were contaminated with remnants of yolk proteins or embryos as seen in previous mass spectroscopy results.

Protein samples from eggs undergoing the new urea buffer extraction process and the respective washes from this protocol were placed on gels to examine if these bands were present from intact egg shells or washes associated with removal of embryo material (i.e. yolk and cytoplasmic contaminates). Figure 2 shows the bands observed in the original wash and subsequent washes (wash 1, 2,3) diminishing in concentration leading the LSU AgCenter's Biotechnology Laboratory to determine that the bands displayed using the previous protocols (Figure 1) were not associated with the egg chorion.

<u>Mass spec results of initial bands</u>

Dr Jeonghoon Lee from the LSU's Chemistry Department completed Mass Spectroscopy performed analysis of two bands within the 3M urea buffer extractions in November of 2013. Using a mascot score histogram, a protein score was generated for identification (Figure 3). A protein score was calculated using the formula -10*Lop(P), where P is the probability that the observed in a random event. Both bands produced scores greater than 70 indicating significance ($p<0.05$) in the identification. The larger of the two bands was identified at vitellogenin-1 from the Gulf killifish's sister species the Mummichog (*Fundulus heteroclitus*). The second band was identified as ribose-5-phosphate isomerase A, which is an enzyme responsible for the conversion of ribose-5-phosphate and ribulose-5-phosphate which is believed to play a large role in carbohydrate anabolism and catabolism (Zhang et al. 2003). These two products are not believed to represent proteins associated with the eggshell.

<u>New protocols developed</u>

In the Spring of 2014 we investigated two new protocols for the solubilization of eggshell proteins using egg samples from our two target species. The two protocols were described in Oppen-bernsten et al. (1990) for Cod (*Gadus morhua*) and Chiou et al. (2004) for Malabar grouper (*Epinephelus malabaricus*). Consultation with the LSU AgCenter's Biotechnology Laboratory on these protocols yielded a protocol similar to Choiu et al. (2004). Eggs are initially cleaned in chilled 0.2 M Phosphate Buffer and centrifuged at 700g for 10 minutes at 4°C. This is repeated 15 times until no cytoplasmic protein is detected within washes. Eggs are then homogenized in TNE buffer (pH 7.2) with 0.1% triton for 15 minutes. This homogenate is then centrifuged at 1000g for 10 minutes at 4°C. The supernatant is removed and chorion fraction is then washed in TNE buffer (by centrifugation) 15 times OR until supernatant did not show cytoplasmic protein. The chorion fraction is then homogenized in S-TNE buffer containing 8 M urea. After centrifugation of this homogenate at 20,000 g for 20 minutes at 4°C the supernatant is collected for protein analysis.

Gels produced from new protocol

Products were identified in gels representing both koi and ballyhoo samples using a more sensitive silver staining as compared to coomassie staining. Figure 4 represents silver stained gels within these species while coomassie stained gels (not shown) did not display visible bands. Although the concentration of these products were low, silver stained bands were used as a marker location for isolation from other replicate gels. Unfortunately, the Mass Spec lab at the LSU Department of Chemistry was not able to process samples in July and August due to maintenance analyzed these samples in September. Samples were processed from these gels in September, however, concentrations were too low for reliable results. Increased numbers of eggs from both species have been received from collaborators and extractions have been carried out in October in an effort to increase protein concentration for mass spectroscopy analysis. Concentrations on Koi chorion proteins were completed and demonstrate 5 potential protein bands, of which 3-4 appear to be sufficient in concentration for Mass Spectroscopy (Figure 5). This analysis will be completed within November.

Objective 2. *Identify the compounds that will de-stick goldfish and ballyhoo eggs from spawning substrate.*

Definitive work on objective 2 within this project has not been completed due to unfulfilled accomplishments in the first objective.

IMPACTS

This project has tested the ability for 20 different compounds to remove koi eggs from spawning mats. These compounds include various concentrations of the following compounds: sodium sulfite, tannic acid, urea, fresh squeezed pineapple juice, bromelain, papaya, papain 4M urea, lithium chloride, cadavarine, lysozyme, acetone, sodium hydroxide, ammonium chloride, ethylene glycol mono-butyl ether, propolyene glycol n-butyl ether, citric acid, and alcalase. Sodium sulfite and trypsin at varying concentrations have been also applied to ballyhoo egg in an effort to remove adhesion with no success.

Previously published characterization of egg shell proteins (Mansour et al 2009) appear to report a protocol that has yielded a high degree of contamination from the contents of the egg itself. As a result, new protocols have been developed for the processing and solubilization of eggshell proteins for Mass Spectroscopy analysis.

PUBLICATIONS AND PRESENTATIONS

None as of November 1st, 2014

REFERENCES

Chiou L, Chung M, Tung P, Hsu T, Yang J. (2004) The use of egg chorion glycolprotein of *Epinephelus malabaricus* for egg identification. Journal of Fish Biology 65: 1614-1621.

Mansour, N., F. Lahnsteiner, and R. A. Patzner. 2009. Physiological and biochemical investigations on egg stickiness in common carp. Animal Reproduction Science 114:256-268.

Oppen-Bernsten D, Helvik J, Walther B. (1990) The major structural proteins of cod eggshells and protein crosslinking during teleost egg hardening. Developmental Biology 137: 258-265.

Zhang R., Andersson C, Savchenko A, Skarina T, Evdokimova E, Beasley S, Arrowsmith C, Edwards A, Joachimiak A, Mowbray S (2003) Structure of Escherichia coli Ribose-5-Phosphate Isomerase: A ubiquitous enzyme of the pentose phosphate pathway and the Calvin Cycle. Structure 11: 31-42.

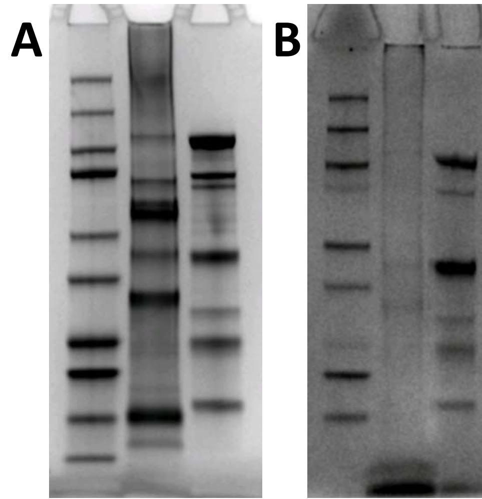


Figure 1. SDS-PAGE with lane 1 as a molecular marker, lane 2 as the proteins from a 3M urea extraction, and lane 3 as proteins extracted from the previous year (Figure 3A). Glycoproteins stained in the new samples did not indicate that any of the extracted proteins where glycoproteins (Figure 3B, lane 2) in comparison to previously extracted samples (Figure 3B, lane 3).

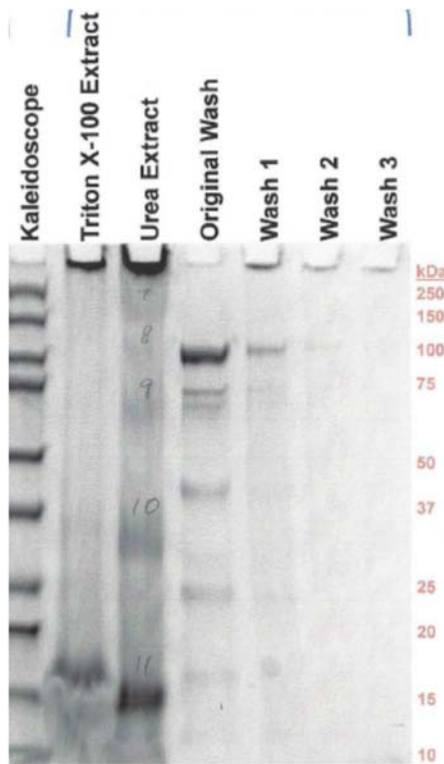


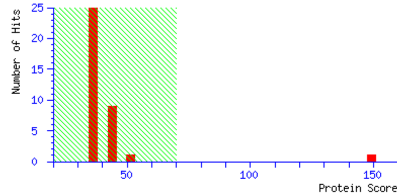
Figure 2. SDS-PAGE Gel with various washes and discard products from the protocol used to produce extracted chorions displayed in figure 1. Original and subsequent washes demonstrate similar protein bands when compared figure 1 indicating possible contamination with this protocol.

MATRIX SCIENCE **Mascot Search Results**

```
User            : JLee
Email           : jeonlee@lsu.edu
Search title    : Glyco-1
Database        : SwissProt 2013_10 (541561 sequences; 192480382 residues)
Timestamp       : 29 Oct 2013 at 20:04:54 GMT
Top Score       : 149 for VIT1_FUNHE, Vitellogenin-1 OS=Fundulus heteroclitus GN=vtg1 PE=1 SV=2
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 | Max. number of hits AUTO

Preferred taxonomy All entries

Re-Search All | Search Unmatched

```
1.    VIT1_FUNHE     Mass: 189402    Score: 149     Expect: 6.8e-10   Matches: 24
      Vitellogenin-1 OS=Fundulus heteroclitus GN=vtg1 PE=1 SV=2
      PGM2_PONAB     Mass: 68741     Score: 41      Expect: 45   Matches: 8
      Phosphoglucomutase-2 OS=Pongo abelii GN=PGM2 PE=2 SV=3
      UPP_PSEA7     Mass: 22909     Score: 37     Expect: 1e+02   Matches: 5
      Uracil phosphoribosyltransferase OS=Pseudomonas aeruginosa (strain PA7) GN=upp PE=3 SV=1
      DUT_ANTLO     Mass: 15687     Score: 37     Expect: 1.2e+02   Matches: 4
      Deoxyuridine 5'-triphosphate nucleotidohydrolase OS=Antonospora locustae GN=DUT1 PE=3 SV=1
```

Figure 3. Mass Spectroscopy results for bands excised from the gels in figure 2. Top protein candidates are listed with a Mascot score >70.

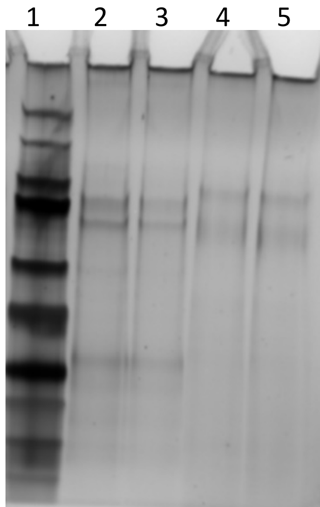


Figure 4. SDS-Page of silver stained egg proteins from koi (lanes 2,3) and ballyhoo (lanes 4,5). Lane 1 represents a protein standard.

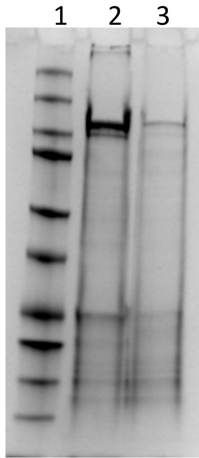


Figure 5. SDS-Page of coomassie stained egg proteins from
koi (lanes 2,3) indicating a concentration from previous samples.
Lane 1 represents a protein standard.