CMER RESEARCH TOPICS - 2005 #3: PREPARATION OF MONOCLONAL ANTIBODIES FOR CYTOCHROME P450 1A (CYP1A) AND VITELLOGENIN (Vtg) PROTEINS THAT ARE SPECIFIC TO BLUEFISH, *POMATOMUS SALTATRIX*, AND ARE APPLICABLE FOR USE IN ENZYME LINKED IMMUNOASSAYS (ELISA) AND WESTERN BLOT. (Proposal)

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Background: The young of the bluefish (*Pomatomus saltatrix*), commonly called snappers, spend their early life in estuaries and waterways that penetrate into the heart of coastal communities and thus experience the more concentrated forms of coastal pollution. The protein Vtg would not normally be found in the serum of the snapper population due to their juvenile status. Vtg in the serum of snappers would be an indicator of high levels of natural (phyto-estrogens) or anthropogenic (human-enhanced) estrogenic compounds in the environment. Young bluefish, snappers, have been shown to accumulate various pollutants in similar amounts to less vagile residents and local food items such as the blue-crab and mummichog (Iannuzzi et al., 2004). The vitellogenic pathway in the liver produces the protein vitellogenin, Vtg. The fish liver responds to environmental estrogens resulting in induction of Vtg, secretion and accumulation of Vtg in the serum of responding males and females. The other pathway, containing cytochrome P450 1A (CYP1A), is one member of the extensive P450 oxidative degradation pathway that is often enhanced by pollutants that need to be cleared from the individuals system. However there is evidence that estrogenic compounds decrease the level of CYP1A in fish hepatocytes (Elskus, 2004). That is, there is a reciprocal effect of estrogen in stimulating Vtg synthesis and at the same time inhibiting CYP1A synthesis. This has been suggested to be due to the huge effect which estrogen has on the liver metabolism, which is turned into a Vtg factory, downscaling other pathways including the P450 pathway. It should be noted that CYP1A is produced in other soft tissues such as gill, heart, and the swimbladder rete mirabile (Schlezinger and Stegeman, 2000), that might not be sensitive to the estrogenic inhibition. The CMER Research Topics announcement states that there are no suitable antibodies currently available for these two critical biomarker proteins from bluefish.

Objectives: I propose developing assays for the two environmental-pollutant-sensitive pathways, CYP1A and Vtg of the bluefish. A protocol examining the contrary effects on liver as well as control tissues will provide a multivariate indicator and more sensitive assay of potential estrogenic effects.

Methods: While monoclonals are possible to use, a polyclonal antiserum for titering Vtg is recommended because Vtg accumulated in the serum can be degraded and produce Vtg fragments that could result in substantial false negatives during testing. This is not the case for CYP1A since it is cellular and its native form represents a current functional protein of which one wants a titer estimate. There are

Table I. Relationships of fish with crossreactive (+) monoclonal or polyclonal antisera available.

Gadus (cod)	**Teleostei**; **Euteleostei**; Neoteleostei; **Acanthomorpha**; Paracanthopterygii; Gadiformes; Gadidae; *Gadus*
Pomatomus, (bluefish)	**Teleostei**; **Euteleostei**; Neoteleostei; **Acanthomorpha**; Acanthopterygii; **Percomorpha**; Perciformes; Percoidei; Pomatomidae; *Pomatomus*.
Pleuronectes (winter flounder)	**Teleostei**; **Euteleostei**; Neoteleostei; **Acanthomorpha**; Acanthopterygii; **Percomorpha**; Pleuronectiformes; Pleuronectoidei; Pleuronectidae; *Pleuronectes*.
Cyprinodon (sheepshead minnow)	**Teleostei**; **Euteleostei**; Neoteleostei; **Acanthomorpha**; Acanthopterygii; **Percomorpha**; Atherinomorpha; Cyprinodontiformes; Cyprinodontidae; *Cyprinodon*.
Oncorhynchus	**Teleostei**; **Euteleostei**; Protacanthopterygii; Salmoniformes; Salmonidae; *Oncorhynchus*.
Salmo	**Teleostei**; **Euteleostei**; Protacanthopterygii; Salmoniformes; Salmonidae; *Salmo*
Cyprinus (carp)	**Teleostei**; Ostariophysi; Cypriniformes; Cyprinidae; *Cyprinus*

Table II. Commercially available monoclonal and polyclonal antibodies to CYP1A.

Source	Antibody	Price	Reactivity
Cayman Chem	Mouse Anti -Cod, Clone NP-7 (Ag: CYP1A, purified from liver microsomes of naphthoflavone-treated cod)	$169/100 ul	(+) Gadiformes, carp (Cypriniformes), (Salmoniformes), and some flatfish (Pleuronectiformes)
Cayman Chem	Mouse Anti -Fish, Clone C10-7 (Ag: amino acids 277-294 of rainbow trout CYP1A)	$169/100 ul	(+) rainbow trout (Oncorhynchus mykiss), carp (Cyprinus carpio), salmon (Salmo salar), Atlantic cod (Gadus morhua), turbot (Scophthalmus maximus), lemon sole (Microstomus kitt), and mudfish (Clarias anguillaris)
Cayman Chem	Mouse Anti -Fish, Clone FA-1 (Ag: aa 277-294 of rainbow trout CYP1A)	$169/100 ul	(+) rainbow trout (Oncorhynchus mykiss), carp (Cyprinus carpio), Atlantic cod (Gadus morhua), sheepshead minnow (Cyprinodon variegatus), gilthead seabream (Sparus aurata), eelpout (Zoarces viviparus), mudskipper (Apocryptes bato), and riverine catfish (Rita rita)
Cayman Chem	Rabbit Anti –Fish CP-226 Polyclonal Ab (Ag: amino acid sequence 190-204 and 282-296 of rainbow trout CYP1A, coupled to the carrier protein KLH	$169/100 ul	(+) rainbow trout (Oncorhynchus mykiss), carp (Cyprinus carpio), salmon (Salmo salar), Atlantic cod (Gadus morhua), turbot (Scophthalmus maximus), lemon sole (Microstomus kitt), and mudfish (Clarias anguillaris)

commercial polyclonal and monoclonal antibodies to CYP1A made against the proteins of other fish species [cod CYP1A or rainbow trout peptides conjugated to Keyhole Limpet Hemocyanin (KLH) or a conserved peptide of rainbow trout] which are predicted to suffice to measure bluefish CYP1A, since the taxonomic differences involved, Table I, are small compared to the range of the antibody cross-reaction. There are several monoclonal antibodies to CYP1A available, Table II, which are rated to be useful in ELISA and Western Blotting for the broad range of fish species tabulated. The reported broad taxonomic cross-reaction (Table II), particularly of the rainbow-trout-CYP1A-aa277-294 based monoclonals, suggests by inference that they would also work with the bluefish.

Purifying CYP1A from bluefish itself would be a challenge and it likely would not be needed. Most successful anti-CYP1A production has used synthesis of a conserved peptide as its source of antigen (Table II). However, an enriched solution of bluefish CYP1A would be useful to serve as a standard for calibrating the immunoassays within and between years. There is a recent technique using reverse phase chromatography which produces purified CYP1A of a Brazilian freshwater fish (DaSilva et al., 2004) in one step. This purification scheme is encouraging and a partial purification using this technique in bluefish could be sufficient or enhanced by a second approach using a counter technique such as gel permeation and/or ion exchange chromatography. The need for relatively pure CYP1A is mainly to serve as an absolute positive control and as a standard for the Western Blot and ELISA assays. Alternately one could devise a CYP1A-enriched standard-tissue-source from a bluefish treated with an inducer such as naphthoflavone or benzopyrene, intraperitonealy injected with corn oil (Schlezinger and Stegeman, 2000). The advantage of a purified standard is the ability to establish absolute levels of the standard, checking

titer with a general protein assay and SDS-PAGE analysis, which allows greater confidence in between-assay and between-year comparisons. A semi-purified standard must allow for degradation of the standard during short and long-term storage from unremoved proteases that are present in all extracts. These degradative forces can be minimized by using cocktails of protease inhibitors during preparation

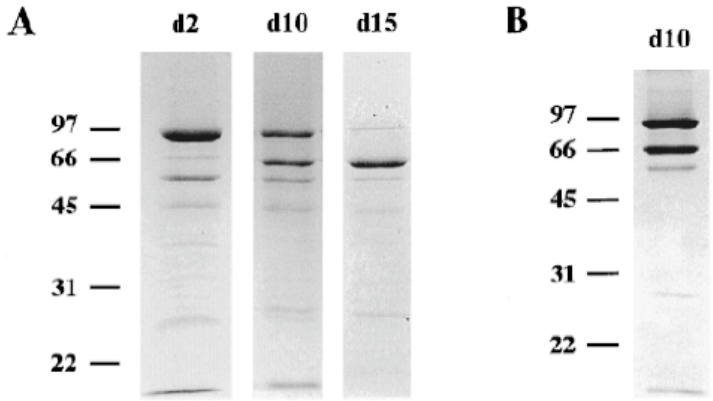

Fig. 1. Proteolytic processing of Lv during winter flounder development. (A) SDS-PAGE of whole extracts of embryos (1994 cultures). Extracts were prepared at: d2, day 2; d10, day 10; and d15, day 15 after fertilization; 50% hatch was at day 18. Ovalbumin (45 kD) was added to each extract as a densitometric standard. (B) Western blot of whole extract of day 10 embryos (1996 culture), using an antiserum raised against Lv from unfertilized eggs. Molecular weight standards in kD are indicated.

and storage of the protein extracts. The Western Blot (Fig 1 from Hartling and Kunkel, 1999) would provide evidence of specificity and titer that would allow more confidence in applying the higher throughput application of the quantitative ELISA test (Fig 6 from Hartling and Kunkel, 1997).

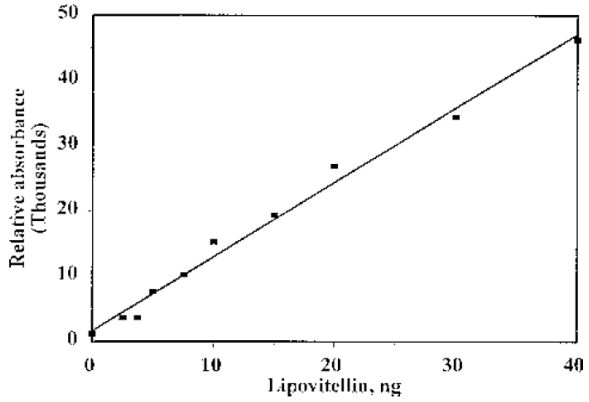

Fig. 6. ELISA standard curve for winter flounder Lv. Each point represents the mean of four samples.

Given the lack of close relatives of bluefish for which we have antibodies to Vtg, it is more logical that a genuine anti-bluefish-Vtg be produced for a bluefish-Vg-immunological assay. Obtaining pure lipovitellin (Lv), the processed form of Vtg in the fish oocyte, from bluefish eggs and its use as an antigen to produce an antibody is perhaps the easiest, most direct and efficient way to produce an anti-bluefish-Vtg. It is relatively rare for a ripe or running bluefish to be taken by the Spring or Fall Groundfish Survey because the timing of the legs is not appropriate for the Spring or Fall spawning periods but developing ovaries with substantial Lv content in their developing oocytes would be suitable. A request for frozen developing bluefish ovaries will be made to the NMFS Fall and Spring Groundfish Survey. I will also offer a bounty from party-boats fishing bluefish for iced ripe ovaries to be delivered at the dock. I will also request that the Sandy Hook NMFS Lab use some of its holding facility to house bluefish that can be induced by estradiol to produce serum Vtg and bluefish that can be injected with naphthoflavone or benzopyrene to produce CYP1A rich liver. My lab has produced specific antisera against particular fish species Vtg using the egg extracted Vtg several times (Hartling et al., 1997; Hartling and Kunkel, 1999).

We have used this approach to produce anti-Vtg against winter flounder, Atlantic cod, American shad and tautog. The anti-cod-Vtg was just as effective in immunological tests with its close Gadid relative, the haddock. It is possible, but not likely, that the polyclonal antibody we have to winter flounder Vtg (Hartling et al., 1997), in the same clade, **Percomorpha** (Table I) as bluefish, crossreacts with the bluefish, but that will need to be tested with bluefish Lv or Vtg.

As an alternate source of bluefish Vtg antigen we have successfully induced large amounts of Vtg to accumulate in the serum of male cod by injecting an adult male cod with estradiol (in olive oil intermuscularly) and bleeding via the caudal vein over several weeks to accumulate large volumes of Vtg laden serum. The resultant Vtg was purified by a combination of purification steps. First, gel-permeation chromatography uses the fact that Vtg is an approximate Mr 500,000 protein, unique in its large size, which is easily separated from the majority of smaller serum proteins. Second, Vtg is generally easily adsorbed to a TEAE-cellulose or DEAE-cellulose column at salt concentrations where most other proteins do not adsorb. My lab is set up to extract, purify and store proteins for use in immunizations (Kunkel, 1987), as assay standards and for the subsequent development of the immunological assays using those antibodies. We can do this for the bluefish if ovarian or egg Lv is not able to be obtained. Vtg is generally a phospho-glyco-lipo-protein and will be denatured by freezing and thawing in its purified form. This will change its reactivity in the ELISA test. We have developed a method for long-term storage of purified Vtg by gently precipitating it in 2/3 saturated ammonium sulfate and storage at 4°C. This avoids the denaturing forces of the freeze thaw cycle, providing a stable standard. Antibodies and protein standards we have developed have been used in several immunological assays including ELISA tests and Western Blot technique, both specified in the sponsor's RFP (Hartling et al., 1997; Hartling and Kunkel, 1999; Figs 1 and 6 above).

Schedule of Events: The project is expected to require two years to develop and test the integrated assays.

The first year will be taken up with (1) obtaining the commercial anti-CYP1A sera and testing them for reactivity to CYP1A induced bluefish tissue extracts. Comparison of the anti-CYP1A sera will be done to find which is the most effective and practical for measuring bluefish CYP1A. (2) Obtain bluefish ovarian Lv and use it to immunize two New Zealand White Rabbits. Obtain the antisera over two months and characterize the antisera. Year one will involve our Sandy Hook sponsors in cooperating to obtain and house adult bluefish for CYP1A and Vtg induction. By the end of year one, September 2006, a report on the availability and suitability of antisera will be provided.

The second year will be spent in developing the protocols for using the chosen anti-CYP1A and developed anti-Vtg-sera to examine the responses of snappers to environmental factors expected to induce CYP1A or Vtg. We will also spend some resources and time having the anti-bluefish-Vtg rabbits boosted and bled to build up the stocks for measurements in future years. Year two will also involve the Sandy Hook sponsors of the project in designing initial experiments and protocols for obtaining snapper serum and tissue samples. By the end of the second year, August 2007, a trip to Sandy Hook to train personnel in the use of the products will be scheduled. A final report due in November 2007 will provide the description of the antisera used, the protocols developed for use of the antisera to study the tissues of the snapper and an inventory list of the antisera and protein standards that are to be transferred to the Sandy Hook Lab.

Flexible timing of efforts is essential since the logical timing of available resources do not coincide with the NOAA/CMER funding cycle. Before funding becomes available on the UMass campus, usually October of the first year, it is expected that when inclusion of our proposal in the Omnibus Request is assured that preliminary catching, housing, injection and bleeding plus tissue harvesting of adult bluefish injected with estradiol and CYP1A-inducers could be carried out in late summer and early fall 2005 prior to the official start of funding. This would require an informal cooperative agreement between PIs and the Sandy Hook lab sponsors using NMFS holding tanks (Sandy Hook or Woods Hole) and injection materials and supplies available to the Co-PI Joe Kunkel. Visits of

Joe Kunkel to the Sandy Hook lab or Woods Hole would be anticipated as needed to firm up plans and carry out protocols that might need his participation such as the injections and bleedings.

Products: It is anticipated that one of the commercial antibodies will be satisfactory for assaying bluefish CYP1A. The anti-CYP1A serum will be characterized with bluefish-CYP1A-enriched-standard and a protocol will be developed for testing organs from control and estrogen treated snappers. The anti-bluefish-Vtg serum will be produced and characterized using serum and organ samples from control and estrogen treated adult and juvenile bluefish. I would provide immunological assay protocols and associated antisera plus standards for bluefish Vtg and CYP1A to carry out within and between year titer comparisons.

Cooperation with NOAA: It is anticipated that we will need to house live adult and juvenile bluefish for producing CYP1A enriched tissue and Vtg enriched serum and tissue. I am aware that the James J. Howard Lab at Sandy Hook and the NMFS Aquarium at Woods Hole have suitable holding tanks. I have used the Woods Hole Aquarium tanks for housing adult cod that were injected with estradiol to induce Vtg. I have also learned that the Sandy Hook lab seasonally has, or can have, adult bluefish in its holding tanks. I request that in the first year of the grant, at least by early fall 2005, four adult bluefish be housed and tagged in two tanks at the Sandy Hook lab or the Woods Hole Aquarium so that they could be injected with estrodiol or CYP1A-inducer. The serum of the estrogen-induced bluefish would be harvested twice by caudal vein puncture, once at 2 weeks and second at 4 weeks after which the tissues of the fish would be harvested and frozen for use as test antigens. Similarly the CYP1A induced fish will be injected and tissues harvested after one month. At harvest the sex and maturity of the adults would be determined. In the second year of the grant the Sandy Hook lab will be requested to house snappers that can be used to establish baselines and methodologies for snapper serum and tissue sampling. Joe Kunkel has experience bleeding smallish fish by caudal vein puncture and will visit the lab with the holding tanks when needed.

Student Participation: For this project I anticipate using a UMass graduate who has substantial experience working in private business dealing and communicating with the public and organizing his own business and work schedule. I have such a student in mind, Nathaniel Clarke, a Biology major with excellent grades who is working in my lab currently as an advanced undergraduate. He graduates this spring and would remain working in my lab as a Project Manager of the Bluefish project. He wants to develop skills in protein purification and the immunological techniques that this project encompasses. He is already proficient in using the basic wet lab instrumentation in my lab. He would be responsible for keeping track of all detailed aspects of this project and be responsible for arranging and coordinating all details with any commercial fishermen and our sponsors at the Sandy Hook lab. The PI, Joe Kunkel, has substantial experience in all aspects of the required technology from harvesting tissues and serum via caudal vein puncture, purification of proteins, developing antisera, characterizing of resultant antisera and developing experimental designs for using the immune assays. This experience will be transferred to the Project Manager over the course of the two years. The work involved in this two year project is too much and too detailed for a graduate student to encompass at the same time as taking courses for a masters degree. It is believed by the PI that this is the most efficient way of accomplishing the project goals as well as educating a student in these field and laboratory skills.

Proposed Budget (Table III):

Salary: Nathaniel Clark, Project Manager, 100% of time devoted to this project. He would earn a yearly salary of $24,960, with no benefits. He would start 10 weeks after the start of the grant the first year representing 42 weeks of work the first year to accommodate acquiring materials before he starts working. This represents $12.00 per hour for a 40 hour week 52 weeks of the year. An MCB master's candidate would cost the same amount in Salary + Benefits and would only provide half the time for research and skills training.

Travel: Three round trips to Woods Hole (300 mi) or Sandy Hook (450 mi) are anticipated per year for the Project Manager and the PI by car for treatment of bluefish adults and harvesting tissue samples. One trip per year by the PI to the Cell Biology Meetings (SF Dec 2005, 2006 by air) is anticipated for the purpose of communication of results.

Equipment: A portable Mac Ibook G4 is requested for use by the Project Manager to allow data and images to be collected in the field and to provide a mobile platform for the Project Manager to organize the project and the acquired data. The Kunkel lab has two Kodak DC 290 cameras one of which can be used on field trips by the Project Manager and is compatible with the Ibook. A printer is requested in the second year to provide the Project Manager a convenient output device in the lab. Currently we depend upon Biology Department printers in an adjacent building. A Disk Drive replacement for a current Kunkel Lab Drive hung on a Departmental SparcStation is requested to ensure the safety of the drive that will hold the archived data of this project.

Supplies: A supplies budget of $4,956 detailed in Table III would provide the necessary lab supplies for several major parts of the project: (1) Ion exchange chromatography and Gel Permeation Chromatography needs media for the columns and chemical buffers and salts to run through the columns and glassware tubes to use in the fraction collector (2) SDS PAGE analysis of the proteins requires supplies for making and running analytical protein gels and staining them; (3) The spectrophotometer requires plastic or acrylic cuvettes and replacement of UV and visible light sources; (4) The ELISA assays require nitrocellulose membranes or microarray plates to house the assays and pipette tips and microcentrifuge tube disposeables to prepare the samples. (5) Sampling the blood of fish requires syringes and sample tubes. (6) Commercial antisera against CYP1A will be purchased for evaluation and when an appropriate antiserum is identified a bulk purchase of that antiserum will be done in year 2 to provide sufficient assays for several years depending on the titer efficiency. (7) Pipetman pipetters are needed to replace existing P1000, P200 and P20 models that are superannuated (8) A deionized water unit in my lab needs periodic changes in its cartridges. (9) Ink-cartridges and paper are needed for the requested printer.

Services: A lab phone in Morrill N426 will service the Project Manager's needs during the two years. Pipetman pipetters in the lab will be refurbished and recalibrated by a commercial service that visits the University yearly. Antisera against purified bluefish Vtg will be produced through either the University Animal Care Service or a commercial service (Invitrogen $850 for 2 rabbits) which allows for those rabbits to be maintained and rebled in subsequent months for additional charge. I have budgeted $2000 for this basic service plus extension. The resultant antisera will become the property of NOAA with a token amount held in the PIs lab.

References:

Da Silva, M. E. Silva, J. A. Marangoni, S. Novello, J. C. Meirelles, N. C. (2004) A new method to purify hepatic CYP1A of *Prochilodus scrofa*, a Brazilian freshwater fish. J. Comp Biochem Physiol C Toxicol Pharmacol Volume 138(1): 67-74.

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Kunkel, JG. (1987). Analytical immunological techniques. In "IMMUNOLOGICAL TECHNIQUES: ARTHROPODS" (L. I. Gilbert and T. A. Miller, Eds.), pp. 1-41. Springer Verlag.

Schlezinger, J.J. and J.J. Stegeman. (2000). Dose and inducer-dependent induction of cytochrome P450 1A in endothelia of the eel, including in the swimbladder rete mirabile, a model microvascular structure. Drug Metab Dispos, 2000. **28**: 701-8.

Table III. Project Budget Details

			Year 1	Year 2	**Total**
Salary	Technician		$20,160	$24,960	**$45,120**
Total salary:			$20,160	$24,960	**$45,120**
Benefits:					
	Fee-waiver		$0	$0	**$0**
	Geo-Health		$0	$0	**$0**
	Health &Wel		$0	$0	**$0**
Total Salary & Benefits			$20,160 (42 weeks)	$24,960	**$45,120**
Travel	3x Amherst to Sandyhook				
	Cell Biology Conference, SF		$2,500	$2,500	**$5,000**
	Printer, HP color	$400			
Equipment	Sparcstation disc drive	$900			
	iBook G4	$999	0	2299	**$2,299**
Supplies:	antibodies, CYP1A x4	$676			
	kimwipes, case	$230			
	centrifuge tubes case	$189			
	deionizer cartridges	$377			
	10 X 25KU heparin	$175			
	Chemicals: electrophoresis	$500			
	chromatography	$500			
	syringes case	$110			
	glassware: flasks, beakers	$100			
	glass test tubes 2 cases	$110			
	BioRad BioGel A1.5	$750			
	gel standards	$139			
	Pipetman 20, 200, 1000ul	$900			
	Office supplies (printer)	$200	$4,956	$4,956	**$9,912**
Services:	2 rabbits immunized		$1,000	$1,000	**$2,000**
	Pipettman calibr @$20 per		$200	$200	**$400**
	Lab Phone		$144	$144	**$288**
Total Direct Cost			$28,960	$36,059	**$65,019**
Indirect Cost	(TDC-Equip)*0.25		7240	8440	**$15,680**
Total Cost			$36,200	$44,499	**$80,699**

BIOGRAPHICAL SKETCH

Joseph G. Kunkel

Born: Oceanside, New York, August 17, 1942
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Education:

Columbia College, New York, New York, Zoology	A.B. 1964
Case-Western Reserve University, Cleveland, Ohio, Biology	Ph.D. 1968

Dissertation: *Control of Cockroach Development*

Society Memberships:
AAAS, SICB; Sigma Xi (Yale); Marine Biological Laboratories, Corporation Member.

Awards and Honors:
NSF Graduate Fellow, Biology, Case Western Reserve University, 1967-68; RCA Scholar in Chemistry, Columbia College, 1961-62; Columbia University Scholar, Columbia College, 1960-64; New York State Regents Scholar, Columbia College, 1960-64; Bnai Brith Scholar, Columbia College, 1960

Positions and Professional Experience:

Sabbatical with W. Nagel, Ludwig Maximillian U., Munich, Germany	2001-02
STEMTEC Cycle II participant, University of Massachusetts at Amherst	1998-2001
Volunteer Scientist, Groundfish Surveys, National Marine Fisheries, Woods Hole, MA	1998-2005
Sabbatical with PJS Smith, National Vibrating Probe Facility, MBL, Woods Hole, MA	1993-94
Owner and Operator, JK_Consulting, research services & software	1986-96
Full Professor, University of Massachusetts at Amherst	1985-present
Adjunct Professor of Entomology, University of Massachusetts.	1985-present
Sabbatical with Beatrice Lanzrein, Zoological Institute, U. of Berne, Switzerland,	1985-86
Member, Cell and Molecular Biology Program	1983-present
Associate Professor of Zoology, University of Massachusetts.	1976-85
Sabbatical in evolution lab of Alan C. Wilson, University of California at Berkeley	1977-78
Assistant Professor of Zoology, University of Massachusetts, Amherst	1970-76
Lecturer in Biology, Yale University	1969-70
NIH Postdoctoral Trainee in Development, with Gerry R Wyatt, Yale University	1968-70
Postdoctoral in Biometry, Case Western Reserve University Medical School	1968
Graduate Research Assistant of Michael Locke, Biology Department, CWRU, Cleveland	1964-68
Research Asst. to Arthur W. Pollister, Zoology Department, Columbia University, NY	1963-64
Research Assistant to Francis J. Ryan, Zoology Department, Columbia University, NY	1963

Professional and Competitive Grants Received:

MIT-SeaGrant seed grant 'X-ray backscatter of Lobster Cuticle Minerals' (D = $10,000)	2004-2005
Deutches Forschung Gemeinshaft: Ion Probe Development, LMU, Munich (D = $7700)	2001-2002
NOAA/CMER 'Serum indicators of American lobster maturation' (D+I = $52,000)	2000-2002
NOAA/CMER 'Morphometric & Biochemical Study of Larval Tautog.' (D+I = $50,000)	1998-2000
Five College Multimedia Project 'Underwing-Moth Multimedia Resource' (D =$2,500).	1996-97
NOAA 'Serum and egg vitellogenin in the Atlantic cod *Gadus morhua*' (D+I = $50,000)	1996-98
Applicable Electronics: Unrestricted gifts (D = $38,292)	1994-98
USDA-APHIS 'Planar geometry of gypsy moth wing venation' (D = $8,000)	1992-93
NOAA 'An immunological assay for winter flounder vitellogenin' (D = $7,500)	1992-93

EcoScience Corporation: Unrestricted gifts (D = $11,000)	1988-93
USDA 'Gypsy Moth Population Biology: Storage proteins' (D+I=$225,000)	1986-93
NSF 'Ionic Currents During Oogenesis' DCB8905552 (D+I=$87,000)	1989-90
Roche Foundation, 'Vitellogenin Receptor' sabbatic (SFr29,000)	1985-86
NSF 'Role of Oligosaccharide Structure in Vitellogenesis' (D+I=$497,800)	1979-89
NIH 'Control of Cockroach Development', AI11269 (D+I=$57,500)	1974-77
U. of Massachusetts Faculty Research Grant, FR-J39-72 (D=$900)	1971-72

Programming Language Skills: Fortran, Basic, Pascal, APL, C, HTML, JAVA, J, R

B. BIBLIOGRAPY OF SIGNIFICANT PUBLICATIONS

- Kunkel JG, MJ Jercinovic, DA Calliham, R Smolowitz, M Tlusty. Electron Microprobe Measurement of Mineralization of American lobster, *Homarus americanus,* Cuticle: Proof of concept. Lobster Shell Disease Workshop, UMass Boston, 2005 (in press).
- Holdaway-Clarke, T.L., N.M. Weddle, S. Kim, A. Robi, C. Parris, J.G. Kunkel, P.K. Hepler., Effect of extracellular calcium, pH and borate on growth oscillations in *Lilium formosanum* pollen tubes. Journal of Experimental Botany. 54:65-72. 2003.
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