Using Serum properties as an indicator of stage of maturation of the American lobster, *Homarus americanus*.

Final Report by Joseph G. Kunkel.

CMER RESEARCH TOPICS - 2000 #12:

(Joe Kunkel, Biology Department, UMass-Amherst (2000-02, to be completed in 2004) "Dr. Kunkel proposed to examine the serum and egg protein titers for American lobsters during the reproductive cycle while simultaneously monitoring various morphometric variables. It is hoped his approach may provide a number of indicators that could be used to assess the reproductive health of individuals and populations from different areas. It is also hoped that some of these indicators can be easily measured in the field. The techniques could also be used to evaluate various disease states of lobsters, such as emerging issues with egg coloration. "

The opportunity to study lobster hemolymph provided by the NOAA/CMER project "**Using Serum properties as an indicator of stage of maturation of the American lobster, *Homarus americanus*.**" sponsored by **NOAA Scientist, Josef Idoine**, was a valuable experience for me and introduced me to the difficulties in studying the lobster using field samples. I read the prior attempts to understand the molting and reproductive cycle and brought with me some preconceived ideas of how the cycles should inter-relate based on my experience with insects. Some of my prior insect knowledge was useful. I became very mindful of the color of the lobster hemolymph and its relation to the developing ovary. I limited the settings in which I could examine the lobster hemolymphs in the ways I wanted. These settings included:
1) The **NOAA NMFS Fall and Spring Groundfish Surveys** legs 3 or 4 covered the Long Island to Gulf of Maine lobster rich regions during two particular narrow time frames, particular weeks of the fall and spring.
2) The impounded lobsters of the **Lobster Conservancy on Friendship Long Island**. It was hoped that we could PIT tag lobsters and be able to release them into the pound, retrieve them from the relatively normal environment at regular intervals and take a serum sample through their molting and reproductive cycles.

What I learned:
A. Diane Cowan's PIT tagged lobsters were largely males and I wanted data on females so I purchased two female lobsters from the Friendship Lobstermen's Association and donated them to Diane. We took an initial hemolymph sample, PIT tagged them and introduced them into the same conditions that Diane was providing for the other tagged lobsters. One female had orange hemolymph and the other had green hemolymph. At the time, Diane was testing the reliability of PIT tagging by maintaining the lobsters in submerged cages around her oceanward dock adjoining her pound. I came back for the next 4 months and took hemolymph samples from all the PIT tagged animals. I was not entirely happy with the caged animals because they tended not to groom themselves, perhaps because they did not get to rub their exoskeletons on the rocks and walls of burrows. We hoped this would reverse when we released them into the pound.

The green hemolymph of the one female persisted over three months. The orange hemolymph faded to colorless by the next bleed. I was able to determine that the orange hemolyph protein traveled as ca. 500K Mr protein based on its gel permeation pattern, Fig 1. This is a typical size for vitellogenins which are usually dimmers of a 200K Mr pro-protein with extra (20%) lipid. The green protein was substantially smaller, perhaps half the size of the orange protein, what I would call monomeric vitellogenin. The ovarian state of these lobsters could not be checked because we were hoping to continue

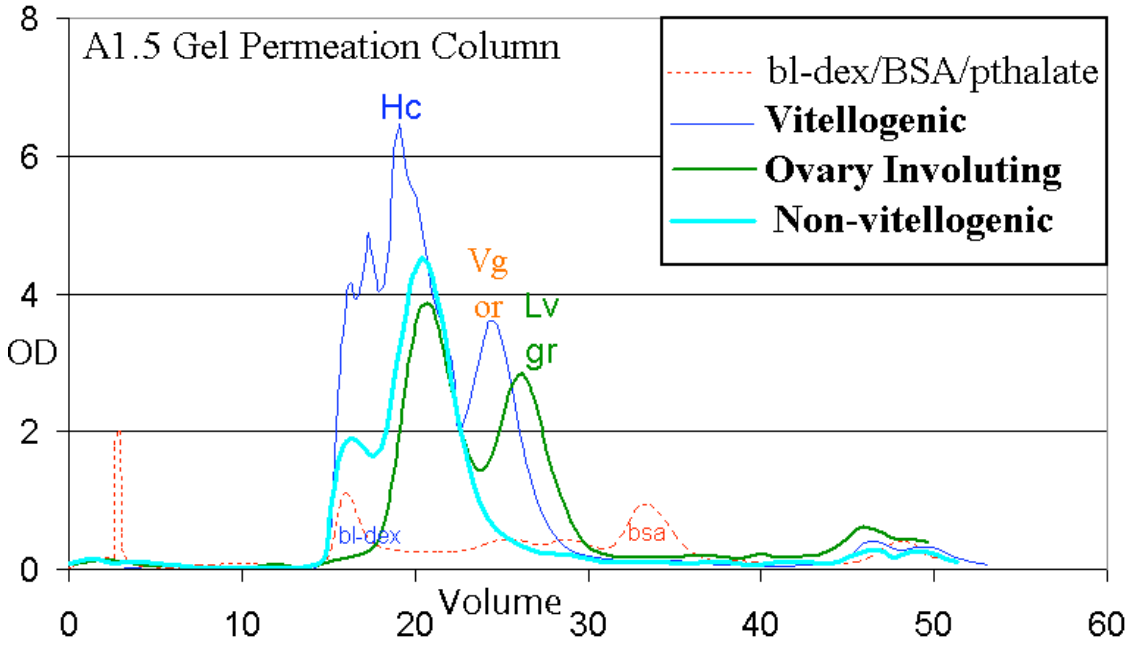

Figure 1. Composite Gel Filtration Chromatogram from 4 runs on the same column. (Red) Blue-dextrin/BSA/pthalate control size standards, (Light blue) non-vitellogenic male, (Dark blue) orange vitellogenic serum, (Green) involuting female hemolymph. OD_{280} was measured in a 1/4 cm flow cell with plotted result multiplied by 4.

sampling them in a minimally invasive way for several months. Neither female ovulated during the study. Neither the green nor orange proteins were related to hemocyanin since it was a bit larger (ca. 600K Mr) and while the color of the HbCy blanched from blue to colorless when flushed with nitrogen, the orange and green proteins did not change in color. My judgement is that the green protein is degraded vitellogenin, perhaps having passed through an ovarian follicle that subsequently involuted.

B. Sampling from **NOAA Ship Albatross IV Spring and Fall Groundfish Survey** trawled lobsters, which had recently been raised from the cold ocean bottom in fall or spring, lobster hemolymph was almost always totally colorless. This contrasted with the pound lobsters from warmer water during summer and early fall since the hemocyanin was often light to dark blue when sampled. The hemolymph of a minority of females was found to be light to middle dark orange and when such lobsters were sacrificed their ovaries were often in the early stage of development, light orange-green to light green and spindly pencil to pinky thick ovarian limbs. The colorless hemolymph females were the ones who had the large thumb thick ovarian limbs. The very rare green hemolymph females in general had the thickest of ovaries, perhaps imminently going to ovulate. One

such green hemolymph female had clearly disintegrating blackish ovaries. I associate the green hemolymph with the end of vitellogenesis and perhaps with involution of follicles that would not or did not ovulate. The orange hemolymph is perhaps an artifact of the female producing vitellogenin at a high rate prior to when the ovaries are able to efficiently remove the vitellogenin from the hemolymph. Later, the vitellogenin may be being produced at a high rate but the follicles are growing faster with greater surface area to clear the vitellogenin from the hemolymph as fast as it is synthesized.

My interest in the hemolymph color characteristics was not leading in any practical direction. The orange hemolymph color did not peak at a time close to ovulation. We also discovered problems with our ability to recapture PIT tagged lobsters that were released in the lobster pound. Simultaneously the incidence of shell disease was increasing such that my NOAA/CMER colleagues asked me if I could bend my interests toward shell disease. I had seen a large selection of shell diseased lobsters being quarantined at the **MV Mass Lobster Hatchery** facility. The disease caught my attention and I developed a hypothesis that fit my strengths and interest in nutrition and hemolymph-cooperation in producing a healthy cuticle. I hatched the cuticle vulnerability hypothesis of shell disease.

I talked with three pathologists, Roxanne Smolowitz of the **MBL**, Charles J. O'Kelly of the **Bigelow Laboratory, ME**, and Andrei Christerov of Louisiana U. They urged me to join them in studying the cuticle's attack by various prokaryote and eukaryote microbes. I was to study the cuticle's role and I added my hypothesis of pathological vulnerability as the reason why the 'same old' microbes were involved in the shell disease lesions. The other three developed a theory of a novel pathological cooperativity of a selection of previously benign microbes. We applied for an **MIT SeaGrant**. We were turned down for a full grant but given a seed grant ($10,000). That amount of money would not provide much if divided among four so Charles, Andrei and Roxanne urged me to take the seed money to pursue my idea of vulnerability. I asked Roxanne to stay on as a consultant to my proposal to look at the mineralization of the cuticle using X-ray

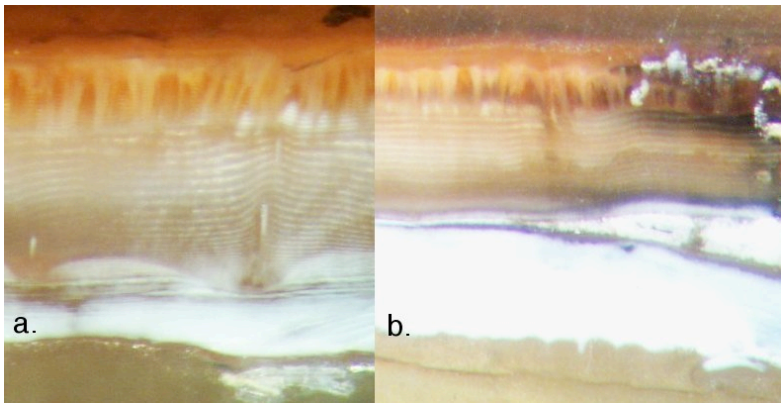

Figure 2. Plastic embedded freeze substituted lobster cuticle with intact minerals.
a. Control cuticle with minimal shell disease. b. Shell disease lesion margin.

backscatter to measure the cuticle composition in freeze substituted cuticle embeded in plastic and treated as a geological specimen. No one has ever done that with lobster cuticle and I felt that the environmental conditions of the LI Sound, Naragansett Bay

and Buzzard's Bay was creating lobsters with mineral alterations at critical points in the cuticle which might make the cuticle vulnerable to previously benign microorganisms. I collected six shell diseased lobsters from the mouth of Naragansett Bay on leg 4 of the **2004 NOAA NMFS Spring Groundfish Survey**, URL: *http://www.bio.umass.edu/biology/kunkel/fish/albatross-iv/al0403/indexlob.html*. The lobster cuticles were freeze fix/substituted and plastic embedded in the **UMass Amherst MCB Microscopy Center**. The embedded specimens, Fig. 2, are being prepared for X-ray backscatter analysis using the **UMass Amherst Department of Geosciences Electron Microprobe / SEM Facility**. The samples of shell diseased lobster were supplemented by an additional, non-shell-diseased lobster collected in the **2005 NOAA NMFS Spring Groundfish Survey**, URL: http://www.bio.umass.edu/biology/kunkel/fish/albatross-iv/al0503/.

Prior to observation in the X-ray backscatter electron microscope the polished plastic embedded cuticle can be examined using various modes of light microscopy including stereo imaging with dissecting microscope and compound microscope optics. This has lead to improved views of the cuticle surface in advance of the polished surface that would be viewed in the X-ray EM. In addition, I discovered that reflecting polarized light is able to resolve additional structural features in the mineralized cuticle which has previously been unable to be clearly resolved in the destructive embedding techniques previously applied to lobster cuticle. This discovery is reported in a chapter (Kunkel et al., in press) which resulted from the Spring 2005 Lobster Shell Disease Workshop held at UMass Boston.

Another input to this project comes from Michael Tlusty of the **NE Aquarium** who has a lobster hatchery and rearing facility. I have sampled lobster larvae from his facility and will use them in studying the development of mineralization of the lobster cuticle through the lobster life cycle in the (now) unique controlled environment of lab reared lobsters. This will get around the issue of a single time point sample from specimens of unknown history.

In addition I will be using Michael's **NE Aquarium** cache of lobsters with shell disease with a history of rearing in his facility. We are investigating approaches to serial sampling of carapace cuticle from a single specimen allowing a longitudinal study with internal controls.

All of these associations and ideas are based strongly on my original catalytic meeting and discussions with Josef Idoine on NOAA Ship Albatross IV cruise AL9910, leg IV, URL: http://www.bio.umass.edu/biology/kunkel/fish/albatross-iv/al9910/jpegs/al9910_all.jpg

My association with **UMASS CMER Director Kevin Friedland** led to discussions in which he brought up the use of the General Additive Model in fisheries biology. He mentioned an upcoming AIFRB (American Institute of Fishery Research Biologists) Workshop on GAMs. I attended and was stimulated by it to devote time to learning GAMs using the recent implementation in the R Computation Environment, which is the

Gnu Public version of S+, where GAMs was originally implemented. I have proceeded to apply GAMs in my collaboration with Peter Hepler on pollen tube growth, which we are in process of publication.

Students supported by the NOAA/CMER lobster project:

1) Arne Christensen (Research Assistant), an MCB Graduate Student, worked for 1 year with me on lobster serum protein purification. He was actively involved in sample collection aboard the Delaware II, he served as a **NOAA NMFS Groundfish Survey Volunteer Scientist** on leg 3 of the 2003 Spring Survey, (I volunteered on leg 4) and he also was involved in early discussions with Charles O'Kelly and Roxanne Smalowitz on how we were going to approach studying microbial vulnerability of the lobster cuticle.

Three student interns participated in the lobster project:
2) J. Xu (Intern) developed an on-line lobster database, which allowed Diane Cowan and I to enter data on particular PIT tagged lobsters from our respective labs.
3,4) S. Hasan (Intern) and J. Schnorbus (Intern) participated in various aspects of lobster serum protein purification and properties.

Summary Statement
Although the original objectives of the NOAA/CMER project were not carried through, the project introduced Joe Kunkel to the problems of using lobsters in research and eventually led him to associations with several lobster scientists which have persisted and have led to publication (Kunkel, in press) of a novel approach to studying mineralized cuticle which acknowledges NOAA/CMER support. These relationships promise to lead to additional research and publications on the normal and shell diseased lobster.

Bibliography

Kunkel, JG, MJ Jercinovic, DA Calliham, R Smolowitz, M Tlusty. Electron Microprobe Measurement of Mineralization of American lobster, *Homarus americanus*, Cuticle: Proof of concept. 2005 Lobster Shell Disease Workshop, UMass Boston. (in press)

Respectfully submitted,
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