2014

Enzymatic Activity in the Chorion for Hatching in the California Grunion

Emily R. Pierce
Pepperdine University

Vince V. Quach
Pepperdine University

Karen L. Martin
Pepperdine University

Enzymatic Activity in the Chorion for Hatching in the California Grunion

Emily R. Pierce, Vince V. Quach, Dr. Karen L. Martin
Pepperdine University, 90263

Summer Undergraduate Research in Biology
Pepperdine University

PEPPERDINE UNIVERSITY

NSF

Abstract

Leuresthes tenuis is a small, silverside fish that spawns on the beaches during some of the highest tides of the summer months. Of the many unique traits to the species including that the eggs develop fully out of water to the point of hatching competence but will not hatch until presented with an environmental cue, which causes them to hatch in less than a minute. The purpose of this study is to better understand the role of enzymes called chorionases, which act to break down the chorion (egg membrane). I hypothesize that the chorion begins to weaken in this species when it is hatching competent but before it receives the stimulus to hatch. Unlike most organisms, the grunion embryo reaches hatching competence when it is fully developed, once it reaches competence, it stalls development and waits for an environmental cue. Before this, the egg is not hatching competent and will not hatch even if the trigger is there. The fact that it hatches so quickly could be evidence that an enzyme is acting to break down the chorion and this is what I hypothesize causes hatching competence. Some related fish have two chorionases while some only have one. To see whether one or two enzymes act in hatching in the grunion, I made a solution of the hatching enzyme, concentrated it, ran both SDS-PAGE and native-PAGE gels to separate out the protein by size, and cut out and sent those bands away for sequencing. To test whether the egg weakens upon hatching competence, I measured change in diameter of eggs that were pressed under a weight. We found no significant difference in pliability between the two groups of eggs using this methodology. If there are two enzymes, one may be acting before the other. If there is only one enzyme, another mechanism is at play.

Figure 1: Picture of a grunion on shore to mate. Photo by Bill Hootkins

Introduction

The California Grunion lays its eggs on beaches (Thompson and Thompson, 1919). There are many unique characteristics of this species, including that the eggs, which develop terrestrially , reach hatching competence but do not hatch until an environmental cue (Martin et al., 2011). This environmental cue happens when the tides rise preceding the new and full moon, and the eggs are washed out of sand into the waves. Another unique trait is that these fish hatch in less than a minute, whereas related fish take 30-60 minutes to hatch (Martin et al., 2011). In medaka, there are two enzymes which break down the inner layer of the chorion, high choriolytic enzyme and low choriolytic enzyme (Yasumasu et al., 2010). The inner layer is thicker, HCE acts first to weaken it, and LCE breaks it down completely, while the outer layer is broken by the fish mechanically. In zebrafish only one enzyme acts to break down the chorion, HCE, so the process is altered (Sano et al., 2008). The purpose of this experiment is to see if both choriolytic enzymes act in the hatching of *L. tenuis*, and if so, why the eggs hatch so quickly. I hypothesize that HCE is released once the grunion reaches hatching competence, and LCE is released once the environmental stimulus occurs. This way, the chorion is already weakened once it comes time for the fish to hatch, and it can do so rapidly.

Methods

Figure 2: In the field collecting grunion eggs the night after a run at Topanga State Beach

Figure 3: Chorions and larvae from eggs hatched to make the hatching liquid.

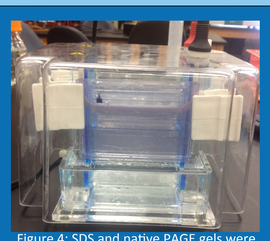

Figure 4: SDS and native PAGE gels were run using hatching liquid in this apparatus.

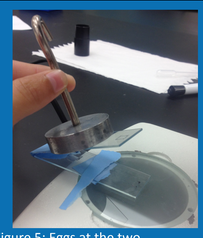

Figure 5: Eggs at the two developmental stages flattened under 0.245 N, change in diameter recorded

Results

Hypothesis one:

6) SDS-PAGE

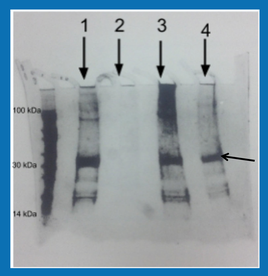

Figure 6: This SDS-PAGE was run with four samples of varying concentration of protein from 0.183 mg/ml of protein to 0.803 mg/ml. 1-4 indicate the lanes in which these four samples were placed The darkest band is measured by the marker to be roughly 30 kDA, the approximate size of chorionase. The band is thick but there is no separation between bands, so it is unclear whether or not there are one or two bands. Those dark bands were cut out and sent off for protein analysis at MS Bioworks.

Preliminary data hypothesis 2:

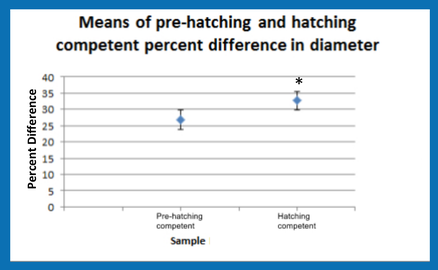

Figure 7: Diameter of eggs before and after hatching competence was measured two ways, before and after pressure of 0.245 Newtons. The difference in diameter was calculated, converted to percent, the arc-sine transformed for normality. The two conditions were significantly different by t-test ($P < 0.0001$), indicating greater flexibility after hatching competence is achieved.

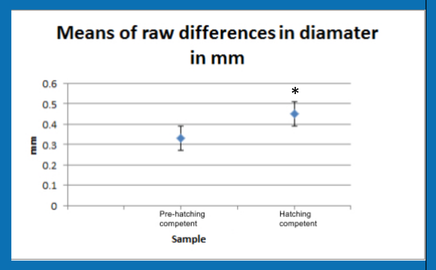

Figure 8: Diameter was measured before and after a microscope slide with a force of 0.245 N was placed on top of the egg. Different clutches were used for the two treatments The initial diameter was subtracted from the final diameter, and they were significantly different by t-test ($P < 0.0001$).

HCE1 in Medaka:
mnlassacllllfllgiaqalpvqneegheegnkeghgeegveegdeddfvdfttriltsnnntdqlllegdlvaptnrnamkcwynscfwkkasngfvvipyvissqysrgevatiegamrafngrtcirfvrrtneydfisvvskngcyselgrkggqqqelslnrggcmysgiiqhelnhalgfqheqtrsdrdsyvrinwkniipasaynfnkhdtnnlntpydyssimhygrdafsiaygrdsitpipnpnbpigqrngmsrwditrsnvlyncr

LCE in Medaka:
mdllakasvlllllslsnaqtdnmeeaengsskeeideseledvssiifrmnmnsmeellegdlvlpktrnamkcfgapdscrwpkssngivkvpyvvsdnyesdeketirnamkefaektcihfvprnneraylsleprfgcksmmgyvgdkqvvvlqrfgcikhaviqhellhalgfyhehtrsdrdqhvkinweniikdfthnfdkndtdnlgtpydygsimhygrtafgkdrketitpipnpkaaigqtermsdidilrvnklykc

Yasumasu et al, 2010, Performed by Dr. Vandergon using protcalc, *O. latipes,* May 23, 2014

Hypothesis two:

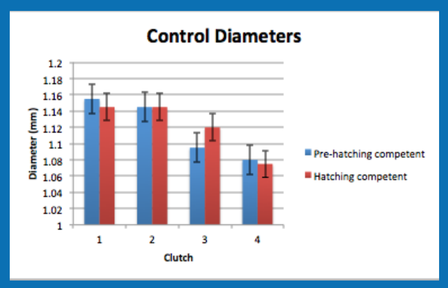

Figure 9: Diameter of the eggs on average does not change between eggs that are hatching competent and those that are not. N=10 for each sample. There is no significant difference over time between the diameters of the eggs within a clutch from one mother.

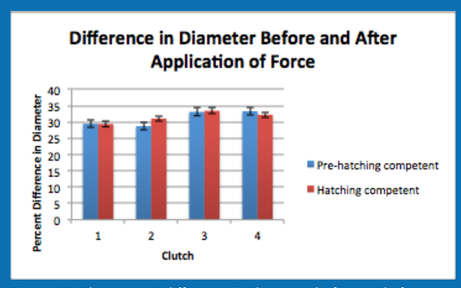

Figure 10: The percent difference in diameter before and after squishing the eggs was made normally distributed using an arcsine transformation. There is no significant difference in rigidity between pre-hatching competent and hatching competent eggs. These values were not significantly different unlike the preliminary study.

	Pre-hatching competent	Hatching competent	P-value
Clutch 1	0.392±0.0752	0.385±0.0595	0.756
Clutch 2	0.371±0.0936	0.427±0.077	0.19
Clutch 3	0.455±0.1004	0.467±0.0795	0.663
Clutch 4	0.441±0.0742	0.427±0.0221	0.589

Table 1: Differences in millimeters in diameter when the eggs are subjected to a weight, for pre-hatching competent and hatching competent eggs. There is no significant difference in plasticity by t-test.

Discussion

We are still waiting on the results of the protein identification. However, in regards to the second part of the hypothesis, a preliminary study suggested that there was a significant difference in egg rigidity between pre-hatching competent and hatching competent eggs. However, due to variation in the population and the fact that the eggs may have been weakened being in the refrigerator and the embryos dying, this data could not be trusted. By comparing a new sample eggs from the same clutch when they were pre-hatching competent, then a few days later when they were hatching competent, we minimized this variability and found that there was no significance difference in rigidity. We still believe that further testing is required. The chorion is supposed to protect eggs from infection. Eggs in the wild have increased rate of infection the longer they stay in the sand, so this could be a sign of weakening. Furthermore, we did not allow the eggs to remain at hatching competence for long, they had just reached hatching competence when these data were recorded. Most of the eggs did hatch, but in the preliminary study, eggs had been hatching competent for at least three days. If enzymes are released once the egg becomes hatching competent, it may take slightly longer for enzymatic activity to begin. We plan on re-measuring these values in a few days. The eggs we tested on were only seven days old. At temperatures near the coast it can take 8-10 days for eggs to become hatching competent (Moravek and Martin, 2011). Lastly, 0.245 N may have not been enough force to show a significant difference. Though these results were not significant, allowing the eggs longer to develop or testing chorion strength with a more sensitive test could show a significance that we cannot with the current method.

Figure 11: A grunion male and female at a grunion run, Photo by Doug Martin

Conclusion

- There may be one or two chorionase enzymes that play a role in hatching of *L. tenuis*, if so they are both close to 30 kDa
- There appears to be no significant difference in egg pliability between pre-hatching competent and hatching competent eggs based on this methodology

References

Martin, K., Bailey, K., Moravek, C., & Carlson, K. 2011. Taking the Plunge: California Grunion Embryos Emerge Rapidly with Environmentally Cued Hatching. *Integrative and Comparative Biology* 51, 26-37.

Moravek, C., & Martin, K. 2010. Life Goes On: Delayed Hatching, Extended Incubation, and Heterokairy in Development of Embryonic California Grunion, Leuresthes tenuis. *Copeia* 2011, 308-314.

Rumble, J. Identification of the hatching enzyme in grunion (Leuresthes tenuis).*Unpublished SURB presentation, 2001.*

Sano, K., Inohaya, K., Kawaguchi, M., Yoshizaki, N., Iuchi, I., & Yasumasu, S. 2010 Purification and characterization of zebrafish hatching enzyme - an evolutionary aspect of the mechanism of egg envelope digestion. *FEBS Journal* 277, 5934-5946.

Thompson, W., & Thompson, J. 1919, The Spawning of the Grunion (*Leuresthes tenuis)*. Fish Bulletin 3:1-29.

Walker, B. A. 1952. Guide to the Grunion. *California Fish and Game*, *38*, 409-420.

Warkentin, K. 2011, Environmentally Cued Hatching across Taxa: Embryos Respond to Risk and Opportunity. *Integrative and Comparative Biology, 51*, 14-25.

Yamagami, K. 1988. Mechanisms of Hatching in Fish. *Fish Physiology* XIA, 447-499.

Yasumasu, S., Kawaguchi, M., Ouchi, S., Sano, K., Murata, K., Sugiyama, H., Akema, T., Iuchi, I. 2010 Mechanism of Egg Envelope Digestion by Hatching Enzymes, HCE and LCE in Medaka, Oryzias latipes. *The Journal of Biochemistry*, *148*, 439-448.

Acknowledgements

Special thanks to Dr. Vandergon, Dr. Henisey, Dr. Brewster, Dr. Joyner, Dr. Davis, Daphne Green, Pepperdine Natural Science Division, Vincent Quach, and my mentor, Dr. Karen Martin

This research was funded by the National Science Foundation, Research Experience for Undergraduates, REU-Site Grant, #DBI-1062721 and the Natural Science Division of Pepperdine University.