PRINCIPAL INVESTIGATOR: Dr. Donald Weston, University of California Berkeley

PROJECT COLLABORATORS :
Dr. Helen Poynton, University of Massachusetts, Boston – Identification of genes responsible for pesticide resistance.
Dr. Richard Connon, University of California Davis – Gene sequencing of Hyalella.
Dr. Michael Lydy, Southern Illinois University – Analytical chemistry of pesticides.
CONSULTANT:
Dr. Gary Wellborn, University of Oklahoma – Speciation within the Hyalella complex.

TITLE: Improving the utility of Hyalella azteca as a tool for monitoring and management.

TOTAL BUDGET: $299,661 in IEP funds. (An additional $20,000 to be provided by SRCSD)

STUDY DURATION: One year. To start as soon as contractual arrangements can be made, but tentatively Nov. 1, 2012 through Oct. 31, 2013.

PRIORITY RESEARCH TOPIC AND QUESTIONS ADDRESSED:
Topic area #2 - Physical and chemical habitat effects on fish populations.

1) How can existing or new monitoring, modeling, or other methods be applied to better define, monitor, and assess fish habitat in the estuary?
2) What is the frequency of occurrence and distribution of acute and chronic toxicity of ambient water to fishes and their food items in the estuary, and how is it affected by the interaction of stationary and dynamic habitat components?
3) What are the distribution, transport, fate, concentration, and effects of contaminants including pesticides, ammonia, and metals that may have lethal or sublethal effects on fishes and their food items in the estuarine low-salinity zone, and how are these affected by the interaction of stationary and dynamic habitat components?

OVERALL PROJECT PURPOSE:

The amphipod, Hyalella azteca, is widely used to test the toxicity of water or sediment samples, and as an indigenous species in the Bay-Delta, also plays a role in bioassessment studies in the region. The species is critical to decisions by management agencies as to whether fish species of concern, and the prey upon which they feed, are threatened by contaminant impacts. Yet new data has emerged (discussed below) that completely change current thinking on the sensitivity of H. azteca to contaminants, and could dramatically influence how toxicity testing and bioassessment data are interpreted. Through further investigation of contaminant sensitivity of the species, the objective of the proposed work is to avoid unreproducible or lab-dependent results, and ensure that the IEP, Delta RMP and other programs have a reliable monitoring tool in H. azteca. The work is also intended to bridge the interpretive gap between monitoring programs that rely on toxicity testing and those using bioassessment, by avoiding conflicting conclusions on habitat health and the potential that conflicting data could misinform management decisions.

PROJECT BACKGROUND AND CONCEPTUAL MODEL:

Toxicity testing is an important monitoring tool in the Bay-Delta to assess habitat quality and potential risks to fish species of concern and other aquatic life. H. azteca, has long been used in the Bay-Delta, as well as nationwide, for assessing sediment quality, and it is being increasingly used for assessing toxicity of water samples as well. The species is particularly important for assessing risks to aquatic life from pyrethroid pesticides, a group that in recent years has been found to often be responsible for toxicity throughout the Bay-Delta and for which H. azteca is better suited for testing than are the other commonly used toxicity testing species. IEP used H. azteca for a large monitoring effort conducted by UCD throughout the Delta in 2006-2010, and it is used in many other studies supported by IEP or its member agencies. It is likely to be critical to water and/or sediment monitoring under the Delta RMP.

Bioassessment is another monitoring tool, and H. azteca plays a role in this approach as well. When interpreting bioassessment data, the presence of the species is generally taken as indicative of good habitat quality, and particularly the absence of any threat due to pyrethroids. For example, the presence of the species in some creeks has been used by pesticide registrants to contest the 303(d) listing of those creeks due to pyrethroid toxicity.

Yet our own studies and anecdotal reports from others have reported some waterbodies to have thriving wild populations of H. azteca, yet when water and/or sediment samples from these same sites are tested in the laboratory, they cause 100% mortality of the test H. azteca from laboratory cultures. Obviously, such results call in to question what toxicity in a laboratory test means, and whether it is of any predictive value in protecting Bay-Delta habitats.

We have tested three laboratory cultures of H. azteca, and seven wild populations from northern California, and found dramatic differences in their sensitivity to pyrethroid insecticides. For example the 96-h LC_{50} for the pyrethroid cyfluthrin for our most sensitive population from a lake near Sacramento is 1.2 ng/L. For our most insensitive population (a creek in Concord), the same compound's LC_{50} is >670 ng/L (highest concentration tested, which still did not cause 50% mortality). This astounding 600-fold variation in contaminant sensitivity could cause great difficulty in using toxicity test results for environmental management. A sample one lab reports as toxic could be non-toxic if tested by another lab using H. azteca from a different source.

Our preliminary studies have shown these differences in sensitivity have their roots in the evolutionary genetics of H. azteca. It has previously been shown by genetic studies that H. azteca is actually a species complex with dozens of members which cannot be differentiated by morphology, but these findings have largely gone unnoticed by the toxicological community. Yet by DNA sequencing of our various populations, we have found that our ten populations are comprised of four groups sufficiently genetically distinct from one another to be considered four different species (though all morphologically conform to "H. azteca"). One of these species is apparently extremely tolerant of pyrethroids, widely distributed throughout northern California, and responsible for the reports of H. azteca thriving in locations that toxicity testing finds to be acutely toxic. Some of the other conclusions of our preliminary work include:

1) If cultured in the laboratory in the absence of pyrethroids, the offspring of the pyrethroid tolerant species are just as tolerant as the parents.
2) A location can contain multiple H. azteca species. LC_{50} estimates from a single locality can vary by orders of magnitude if investigators unwittingly sample different microhabitats or collect at different times, preferentially obtaining one species over the over.

3) We have found a population that is not only extremely tolerant to pyrethroids, but has also achieved tolerance to the organophosphate chlorpyrifos, even though these two compounds kill by entirely different modes of action.
4) It is not uncommon that laboratories culturing H. azteca would collect local wild animals and add them to the cultures occasionally if, for example, the original cultures were not producing well. Unknowingly, these efforts most likely have added a different species, making it uncertain what that lab is now testing with. If toxicity labs testing with H. azteca are not all using the same species, the immediate question this finding raises is whether a monitoring program's findings are a function of which laboratory did the testing and whether they reported toxicity because they used a sensitive member of the H. azteca complex, or missed toxicity because an insensitive one was used.
5) We have tested three laboratory cultures so far, all of which we believe can be traced to a common ancestor in the 1980s. These three have similar pyrethroid sensitivity (pyrethroid LC_{50}s within a factor of two). Yet there are several other commercial suppliers of H. azteca, supporting labs testing samples from the Bay-Delta, and the ancestry of their H. azteca has not been traced, nor their contaminant sensitivity tested.

The work described above has been done over the past year by most of the participants in the proposed study, and the findings are now being prepared for publication. It was done without funding because we believed there was a need to understand how H. azteca was living in sites known to be toxic when tested in the lab. The preliminary data indicate the topic merits further investigation, and the proposed study will allow us to examine the contaminant sensitivity of additional populations, particularly the cultures of several commercial vendors currently supplying H. azteca to monitoring programs in the Bay-Delta. We will also expand the work to additional current-use pesticides beyond pyrethroids, and through genomic studies, understand the mechanisms by which high pesticide tolerance in some populations is attained.

NUMBER OF FESA AND ESA-LISTED FISHES CAPTURED: None; no fishes will be captured.

PROJECT DESCRIPTION

Task 1 – Comparative pesticide sensitivity of H. azteca from various sources

Investigators responsible – D. Weston and M. Lydy.

Specific questions addressed – This task is designed to determine if the extraordinary range in sensitivity to pyrethroids seen among H. azteca populations extends to other pesticide classes, and if tolerance of a given population to one pesticide class confers tolerance to the other classes. In particular, we will determine if H. azteca obtained from a variety of suppliers, and now in use by the primary labs testing samples from the Bay-Delta, have similar pesticide sensitivity, or if toxicity testing results could be strongly influenced by the supplier from which organisms were obtained.

Approach and methods – IEP member agencies contract with many labs for testing of sediment or water samples with H. azteca, and based on the preliminary data we have, it appears essential to determine if results could be a function of the choice of lab and where they obtain their H. azteca. The primary labs working in the Bay-Delta will be contacted to identify their suppliers, and for budgeting purposes we assume five suppliers will be identified (e.g., Aquatic Research Organisms in New Hampshire, Chesapeake Cultures in Virginia, Aquatic Biosystems in Colorado, and the UC Berkeley Weston lab which cultures its own H. azteca are likely to be

four of the five suppliers). For each of these sources, we will attempt to construct a "family tree" to determine where the animals used to start the culture originally came from, when a given culture diverged from the source population, and if additional local animals have been added to the cultures over the years. The history of these cultures can be quite complex. For example, the UC Berkeley culture was obtained from the U.S. Army Corps of Engineers in 2002, who obtained their animals from the University of Mississippi in the late 1990s, who obtained their animals from the US Fish and Wildlife Service in 1994, who obtained their animals from a 1982 wild collection in Oregon, and in 1990 supplemented the Oregon animals with local animals collected in Missouri. We have already determined the ancestral relationships of the UC Berkeley and Chesapeake Cultures animals, and will do so for all other cultures used in this study.

In addition to the five laboratory cultures, four wild populations from northern California will be used for these comparative studies. The intent will be to collect H. azteca populations likely to span a broad range of contaminant sensitivity, and our preliminary studies will be very helpful in this regard. We already have pyrethroid sensitivity data from seven sites to choose among, and if collections from additional sites are needed to obtain suitable animals, we have a listing of over 300 California sites where the DFG bioassessment group has collected H. azteca in the past.

Using these nine populations (five cultured, four wild), we will determine their sensitivity not only to a pyrethroid, but to chlorpyrifos and fipronil sulfone. Testing with a pyrethroid is an obvious choice because there have been numerous published studies showing this group is responsible for much of the H. azteca toxicity observed in testing throughout California (Weston et al., 2004; Holmes et al., 2008). Our preliminary work has provided much of the pyrethroid data needed, and we expect to only need to additional data on a few of the populations we have not previously tested. We have data on both cyfluthrin and bifenthrin, and one of these compounds will be used for the additional testing. Chlorpyrifos was selected because it too has been shown responsible for a significant amount of toxicity (i.e., about 8% of Central Valley agriculture-affected sediment samples in Weston et al., 2008). Fipronil sulfone is an environmental degradate of the pesticide fipronil. While we could do the testing with fipronil itself, we believe the sulfone degradate is a better choice. We have found the sulfone is present in the environment at concentrations comparable to that of the parent compound, yet it is three times as toxic to H. azteca and thus more likely to be responsible for observed toxicity. Since fipronil is an emerging pesticide that has replaced pyrethroids in some uses, and is likely to continue to do so, we believe inclusion of fipronil and degradates is important to keep up with changing pesticide use patterns. All these pesticides cause toxicity by differing modes of action: pyrethroids by interfering with sodium transport, chlorpyrifos by inhibiting acetylcholinesterase, fipronil and its sulfone by blocking the GABA-regulated chloride channel. Therefore, there is no reason to presume tolerance to one will ensure tolerance to the others, though we are intrigued by one population we have found which has achieved tolerance to both pyrethroids and chlorpyrifos.

The outcome of this task will be EC_{50} and/or LC_{50} values for nine populations exposed to three pesticides, with all EC_{50}/LC_{50} determined in duplicate to demonstrate reproducibility (total of 54 EC_{50}s/LC_{50}s, of which approximately ten for pyrethroids and two for fipronil sulfone are already done). Chemistry samples will be taken from each test so that results can be expressed based on actual, rather than nominal, concentrations. The key questions of interest are the degree to which EC_{50}/LC_{50} estimates vary among the cultured populations used for Bay-Delta

monitoring, and whether the sensitivity of these cultured animals is comparable to wild populations.

Interaction with existing monitoring surveys or other studies – As noted above, we have data with pyrethroid pesticides that show a 600-fold variation in sensitivity, depending on the source of animals used for testing. The proposed work expands upon these studies to investigate ramifications to Bay-Delta monitoring and management, and broadens the investigation to other current-use pesticides. In addition, all studies employing H. azteca will benefit from the findings of this study, as we will ensure that all suppliers of amphipods to recent and on-going Bay-Delta monitoring efforts are included so that environmental managers will know if findings are biased by the source of animals that have been used.

Feasibility – Since we have done similar work with pyrethroids, testing three lab cultures and seven wild populations, there should be no problems encountered in expanding these efforts.

Task 2 – Testing of field samples

Investigators responsible – D. Weston and M. Lydy.

Specific questions addressed – There is an implicit and essential expectation of environmental managers that a field sample collected for toxicity testing will yield similar results regardless of which laboratory performs the test. Our preliminary data provides justification to question this assumption, and Task 2 will explicitly test it by establishing if a determination of toxicity or non-toxicity is a function of the source of animals used for testing.

Approach and methods – To determine if the results of a toxicity test could be influenced by the many suppliers, each providing different members of the H. azteca species complex with varying pesticide sensitivities, side-by-side tests will be performed with organisms from the five lab cultures evaluated in Task 1. Field samples will be collected from locations and at times that toxicity is likely, based on our past experience. Samples may include urban runoff, agricultural runoff, or municipal wastewater effluent. While our primary emphasis will be on water samples (because we will have water LC_{50} data for all the contaminants of interest), sediment samples will also be considered as all three compounds are hydrophobic enough to be in both media. After initial screening to confirm toxicity, approximately five samples will be selected for the side-by-side testing with organisms from the five cultures, using standard testing protocols that will be identical across the tests in all respects with the exception of the source of animals used.

To assist in interpretation of results, all samples will be tested for pyrethroids, chlorpyrifos and fipronil and its degradates. We will also use animals from one culture source to perform five simultaneous tests on each environmental sample. The purpose of this step is to establish the normal magnitude of variation of results within a single treatment, so that any variation in toxicity between treatments (the various H. azteca sources) can be attributed to a treatment effect

Interactions with existing monitoring surveys or other studies – Obtaining the toxic field samples for comparative testing will be made easier by the many past and on-going monitoring programs throughout the Bay-Delta which utilize H. azteca. We know where and when to obtain these samples through our own sampling on a variety of past projects (e.g., IEP-funded work in Cache Slough area, SWAMP special studies, Irrigated Lands Regulatory Program). Similarly on-going monitoring of other parties may also alert us to potential sampling locations.

Feasibility – In order to fully compare the toxicity response of animals from the various cultures, we would ideally like all three contaminants of interest to this project (a pyrethroid, chlorpyrifos, fipronil sulfone) to be the cause of toxicity in one or more of the five environmental

samples used for comparative testing. We have analyzed many hundreds of samples throughout the Central Valley for pyrethroids and chlorpyrifos, and are confident we can find environmental samples containing these toxicants. However, it may be more difficult to locate a sample containing toxic concentrations of fipronil sulfone since fipronil and its degradates are relatively new pesticides, no one has done much monitoring for the group, and we have sampled from only about two dozen locations. Conducting Task 2 with a pyrethroid and chlorpyrifos-contaminated material alone would be an acceptable outcome, but if need be, we can create a toxic sample for the comparative studies by spiking clean material with fipronil sulfone.

Task 3 – Determining Hyalella speciation based on genetic similarity

Investigators responsible – G. Wellborn

Specific questions addressed – The discovery that H. azteca is a complex of many distinct species, together with our findings that pyrethroid sensitivity can differ dramatically among its members, implies that it is imperative for toxicology and biomonitoring studies to consider the relationship of their specific organisms to other species within the complex. Evolutionary geneticists have distinguished dozens of members within the complex based on patterns of genetic similarities and differences. In Task 3 we will explore whether these same genetic patterns underlie the differences in pyrethroid, chlorpyrifos, and fipronil sulfone sensitivity we observe among laboratory cultures and natural populations investigated in this project.

Approach and methods –What is now known as H. azteca was described over 150 years ago, and for most of the time since then, has been considered a single species. Only within about the past decade has it been recognized to be a species complex. Its members are generally indistinguishable by morphology, and often can be differentiated only by genetic techniques. In particular, there are specific fragments of mitochondrial and nuclear DNA that can be sequenced to establish if any given individual shares genetic similarity with any of the dozens of previously characterized species within the complex, or if it the gene sequence is sufficiently different to represent a new species. In our preliminary pyrethroid studies, the approach has been extraordinarily successful, showing that populations from four locations separated by over 250 km, and all extremely insensitive to pyrethroid toxicity, were all members of the same genetically-defined species, and were genetically distinct from all the our pyrethroid-sensitive populations. In other words, we could accurately predict a given individual's pyrethroid tolerance solely on the basis its species identity as determined by DNA sequences.

These same mitochondrial and nuclear DNA fragments will be sequenced in all nine populations (five cultured, four wild) evaluated within Task 1. The fragments will be PCR-amplified, sequenced, and genetic differences among and within populations determined. Not only will the data be helpful in interpreting pesticide sensitivity in light of genetic relationships, but it may prove invaluable in completing the "family tree" of Task 1 showing the ancestral relationships of the various laboratory cultures. Many of these cultures likely were started over 20 years ago and current staff at the labs may not know from where the cultures originated. Genetic similarities may help establish where the cultures originated even if this information has been forgotten by employees currently working at the various firms that supply H. azteca.

Interactions with existing monitoring surveys or other studies – This study allows us to place our results into the broader species diversity of the H. azteca species complex that has been documented through DNA analysis over the past decade. For example, the natural geographic range of many species within the complex is documented in many cases, and these range data

will allow us both to determine the native range of Hyalella that are sequenced for this project, and to assess any natural geographic patterns of pyrethroid resistance.

Feasibility – Feasibility of this task is very high. Dr. Wellborn has extensive experience in genetic analysis of Hyalella amphipods, including DNA sequencing and analysis of DNA sequence data. We have developed laboratory protocols, including development of a library of PCR primers for Hyalella, that yield very high success rates in recovering high quality sequence data.

Task 4 – Mechanisms of H. azteca pesticide resistance and Diagnostic Assays of Resistance

Investigators responsible – H. Poynton and R. Connon

Specific questions addressed – Mechanistically, why are some populations of H. azteca more or less resistant to pesticide exposure than others? Can we screen a population genetically to determine which pesticides it may be resistant to? Testing numerous additional populations by comparative LC_{50} tests, as described in Task 1, would be a formidable effort. Therefore, in this task, we propose to determine the mechanism of pesticide resistance (e.g., increased metabolism of pesticide or mutation in target site) and develop screening assays, H. azteca Diagnostic Assays of Resistance (Ha-DARs), to quickly determine if other field populations or commercial lab strains are resistant to one or more pesticides.

Approach and methods – Organisms generally become resistant to pesticides by either metabolic and target site resistance (Perry et al, 2011; Ffrench-Constant et al., 2004). **Metabolic resistance** occurs when an organism gains the ability to metabolize and excrete a pesticide keeping the pesticide from accumulating at its target site. This mechanism involves a rise in metabolic enzyme levels, which often occurs through increased gene expression. For example, increased expression of cytochrome P450 *cyp6d1*, glutathione S-transferase, and esterase genes all have been implicated in pyrethroid resistance (Tomita and Scott, 1995; Vontas et al., 2001; Field et al., 1993). **Target site resistance** occurs when organisms acquire mutations at the site of action for the pesticide, inhibiting the binding of the pesticide to its target protein. In our studies of pyrethroid resistance in H. azteca, we have identified two mutations in the voltage-gated sodium channel (*vgsc*), the target for pyrethroid toxicity. Similar mutations have been found in the same protein in pyrethroid-resistant insects (Williamson et al., 1996; Morin et al., 2002).

Next generation sequencing (NGS) technologies offer an innovative solution for uncovering the mechanisms of pesticide resistance. By sequencing the mRNA produced in exposed populations, we can identify genes with elevated expression in resistant populations as well as detect any mutations in target gene sequences. Therefore, our approach will be to use NGS to sequence the most sensitive and most tolerant H. azteca populations to each pesticide to identify potential mechanisms of pesticide resistance (Task 4A). Targeted molecular studies on candidate genes in all nine laboratory and field populations will be used to verify that the gene expression or mutation analysis corresponds with pesticide tolerance and determine if there are differences in resistance mechanisms across the populations (Task 4B). Finally, after establishing mechanisms of resistance for each pesticide, we will develop screening assays to quickly identify resistant populations in field-collected or laboratory-cultured H. azteca (Task 4C).

Task 4A - Identifying genes potentially involved in pesticide resistance. NGS will be performed on RNA isolated from the most sensitive and the most resistant H. azteca populations identified in Task 1 for each pesticide class (a pyrethroid, chlorpyrifos, and fipronil sulfone). For each population, we will expose the H. azteca to the population-specific No Observable Effect Concentration. RNA will be isolated from unexposed and pesticide-exposed individuals. mRNA-

seq libraries will be constructed at the UC Davis Genome Center using TruSeq RNA Sample Preparation Kit for sequencing on the Illumina HiSeq 2000 using paired-end sequencing for maximum length reads and coverage. Sequence assembly and gene expression analysis will be performed by the Bioinformatics Core at the UC Davis Genome Center using Illumina software (e.g. ELAND) and an in-house bioinformatics pipe-line.

Genes potentially involved in pesticide resistance and sensitivity will be identified through several different avenues by analyzing the NGS data:

(1) Genes that are highly differentially expressed in the resistant or sensitive populations may be related to the insecticide resistance. Sequences identified by NGS in this category will be annotated using homology-based searches including Blast2Go and also through investigation of potential protein domains thought the NCBI GenBank database. Sequences with homology to genes involved in detoxification (carboxylesterases, glutathione-S-transferases, and cytochrome P450 monooxygenases) will be selected as candidates of insecticide resistance.

(2) Genes with alternate splicing or significant nucleotide mutations, resulting in amino acid substitutions, in the resistant or sensitive populations may be related to insecticide resistance. Sequences identified by NGS in this category will be annotated as described above. Sequences with homology to genes involved in the mechanism of action for these pesticides (*vgsc*, acetylcholinesterase, GABA-regulated chloride channel, and others found through literature review) will be selected as candidates of insecticide resistance.

Task 4B - Characterizing candidates genes and revealing their association with pesticide resistance. Additional molecular studies will be conducted on the other seven populations not addressed by Task 4A. These studies will be partially dependent on the number of candidate genes found and the annotation of these genes, but may include: (1) reverse transcription quantitative PCR (RT-qPCR) to determine the expression level of these genes in other seven populations; and (2) targeted sequencing of these genes (e.g., Sanger) to identify the presence of mutations in the other seven populations.

Task 4C - Developing molecular diagnostic assays (Ha-DARs) for the presence of resistance in H. azteca populations. We will evaluate RT-qPCR and PCR-based methods targeting the genes identified as responsible for pesticide resistance in task 4B. We have extensive experience developing RT-qPCR assays for the assessment of contaminants exposure in aquatic fish and invertebrates (Connon et al., 2009; 2011; Beggel et al., 2011, 2012; Poynton et al., 2007; 2008; 2011; 2012). We will use similar methods as described previously to develop RT-qPCR assays for the induction of metabolic enzyme gene expression.

Although many sophisticated high-throughput methods for single nucleotide mutation, or polymorphism (SNPs), detection exist, our goal is to develop an accessible and inexpensive assay for detecting insecticide resistance mutations. PCR assays with mutation specific primers, referred to as PCR amplification of specific alleles (PASA) are commonly employed, inexpensive methods for detecting SNPs in insects (Black and Vontas, 2007). Alternative methods can also be used, such as PCR-RFLP, which employs restriction enzyme digestion following PCR, and has been recently used to identify pyrethroid-resistant insect populations in Crete (Tsagkarakou et al., 2009).

Interactions with existing monitoring surveys or other studies –The work proposed in Task 4 expands upon the results of Task 1 by providing information on the mechanism of resistance of H. azteca populations in the Bay-Delta area and those used by laboratories involved in monitoring in California. In addition, monitoring efforts in the Bay-Delta will benefit from the development of Ha-DARs (Task 4C), which will enable screening of field populations as

well as commercial populations of H. azteca for possible resistance that would bias monitoring efforts.

Feasibility –Drs. Poynton and Connon have extensive expertise in conducting the proposed research. For example, in our preliminary work we have identified two mutations in *vgsc* target site for pyrethroid pesticides, that cause amino acid substitutions at critical sites and provide resistance to the pesticide by inhibiting its binding to the target site. Our experience, along with collaboration with the UC Davis Genome Center, provides optimal means towards the identification of mechanisms of pesticide resistance in H. azteca. We will use PCR to identify point mutations and RT-qPCR to examine differences in gene expression, and both are well-established and highly successful conventional molecular biology techniques for these purposes.

DELIVERABLES – Deliverables are addressed here for the project as a whole, since comparable methods will be used to disseminate study results regardless of the specific task. We anticipate great interest in the study because its results have ramifications to so many programs and stakeholders utilizing H. azteca for environmental monitoring. We anticipate two or more oral presentations. At least one will be in a regional venue, for example the annual IEP Workshop, and the second may be at a national conference, such as the annual meeting of the Society for Environmental Toxicology and Chemistry. We would also be happy to make presentations to IEP's Interagency Management Team and the Regional Board's Contaminant Work Team.

We anticipate the final report will in a format suitable for submission to a peer-reviewed journal. We will submit the manuscript prior to the end of the project period, though actual publication is likely to take 3-6 months and occur after project completion. We also anticipate one newsletter article, appearing in a publication such as the IEP Newsletter or Pulse of the Delta.

BUDGET – The results of this study will be of considerable importance to all parties that test environmental samples with H. azteca, or rely upon such data. As an indication of this interest, the Sacramento Regional County Sanitation District (SRCSD), which is evaluating the species for testing of its effluent, has indicated a willingness to support this study. The District will commit $20,000 to this project, if funded by IEP. The budget below assumes the District's commitment is applied to Task 1, and only shows the additional IEP costs.

	Task 1	Task 2	Task 3	Task 4	TOTAL
Salaries	51624	8368			59992
Fringe benefits	12801	2181			14982
Supplies and Expenses	10340	4200			14540
Prof. & Consulting Services			3000		3000
Travel	1800	1000			2800
Subawards					
UC Davis (Connon)				54186	54186
U Mass. (Poynton)				48638	48638
S. Illinois Univ. (Lydy)	14668	7404			22072
Total Direct Costs	91233	23153	3000	102824	220210
Indirect Costs	50908	12919	1674	13950	79451
Total Costs	142141	36072	4674	116774	299661

1. Severability of tasks – The package of tasks we propose will allow us to thoroughly investigate the questions, and provide insight on a range of topics from the genetic mechanisms by which contaminant tolerance is achieved, to its very practical consequences of whether environmental monitoring results are biased by differential tolerance. Therefore we hope the work can be funded in its entirety, but in accordance with instructions, we address here the independence of each task. Task 1 is essential to the performance of all subsequent tasks. Task 3 is an independent task, but it will be extremely helpful in interpreting Task 1 findings, and its value added far outweighs its small additional cost. Both Tasks 2 and 4 are discrete units, to be done secondarily to Task 1. Task 2 demonstrates how different pesticide sensitivities can influence monitoring results, while Task 4 identifies the mechanisms underlying Task 1 findings.

2. Gary Wellborn's participation will be handled through a contract with him individually, rather than a subaward to his institution, so is budgeted under Professional & Consulting Services.

3. Indirect costs – The federally-negotiated indirect cost rates at UC Berkeley are 55.5% for the first eight months of this study (through 6/30/13) and 56.5% for the last four months (until project end 10/31/12). Therefore, the rate at which costs are incurred is assumed constant over the whole project period and a weighted average indirect rate of 55.8% has been used. This rate is applied to all of the costs other than subawards. For subawards, this rate is applied to only the first $25,000 to University of Massachusetts and Southern Illinois University. UC Berkeley applies no additional indirect costs on subawards to other UC campuses, such as UC Davis.

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