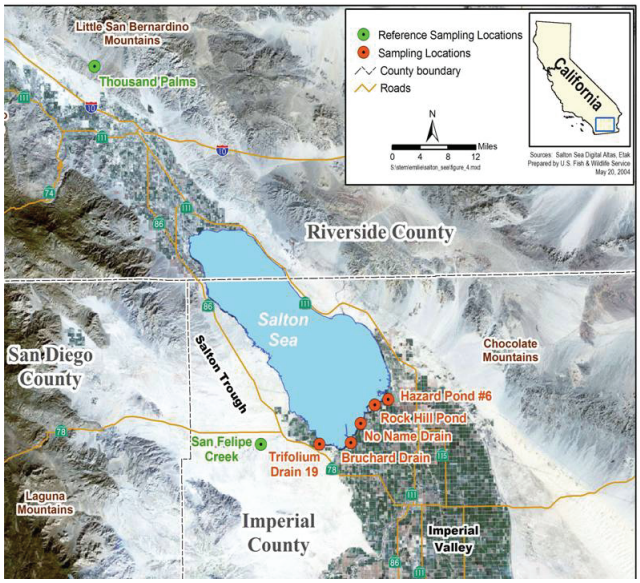
USGS
science for a changing world

National Wetlands Research Center

Bioindicators from Mosquitofish (*Gambusia affinis*) Sampled from the Imperial Valley in Southern California

By Jill A. Jenkins and Rassa O. Draugelis-Dale



Open-File Report 2006-1307

U.S. Department of the Interior
U.S. Geological Survey

U.S. Department of the Interior
DIRK KEMPTHORNE, Secretary

U.S. Geological Survey
Mark D. Myers, Director

U.S. Geological Survey, Reston, Virginia 2006

For more information on the USGS—the Federal source for science about the Earth, its natural and living resources, natural hazards, and the environment: World Wide Web: http://www.usgs.gov
Telephone: 1-888-ASK-USGS

Outside front cover photograph: Imperial Valley, California, showing sampling sites. Figure contributed by the Environmental Contaminants Division, USFWS, Carlsbad, California.

Suggested citation:
Jenkins, J.A., Draugelis-Dale, R., 2006, Bioindicators from mosquitofish (*Gambusia affinis*) sampled from the Imperial Valley in southern California: U.S. Geological Survey Open-File Report 2006-1307, 48 p.

Contents

Figures

Tables

Background Information

The Sonny Bono Salton Sea National Wildlife Refuge (SSNWR) is located 64 km north of the Mexican border at the southern end of the Salton Sea in California's Imperial Valley. Freshwater ponds and managed habitats at the SSNWR, Calipatria, Calif. are supplied with Colorado River water that carries compounds from upstream sources. Components include municipal and industrial discharges, agricultural drainage, and sewage plant inputs. Aquatic animals in these ecosystems are continuously exposed to multiple constituents, several of which have been demonstrated to be associated with hormonal disturbances (Carey and Bryant, 1995; Gross and others, 2003). To investigate possible endocrine impacts to fish in the Imperial Valley, Calif. these experiments addressed the null hypothesis that aquatic species in impacted sites did not exhibit evidence of endocrine disruption as compared with those from nonimpacted sites.

The results presented are intended to provide managers with science-based information and interpretations about the condition of the animals in their ecosystems for the minimization of potential adverse effects to trust fish and wildlife resources and for the maximization of available water resources. These data address IAG FWS#11430-10HO32 in the USFWS proposal entitled "CA – Effects of Endocrine Disruptors on Aquatic Species Using Pond Habitats at the Sonny Bono Salton Sea National Wildlife Refuge."

Many human-made chemicals can disrupt endocrine systems of wildlife (Colborn and Clement, 1992) and can affect tissues at concentrations well below detectable levels. Hence, for investigating causal effects, the use of biomarkers for assessing contaminant exposure is necessary (Carey and Bryant, 1995). Bioindicator data are measurable and directly reflect the condition of the animal. Two important features of bioindicators are the ability to detect interactions between stressors and organisms and the capability of quantifying sublethal effects. Obtaining data from more than one biomarker can furnish a more precise assessment of the physiologic effect and bioavailability. Xenobiotic pollutants may disrupt reproductive endocrine function by acting at the level of several organs, such as the hypothalamus, gonad, and liver. Disruption at any of these organs may result in either changes in the rate of gonadal development or in the viability of the gametes, as well as the arrest of gametogenesis (Kime and Nash, 1999) or altered

organosomatic indices. Most endocrine disrupting studies have focused on reproductive impacts; however, other effects have been documented, including those on growth, metabolism, thyroid, and immune function (Gross and others, 2003).

A large part of this report highlights studies on sperm cells from mosquitofish (*Gambusia affinis*) from Imperial Valley sites. A sperm cell consists of several membrane compartments, and cell competency requires that each of these membrane compartments be intact (Graham and others, 1990). The combination of multiple assays on sperm is a better predictor of male fertility than any individual test. The classic method of assessing the viability of sperm is to determine the percentage of progressively motile cells by using a microscope (Jenkins, 2000). This is an indirect method of assessing metabolic activity, and results reflect inherent variability because of the subjective nature of collecting data on motility by individual researchers using microscopy. In this study, sperm membrane integrity was assessed by cell viability staining, where nucleic acids inside intact membranes were stained fluorescent green by the stain SYBR-14, whereas nonintact or permeable membranes allowed a red counterstain (propidium iodide) to enter the cells so that "dead" or moribund cells stained fluorescent red. Upon cell death, propidium iodide (PI) rapidly overwhelmed the fluorescence exhibited by SYBR-14. Both the sperm viability assay and mitochondrial function assay assessed the functional capacity of sperm in an objective manner. This fluorometric staining combination of SYBR-14 and PI has been shown to be a rapid and reliable means for determining the proportions of living and dead sperm in several species across taxonomic lines (Ericsson and others, 1990; McNiven and others, 1992; Garner and Johnson, 1995; Donoghue and Donoghue, 1997; Thomas and others, 1998; Segovia and others, 2000; Adams and others, 2003; Salinas-Flores and others, 2005; Lezcano and others, 2004). In this study, flow cytometry technology was used for sperm viability and mitochondrial function as well as for sperm counts per mg testis and testis cell meiotic stage of maturation.

The field of flow cytometry had practical research and clinical applications for immunotoxicology (Hudson and others, 1985), for testing in vitro toxicity of chemicals (Tuschl and Schwab, 2004), and for male human infertility diagnostics. It is increasingly being used in sperm cryopreservation studies. This is multiparameter analytical instrumentation for assessing nuclear and cellular components of interest in freely

flowing cells. Individual cells or nuclei of any tissue from any species can be probed by a laser light, and emitted light signals are transferred quickly into electronic measures so that typical sample sizes approximate 10K – 50K events and data are collected within ~1 min per sample. This report is the first such comprehensive multiparameter study of potential sperm impacts in an environmental field study. This report documents the use of this technology for detection of potential cellular and molecular impacts on sperm from mosquitofish in ecosystems in the Imperial Valley.

Just as the study of more than one biomarker per individual increases confidence in health assessments, in addition to reproductive endpoints, other measures of animal condition (such as hepatosomatic index – HSI) were included in this study. In these studies, we assessed the quality of sperm and morphological indices and analyzed hormone concentrations from multiple years. Morphological indices included gonopodium length, hepatosomatic indices, and gonadosomatic indices. Contaminants data obtained from fish and the sites were analyzed. Multiple researchers and other personnel contributed data and efforts in this study.

Materials and Methods

Collections

Gambusia affinis were collected by personnel in California by using pulsed DC electroshocking, hoop nets, or seining. Blood samples were collected (Goodbred and others, 1997). Fish, blood, and soil samples were sent out for respective analyses, including live fish shipped overnight in July 2003 for analyses at the National Wetlands Research Center (NWRC). Samples were received at the NWRC from the following sites: "Hazard Pond 6" on July 15, 2003, "Trifolium Drain 19" on July 16, 2003, and Thousand Palms on July 17, 2003. Thousand Palms was a reference site where male mosquitofish were available.

Overall, samples were obtained in 2001, 2003, and 2004. In 2001 (June 11-13), fish were collected for analyses from "Rock Hill Trail Pond," Hazard Pond 6, "No Name Drain," Trifolium 19 Drain, "Bruchard Drain," and the reference site San Felipe Creek. No males were available from San Felipe Creek in 2001. In 2004, the five sites sampled

were Prado Dam, “Rialto Dam,” “Rix,” “Sunny Slope,” and Thousand Palms. This report includes statistical analyses of data obtained over these three sampling years.

Preliminary Experiments at NWRC

Dissection methods, buffer choice, and sperm cell preparation methods were optimized. The duration of motility of sperm cells was sometimes over 26 min; hence, duration of motility as a metric was not feasible for use as a biomarker, and percentage motility as a metric did not prove to be a powerful metric for condition as substantiated by statistical analysis (see results below). Less subjective sperm motility analyses may be investigated by using computer assisted sperm motion analysis (CASA), as routinely applied with domestic mammalian sperm (Xu and others, 2001), and from which we have data using sperm from fathead minnows (*Pimephales promelas*) whereby progressive motility was significantly positively related to gonadosomatic index (GSI) (Jenkins, unpublished data).

The sperm cell viability assay based on a membrane integrity principle, whereby membrane-intact cells are considered viable, was employed with *Gambusia affinis* sperm. With individual fish (n=3) obtained locally in Louisiana, standard curves were generated by using known live and dead cell mixtures. Sperm at 1 x 106 per mL (3 mL in a 10 mL tube) were killed by heating in a waterbath at 80EC for 10 min. The assay was verified for identifying viable and nonviable cells of known live and dead mixtures (fig. 1) in triplicate. The R^2 values for each of the curves were 0.9911 (fig. 1), 0.9867, and 0.9951. The buffer used for analyses was Hank’s Balanced Salt Solution (HBSS) (Glenn, 1998) without Ca++ or Mg++ at pH 7.5 at 311 mosm/kg (HBSS).

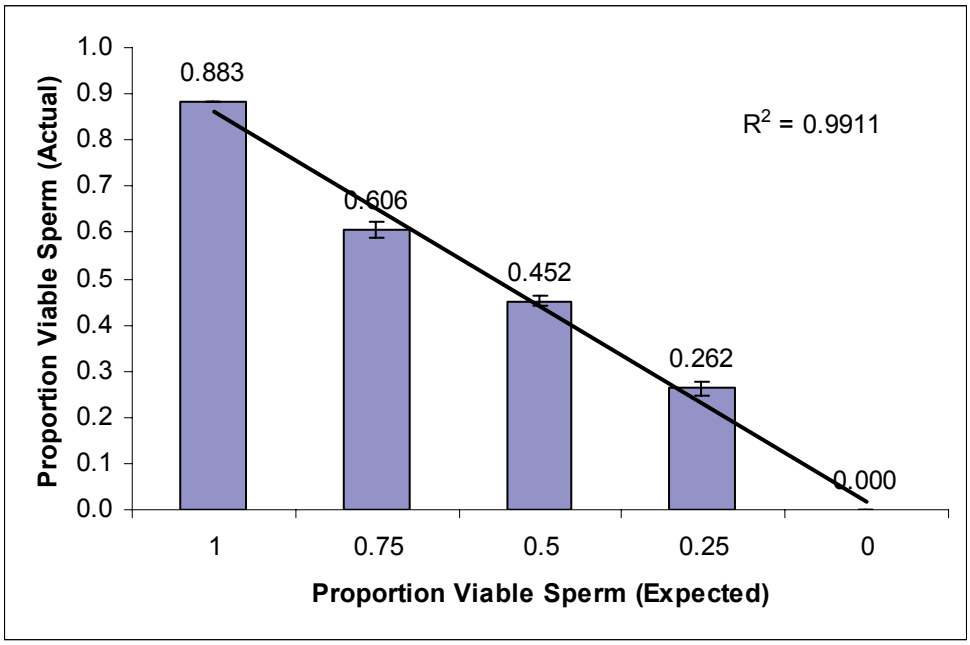


Figure 1. Standard curve demonstrating verification of a flow cytometric membrane integrity (viability) assay for use with mosquitofish (*Gambusia affinis*). Data were generated by using known proportions of live and heat-killed sperm from one fish. Representative data are presented from one fish.

A flow cytometric assay for sperm counting was verified for use with dilutions of fresh sperm from Nile tilapia (*Oreochromis niloticus*), green swordtail (*Xiphophorus helleri*), and *Gambusia affinis* (fig. 2) with R^2 values of 0.9364, 0.9941, and 0.9456, respectively. By using *Gambusia affinis* sperm from the July 17, 2003 collection, counts obtained by both microscopy and flow cytometry methods were compared statistically (see statistical results below).

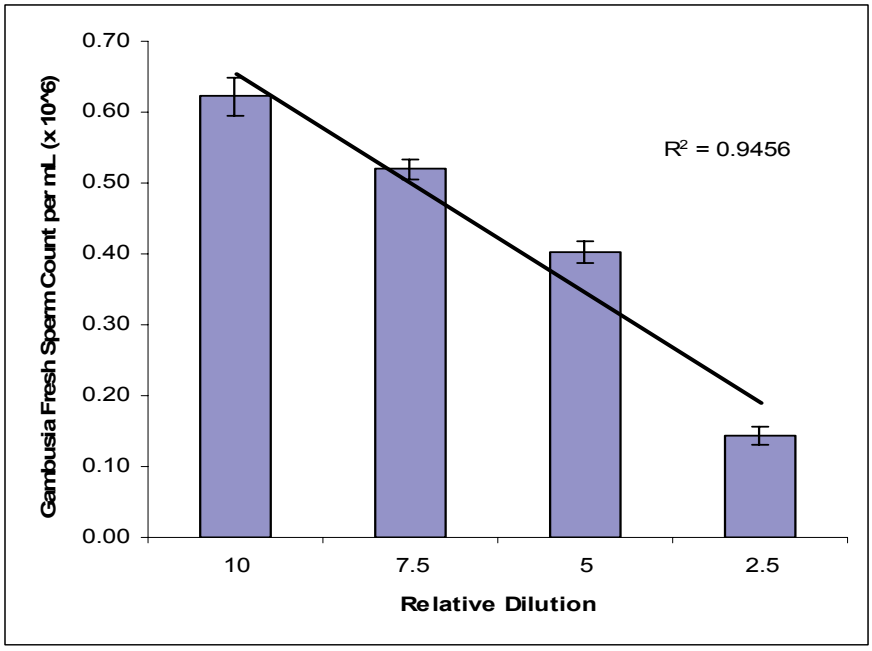


Figure 2. Standard curve demonstrating verification of flow cytometric particle counting methodology developed for use with sperm from mosquitofish (*Gambusia affinis*) generated by using known dilutions of fresh sperm.

Morphological Measures

Fish arrived by overnight mail, live fish were processed completely, and morphological measures were made by using intact but not yet decomposed dead fish. Live fish were euthanized with tricaine methane sulfonate (Nickum and others, 2004). Total fish length and gonopodia lengths (fig. 3) were measured with digital calipers, and fish were weighed. Gonopodia were severed and stored in buffered formalin at 4EC for potential future analysis of gonopodial rays and hooks as secondary sex characteristics (Toft and others, 2003). Fish were dissected with microtools by using a dissecting microscope, and liver and testis (fig. 4) were extracted and weighed. Condition and color of organs were noted. During the few minutes between organ handling and sperm cell processing, the testis was placed in a humidified chamber until it was cut in two pieces. One piece was weighed for sperm count analysis, and the other portion was used for assessing sperm motility, mitochondrial function, and viability.

Biomarker data were collected on GSI and hepatosomatic index HSI.

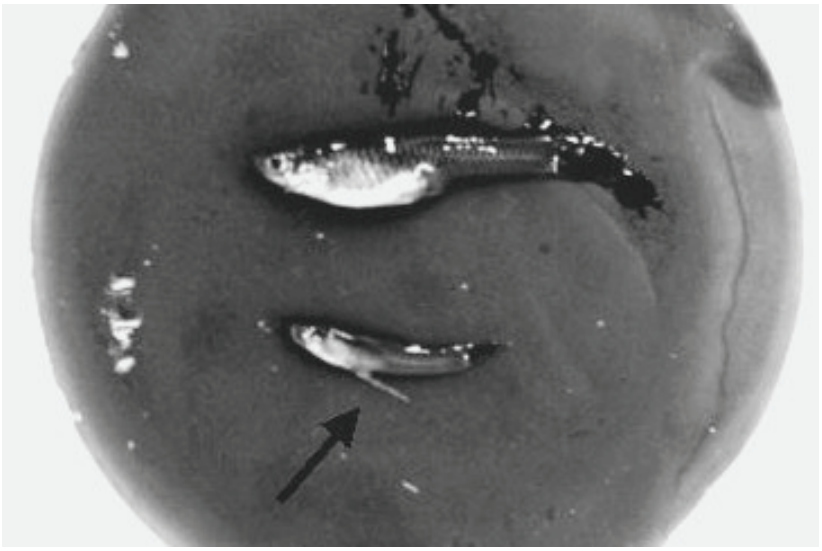


Figure 3. Gonopodium on male mosquitofish (*Gambusia affinis*).

Figure 4. Dissected testis of mosquitofish (*Gambusia affinis*) overlaying the bottom half of scissors.

Sperm Counts

For sperm extraction prior to counting, the weighed testis half was placed in 100 µl 0.175 mM salt concentration KCl (13.046 mg/L) (Toft and others, 2003). The tissue was minced for exactly 1 min, maintained in the KCl solution for 10 minu, 100 µl buffered formalin was added, and the tube stored at 4 °C until counting. After thorough resuspension of stored cell suspensions, sperm cells, partial sperm packets, and whole sperm packets were enumerated in triplicate with a hemocytometer and after trypan blue staining for ease of cell visualization by using microscopy, the following formulae were used:

For sperm:

$$\frac{\textit{Number sperm counted in 5 small squares}}{80} \; X \; (4.0 \times 106) \; X \; \textit{(dilution factor)}$$

For packets:

$$\frac{\textit{Number sperm counted in 4 large squares}}{64} \; X \; 1000 \; X \; 160 \; X \; \textit{(dilution factor)}$$

By flow cytometry, sperm cells and cells from mashed testes were enumerated by using a Bacteria Counting Kit (Molecular Probes, Eugene, Oreg.), whereby florescent SYTO BC dye and a known concentration of polystyrene microspheres were included with 10 Φl sperm cell aliquots in 250 sheathe buffer (Becton Dickinson Immunocytometry System [BDIS], San Jose, Calif.) (fig. 5). Cell count results were expressed as both cells per mL of buffer and cells/mg testis. Both microscopy and flow cytometry counting methods were employed with samples received from Thousand Palms July 17, 2003, and statistical analyses were performed to compare counts by the methods (see Number 1, below in Results).

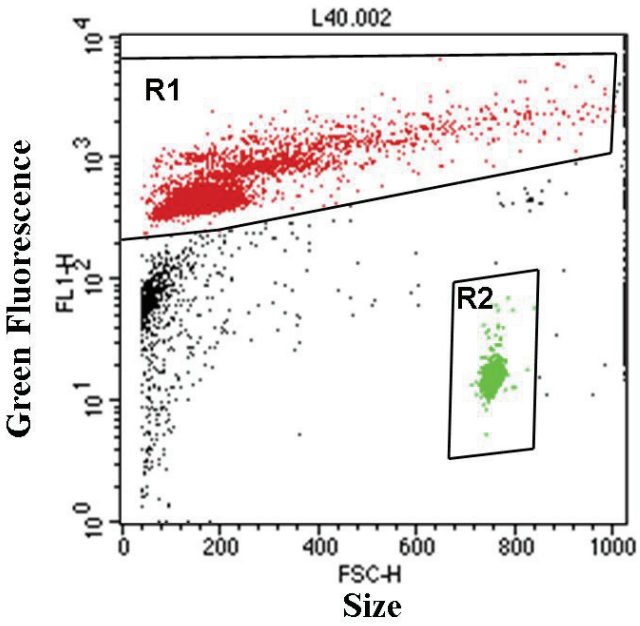


Figure 5. Flow cytometric dot plot illustrating the experimental, unknown sperm cell concentration in Region 1 (R1) compared with a known number of microspheres in R2. Particle size is on the x-axis (forward scatter or FSC-H) parameter, and green fluorescence of stained sperm and microspheres is on the y-axis (fluorescence detector FL1-H).

Sperm Motility

The percentage of sperm showing forward movement was assessed by visualization with darkfield microscopy at 100X magnification. An aliquot of 0.25 Φl sperm was mixed with 25 Φl HBSS, and progressive movement in triplicate by three separate readers was estimated to the nearest 5%. Sperm morphology (figs. 6 and 7) and obvious parasites or bacteria, if present, were noted.

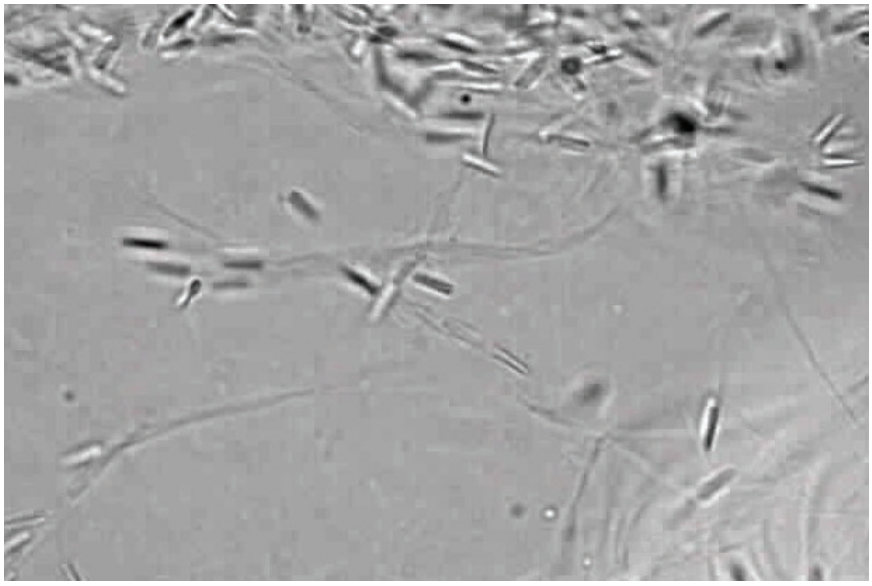


Figure 6. Mosquitofish (*Gambusia affinis*) sperm magnified 600 X by using phase microscopy. Individual sperm heads with tails are noted.

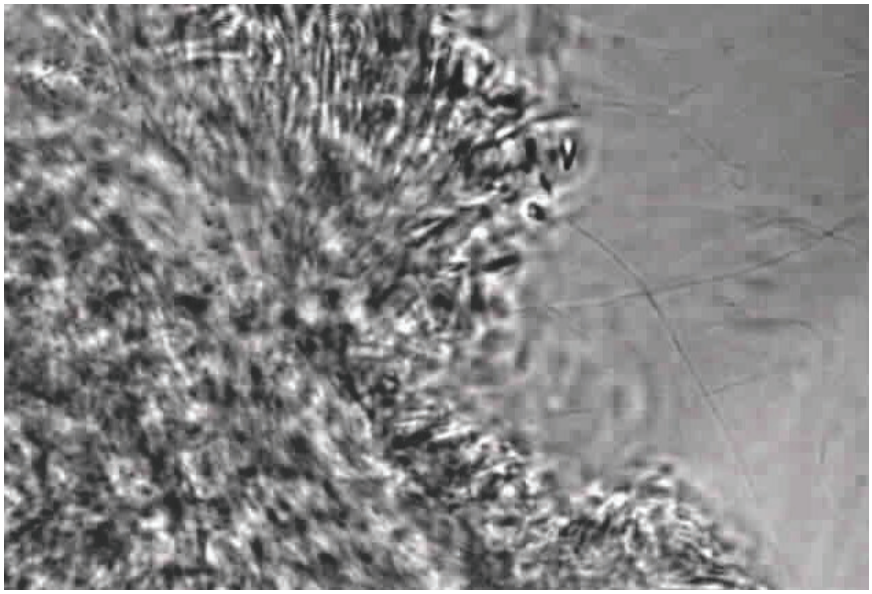


Figure 7. Edge of a sperm packet, or spermatozeugmata, where sperm are closely aligned.

Sperm Cell Viability and Mitochondrial Function

Cell suspensions were filtered with 30 Φm nylon mesh (Small Parts, Miami Lakes, Fla.) prior to dilution to 1 x 106 /mL. Suspensions of cells at 250 Φl aliquots were stained by using a live/dead sperm viability kit (Molecular Probes, Eugene, Oreg.) with a starting stock solution of 1:100 SYBR-14 (Segovia and others, 2000). For mitochondrial membrane potential, dual fluorescent staining with Rhodamine 123(R123) (Sigma-Aldrich Chemical Company Co., St. Louis, Mo.) and PI was used according to Segovia and others (2000). The analytical instrumentation used to obtain data was a flow cytometer (FACScan, [BDIS]). For the verification of cell condition and probes employed, epifluorescence microscopy (Leitz Diaplan) was used in conjunction with flow cytometry.

Sperm Maturity

Stored cell suspensions were stained with PI, RNAse A at 1 μg/mL, and 0.1% (v/v) Triton X-100 for 30 min at 24°C. Stained cells were filtered through a 30 μm nylon mesh, and distributions of nuclei in different meiotic stages were analyzed with a flow cytometer (FACScan [BDIS]). Nuclei were analyzed at 1 x 10^6 per mL at a rate of 300 per second, and 5-10 K events were collected by using a 1024-channel FL2 parameter at 340 linear, with linear size and scatter parameters, and doublet discrimination mode. Samples were run in triplicate. Histograms, dot plots, and density plots were generated by using CellQuest software (BDIS), with each sample analyzed in three ways: FSC-H versus side scatter (SSC), fluorescence detector 2 area (FL2A) histogram, and fluorescence detector 2 width (FL2W) versus FL2A in doublet discrimination mode (DDM). Nuclei were gated in DDM so to disregard aggregated nuclei in the analyses, and gated FL2A histograms were used to obtain event numbers under the peaks representative of different meiotic stages.

Contaminants Analyses

Whole Body Collections and Tissue Analyses

The two replicate composites per site of male and female mosquitofish of random lengths and of a minimum weight of 15 g were analyzed for 28 chlorinated compounds having a Log octanol-water partition coefficient (K_{ow})>4 by using capillary-column gas chromatography (GC) and electron-capture detection (Leiker and others, 1996). The fish composites had been placed into chemically cleaned jars, frozen on dry ice in the field, and stored at -20°C until shipment to the USGS National Water Quality Laboratory (NWQL) in Denver, Col. Processing, analyses, quality assurance parameters, recoveries, and methods performance standards are detailed in Leiker and others (1996).

Bed Sediments

From 10 locations within each fish sampling site, bed sediments were collected by using a modified procedure (Shelton and Capel, 1994). A chemically cleaned teflon scoop was used to collect two replicate bed sediment samples from the first 2 to 3 cm of the bottom material and composited. The samples were thoroughly mixed in chemically cleaned glass jars, frozen on dry ice, and stored at -20 °C until ready for analyses at the NWQL. Chemical analyses for 31 organochlorine compounds and polychlorinated biphenyls (PCBs) were done by dual capillary-column GC with electron-capture detection (Foreman and others, 1995).

Polar Organic Chemical Integrated Samplers (POCIS)

In order to sequester very hydrophilic compounds such as pharmaceuticals, personal care products, hormones, and newer generation pesticides having low Log K_{ow}s, POCIS were deployed at each site for 30 days in June/July 2003. Passive samplers integrate environmental contaminants during deployment, therefore compounds at very low levels, or present episodically, are enriched. Thus, these contaminants are better able to be detected than by using other sampling methods. The POCIS, made with an admixture of solid phase sequestering media encased in a microporous polyethersulfone membrane (Alvarez and others, 2004), were prepared by the USGS Columbia Environmental Research Center (CERC), Columbia, Miss. The two different types of sorbents constructed to maximize the retention and recovery of a wide variety of analytes were the Type “A” sorbent tailored for pharmaceuticals and the Type “B” sorbent containing the triphasic sorbent admixture tailored for most

pesticides, herbicides, and hormones. The discs with each sorbent were placed in stainless deployment devices and secured at each site to ensure it was underwater during the entire sampling time. A field blank was opened during both deployment and retrieval of the POCIS to account for any potential atmospheric contamination. After the 30 days, samplers were retrieved, put in air tight cans on ice, and shipped to CERC for enrichment and dialysis according to procedures in Petty and others (2004).

Methodologies and Results

Physical conditions at the three sites are presented in table 1.

Table 1. Water quality data per site on collection dates (July 14-16, 2003)

	Temperature (^{B}C)	Specific Conductivity (ms/cm)	Dissolved Oxygen (mg/L)(%)	pH	Turbidity NTU*
Hazard Pond 6	28.53	1.126	5.93 (76.2)	7.98	75.5
Trifolium Drain 19	27.88	6.420	6.67 (88)	6.92	5.3
Thousand Palms	29.21	1.881	6.23 (81.7)	7.69	0.5

* Nephelometric units.

In this report, the scientific hypothesis is provided per experiment, there is a brief description of methods, and statistical results are presented. Statistics were run with reference to analyses in Toft and others (2003), with an alpha level at 0.05. All statistics were performed by using SAS (1990).

1. Comparison of Microscopy and Flow Cytometry Methods for Counting Cells

H_{01}: There is no difference in cell counts as determined by hemocytometer use and flow cytometry.

Cells were extracted from fish from Thousand Palms, suspended, stored, and then diluted for duplicate counts by each method (microscopy and flow cytometry). Respective formulae for back-calculating sperm per ml were employed, and testis weight was used to adjust values to obtain counts per mg testis.

The general linear models procedure determined that there was no difference in counts/mg testis when using both counting methods ($p=0.9271$)($F_{(1,40)}=0.01$; $P = 0.9271$) (fig. 8.) All statistics were performed by using SAS (1990). Therefore, count data for 2003 were obtained by using flow cytometry.

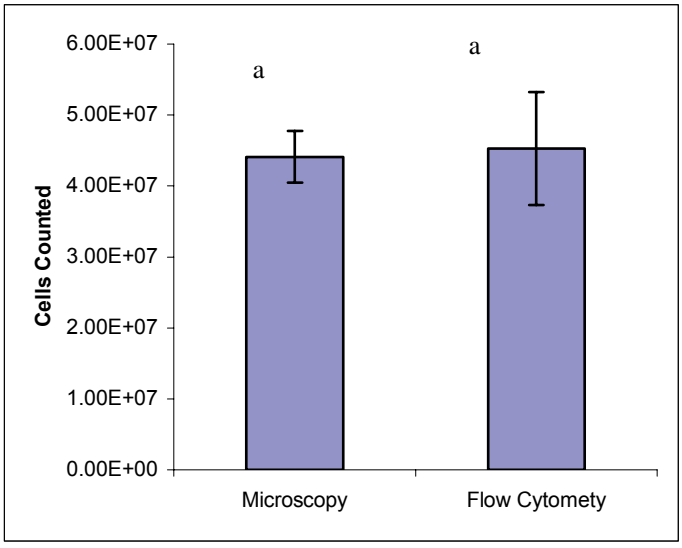


Figure 8. Average sperm counts per mg testes (n=21) of mosquitofish (*Gambusia affinis*) received July 17, 2003 from Thousand Palms by using two different methods (*P*=0.9271).

2. Comparison of 2003 Sperm Counts per Site Determined by Using Flow Cytometry

H_{01}: There is no difference in *Gambusia affinis* sperm counts per site.

By using samples received July 15-17, 2003, cells per mg testis were determined by using flow cytometric methods.

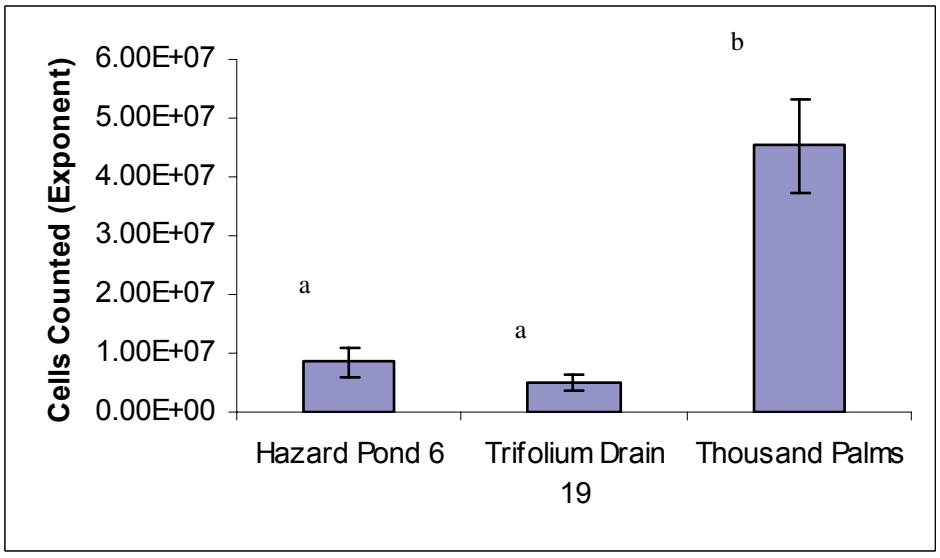


Figure 9. Average sperm counts (raw data) per mg testes (n=12, 13, 21 of mosquitofish [*Gambusia affinis*] per site, respectively) for 2003 (*P*<0.0002).

The data on sperm counts/mg testis were log transformed. The covariates fish length, gonopodium length, and weight were not correlated with log (count) as determined by a correlation analysis; however, an analysis of covariance run with these values as covariates resulted in significant differences in sperm counts between sites ($p=0.0002$). By using Duncan's and Tukey's multiple comparisons tests, sperm counts at Thousand Palms were found to be significantly higher than those at both Hazard Pond 6 and Trifolium Drain 19 sites, with both grouped alike. Covariates were not significant, so there was no need for them to serve as covariates in analysis of variance. The results from an ANOVA achieved by using nontransformed values are reportable.

3. Comparison of 2003 Sperm Mitochondrial Function and Sperm Viability per Site

H_{01}: There is no difference in *Gambusia affinis* sperm mitochondrial function (membrane potential) and membrane integrity (viability) and per site.

For mitochondrial function, statistical analyses were performed both with proportions of functional or live cells (in other words, the proportions of sperm that had functioning mitochondria/[functional + nonfunctional]), where values were arcsin[sqrt] transformed for a one-way ANOVA and analyzed with the exact proportions formulae (Zar, 1984) between pairs of sites [pairwise comparisons between each pair of sites]. No covariates were used in the model.

The results of the ANOVA found that site was significant, $F_{(2,129)} = 40.25$, $p<0.0001$. Both Duncan's multiple range test and Tukey's studentized range (HSD) test showed significant groupings of both contaminated sites having higher proportions of sperm with functioning mitochondria than the control site.

By the exact proportions formulae, pairwise comparisons are:

Hazard Pond 6 vs Thousand Palms	$t=244.138$, $p<0.0000$
Trifolium Drain 19 vs Thousand Palms	$t=235.743$, $p<0.0000$
Hazard Pond 6 vs Trifolium Drain 19	$t=8.9140$, $p<0.0000$

For sperm viability, statistical analyses were performed similarly (one way ANOVA with arcsin[sqrt] transformed data) without covariates, with similar results, in that the fish from the reference site showed significantly lower viable sperm than did that from the experimental sites, which were similarly grouped, with $F_{(2,129)}$ =29.33, p<0.0001.

By the exact proportions formulae:

Hazard Pond 6 vs Thousand Palms	t=258.006, p<0.0000
Trifolium Drain 19 vs Thousand Palms	t=225.399, p<0.0000
Hazard Pond 6 vs Trifolium Drain 19	t=32.8186, p<0.0000

In a second analysis, covariates were used in a one-way ANCOVA for both mitochondrial function (table 2) and viability (table 3). The covariates included fish length, gonopodia lengths, and fish weight. Correlations of proportions (arcsin[sqrt] transformed) were checked, and it was found that for mitochondrial function, there were some significant correlations with length and weight:

Table 2. Correlations (*P* values) of transformed mitochondrial function values with covariates for all sites combined and for each site

	All Sites	Hazard Pond 6	Trifolium Drain 19	Thousand Palms
Length	**0.25 (0.0033)**	**-0.36 (0.0325**)	0.21 (NS)*	0.12 (NS)
Gonopodium length	0.12 (NS)	**-0.43 (0.0095)**	0.097 (NS)	**0.31 (0.0206)**
Weight	**0.43 (0.0001**)	-0.18 (NS)	0.22 (NS)	0.24 (NS)

*NS= not significant.

Table 3. Correlations (*P* values) of transformed viable sperm values with covariates for all sites combined and for each site.

	All Sites	Hazard Pond 6	Trifolium Drain 19	Thousand Palms
Length	**0.2 (0.0151)**	**-0.29 (0.0852**)	0.31 (NS)*	0.03 (NS)
Gonopodium length	0.11 (NS)	-**0.40 (0.0162)**	0.207 (NS)	**0.27 (0.0421)**
Weight	**0.40 (0.0001**)	-0.09 (NS)	**0.34 (0.0351**)	0.18 (NS)

*NS= not significant.

For mitochondrial function, length and weight were significant, thereby explaining some of the variance, and site remained significant ($F_{(2,126)}$=5.89 P=0.0036). By using both Duncan's multiple range test and Tukey's studentized range (HSD) test,

there was significant grouping of both contaminated sites with higher proportions than the reference site.

For viability, length and weight were significant thereby explaining some of the variance, whereby site did not remain significant ($F_{(2,126)}$=2.62, NS). In other words, sperm viabilities were not significantly different between the three sites.

Therefore, the analyses were redone without using gonopodia as a covariate. By using both Pearson (parametric) and Spearman (nonparametric) correlations of proportions (arcsin[sqrt transformed]) for data from all sites, both weight and length were positively correlated, whereas gonopodia data were not. For mitochondrial function, a proportions (arcsin[sqrt] transformed) one-way ANCOVA was run with covariates of length and weight. The analysis was run on all three sites, then with sites recategorized as only experimental (Trifolium Drain 19 and Hazard Pond 6) and reference (Thousand Palms).

Table 4. ANCOVA (Arcsin[sqrt]) results using mitochondrial function adjusted means.

	THREE-SITE		TWO-SITE	
Site	$F_{(2,127)}$=7.45	*P*=0.0009	$F_{(1,128)}$=15.01	*P*=0.0002
Length	$F_{(1,127)}$=9.52	*P*=0.0025	$F_{(1,128)}$=10.32	*P*=0.0017
Weight	$F_{(1,127)}$=10.02	*P*=0.0019	$F_{(1,128)}$=10.15	*P*=0.0018

Covariates length and weight were significant and explained some of the variance, whereby site still remained significant in both the three-sites and two-site analyses of mitochondrial function. Pairwise comparisons for the three sites were performed by using least squares means, where Hazard and Trifolium were not significantly different from each other, but both Hazard and Trifolium differed significantly from Thousand Palms (*P*=0.0004 and 0.0015, respectively, *P* =0.05/3=0.0167.) Adjusted means (average proportions) and standard errors are plotted (fig. 10) for the three-site and two-site analyses.

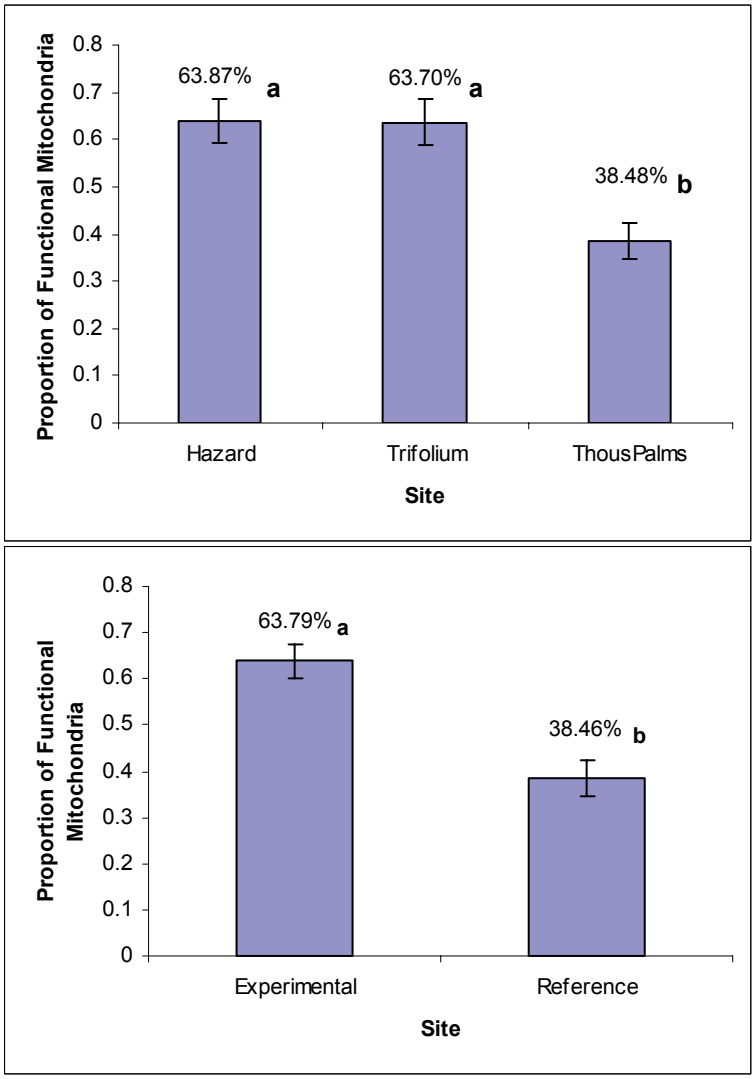


Figure 10. Adjusted means of sperm mitochondrial function (SE) from mosquitofish (*Gambusia affinis*) in the three site (top) (*P*=0.0004) and two site statistical analyses (bottom)(*P*=0.0015).

Thus, for mitochondrial function, site was significant in both the three-site and two-site analyses, and weight and length were significant covariates.

For viability, a proportions (arcsin[sqrt]) transformed) one-way ANCOVA was run with covariates of length and weight. Site remained significant in the presence of these covariates.

Table 5. ANCOVA (Arcsin[sqrt]) results using viability adjusted means

	THREE-SITE		TWO-SITE	
Site	$F_{(2,127)}$=3.15	*P*=0.0462*	$F_{(1,128)}$=6.07	*P*=0.0151
Length	$F_{(1,127)}$=13.93	*P*=0.0003	$F_{(1,128)}$=16.48	*P*<0.0001
Weight	$F_{(1,127)}$=15.42	*P*=0.0001	$F_{(1,128)}$=15.93	*P*=0.0001

Again, in a pairwise comparison, Hazard and Trifolium did not differ significantly from one another, but only Hazard differed significantly from Thousand Palms (p=0.0140). Trifolium did not significantly differ from Thousand Palms (p=0.077), where =0.05/3=0.0167. Adjusted means (average proportions) and standard errors are plotted (fig. 11) for the three-site and two-site analyses.

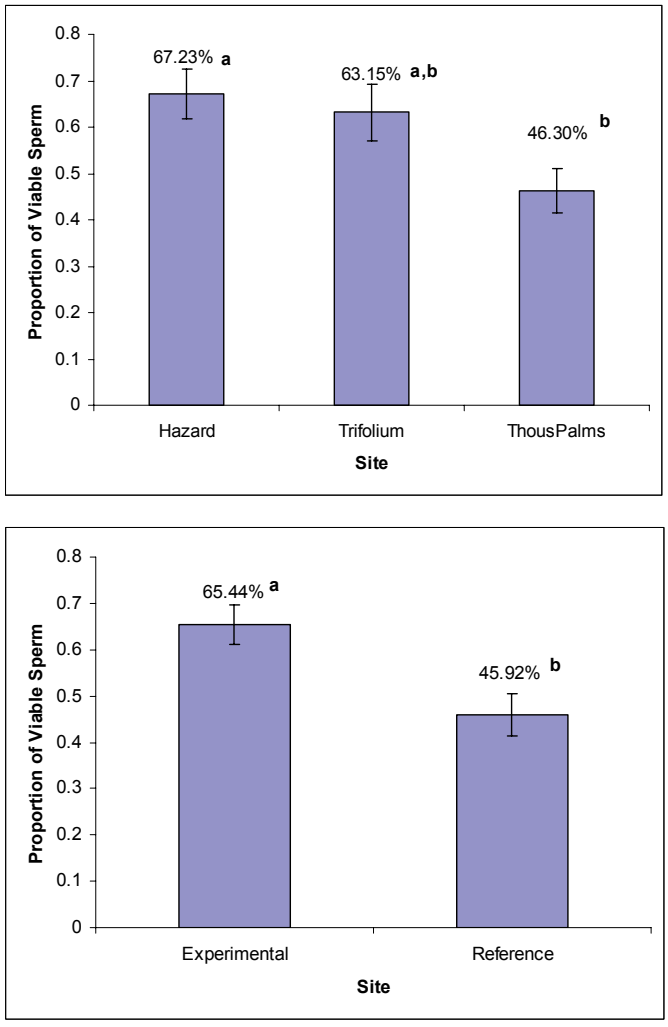


Figure 11. Adjusted means of viability (SE) of sperm from mosquitofish (*Gambusia affinis*) per site in the three site (top) (P=0.0140) and two site analyses (bottom)(P=0.0151).

Summarily, site was significant for both sperm mitochondria and viability analyses in both the three-site and two-site analyses using ANCOVA with fish length and weights as significant covariates. Mean proportions were adjusted with corresponding error terms (fig. 11).

4. Comparison of Sperm Maturity per Site

H_{01}: There is no difference in counts of immature sperm from *Gambusia affinis* per site.

Sperm were grouped into two categories, immature (M1) or mature (M2) (fig. 12).

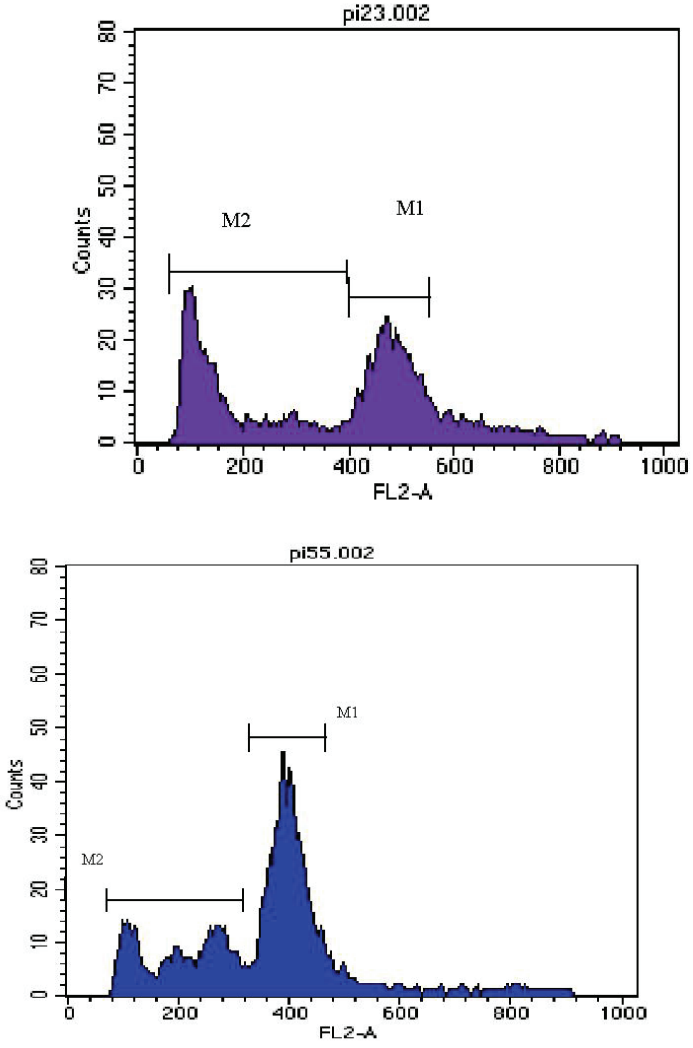


Figure 12. Flow cytometric histograms with DNA fluorescence intensity on the x-axis. More mature nuclei with less DNA are under Marker (M)2. Sperm of lesser maturity are delineated by M1. The top sample was fish 23 from Trifolium Drain 19, and the bottom sample was fish 55 from Thousand Palms.

Numbers of immature sperm (M1) and mature sperm (M2) were identified according to M1/(M1+M2) for determining proportions of immature sperm. For statistical analyses, Proc ANOVA was run on arcsin[sqrt]proportion data. There were significantly higher numbers of immature sperm at Hazard Pond 6 than those at either Thousand Palms or Trifolium Drain 19. By Duncan's and Tukey's multiple comparisons, Trifolium grouped with both of the other sites: Therefore, for counts of immature sperm, Hazard≥Trifolium≥Thousand Palms, and Hazard>Thousand Palms. Raw data are presented in figure 13.

Correlations were examined for the count response with length, gonopodium length, and weight as covariates, and no significant correlations were found over all sites. Therefore, no analysis of covariance was needed.

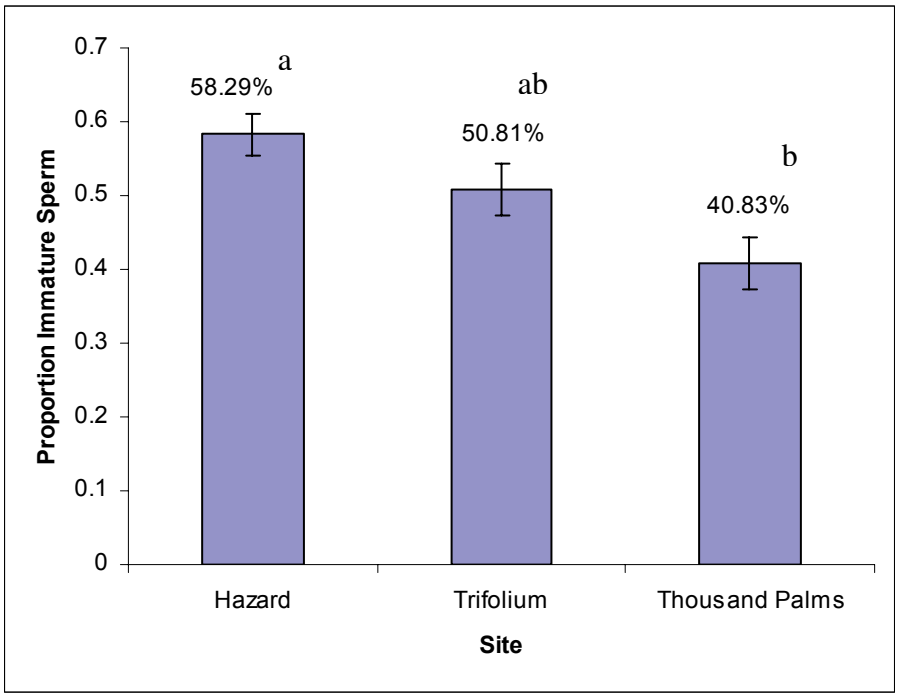


Figure 13. Average proportions of immature mosquitofish (*Gambusia affinis*) sperm per site (*P*=0.0031).

5. Comparison of GSI, HSI, and Motility per Site (2003 data from fish received at NWRC)

H_{01}: There is no difference in *Gambusia affinis* GSI among sites.
H_{02}: There is no difference in *Gambusia affinis* HSI among sites.
H_{03}: There is no difference in *Gambusia affinis* sperm motilities among sites.

The GSI was calculated as = (testis weight/fish weight) x 100. The HSI was calculated as = (liver weight/fish weight) x 100, and sperm motility was estimated as the percentage (to 5%) of progressively moving sperm as determined visually by darkfield microscopy observations in triplicate by three independent readers.

Table 6. Average values (SE) of morphological characteristics of mosquitofish (*Gambusia affinis*) collected July 14-16, 2003

Site	Hazard Pond (n = 12)	Trifolium 19 (n = 13)	Thousand Palms (n = 21)
GSI	3.17 (0.36)[a]	2.44 (0.13)[a]	1.60 (0.11)[b]
testis weight (mg)	3.85 (0.49)	4.12 (0.37)	1.38 (0.14)
fish weight (mg)	123.61 (9.05)	167.53 (11.18)	85.23 (5.00)
gonopodium length (mm)	6.483 (0.161)	7.238 (0.157)	6.568 (0.095)
liver weight (g)	1.77 (0.23)*	1.71 (0.17)	0.76 (0.07)
fish length (mm)	23.998 (0.628)	27.365 (0.605)	23.153 (0.432)

[a,b] = Different superscripts per row indicate significant differences in log transformed GSI data.

* n = 3 for liver weights for this site.

The GSI was not normally distributed, therefore a log transformation was applied to achieve normality and homogeneity. A one-way ANOVA was applied on log (GSI), where site was strongly significant $F_{(2,43)}=14.91$; $P<0.0001$. Duncan's and Tukey's multiple comparison tests grouped both contaminated sites with higher values than those of Thousand Palms. An additional one-way nonparametric analysis on untransformed GSI led to the same conclusion (i.e., Kruskal-Wallis test, $p<0.0001$)(see fig. 14).

A discriminant analysis was performed in order to obtain the rates of misclassification into the sites based on the log GSI data. For Hazard Pond 6 (n=12), the rate of misclassification was calculated to be 0.1667 (or 16.67% of the time). For Trifolium Drain 19 (n=13), the rate of misclassification was found to be 0.6154 (or 61.54%), and Thousand Palms (n=21) had a rate of 0.2381 (or 23.81%).

Both experimental sites were grouped together by Duncan's and Tukey's tests, so the analysis was rerun on recategorized sites where both experimental sites were grouped as "contaminated." Again, the one-way ANOVA on log GSI showed significant site differences with values of log GSI in experimental sites more than twice that of the reference site. A nonparametric analysis result was the same. The discriminant analysis gave strong evidence of correct classification, whereby log GSI correctly identified the sites more than 80% of the time. The rate of misclassification found by using log GSI

was 0.1600 (or 16.00% of the time) for the experimental sites, and 0.1905 (or 19.05%) for the reference site.

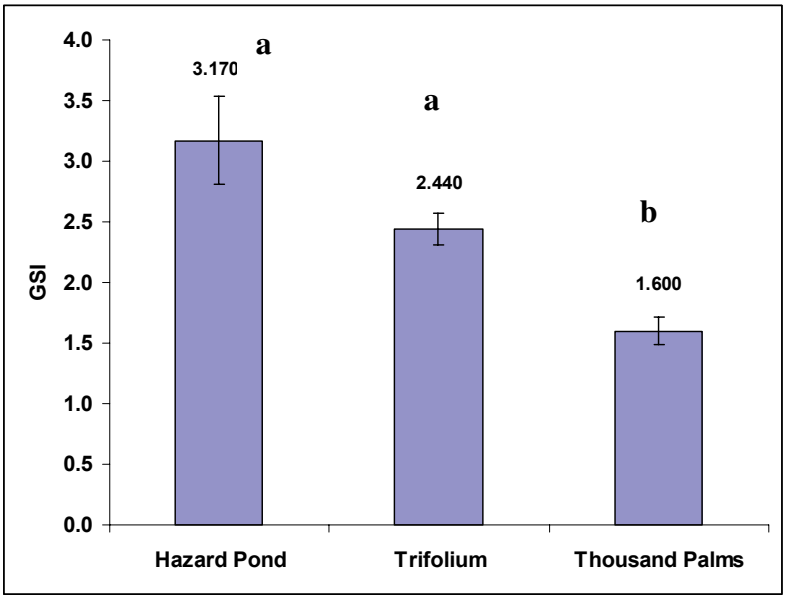


Figure 14. Average, nontransformed gonadosomatic index (SE) of mosquitofish (*Gambusia affinis*) per site (*P*<0.0001).

Most liver weights were missing from the Hazard Pond 6 site, so the analyses were run only on recategorized sites, where Hazard Pond 6 was put together with Trifolium Drain 19. The HSI data were not normally distributed, and a log transformation was applied to achieve normality and homogeneity. A one-way ANOVA was applied on log HSI, and site was strongly significant ($F_{(1,43)} = 8.11$; $p < 0.0067$)(fig. 15). Duncan's and Tukey's multiple comparison tests verified this result whereby experimental site values were significantly higher than the values from the reference site. An additional one-way nonparametric analysis on untransformed HSI led to the same conclusion (i.e., Wilcoxon 2-sample test, $p<0.0017$ and Kruskal-Wallis test, $p < 0.0016$). The discriminant analysis gave strong evidence of correct classification, where log HSI correctly identified the sites about 73% of the time, with a misclassification rate of 0.2609 (or 26.09% of the time) for the experimental sites, and a misclassification rate of 0.2727 (or 27.27%) for the reference site.

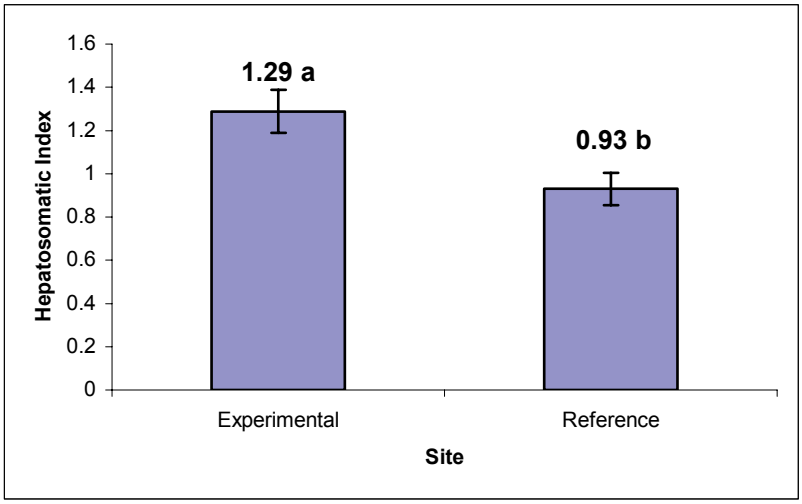


Figure 15. Average hepatosomatic indices from mosquitofish (*Gambusia affinis*) at recategorized sites, where dissimilar letters indicate significant differences (P<0.0067). For the experimental sites of Hazard Pond 6 and Trifolium Drain 19, n = 23, and for the reference site of Thousand Palms, n = 22.

Sperm motility average percentages obtained by the three readers were converted to proportions and arcsin[sqrt] transformed. Although many values fell within the 30-70% range in a manner that transformation would not be needed (Zar, 1984), the extreme values necessitated transformation of the data. One-way ANOVA indicated no significant differences among sites $F(2,41)=2.12$; $P = 0.1325$, thus no further analyses were performed. The average sperm motility for Hazard Pond 6 was 51% (SE 5), for Trifolium Drain 19 was 55% (SE 2), and for Thousand Palms was 39% (SE 3), with no significant differences in motilities among sites.

Weights of fish are presented in figure 16.

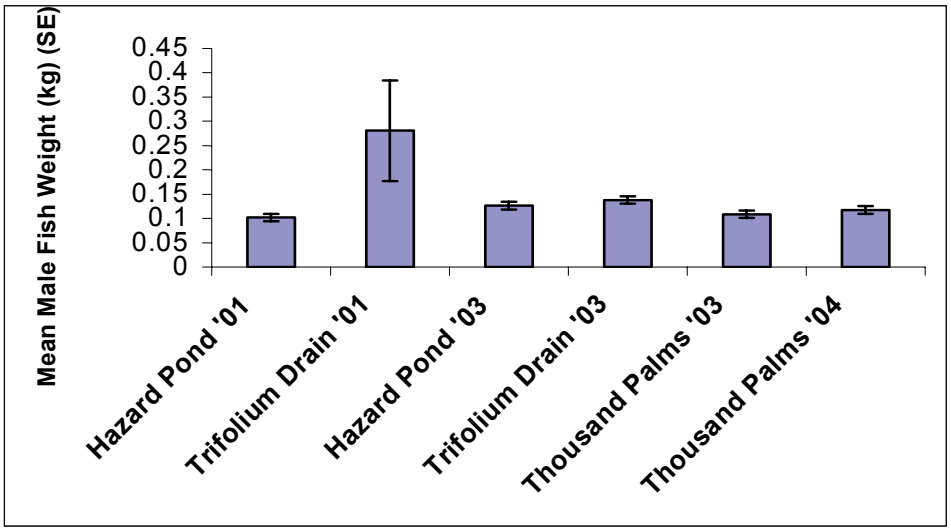


Figure 16. Mean male mosquitofish (*Gambusia affinis*) body weights from sites sampled in 2001, 2003, and 2004.

6. Comparison of Gonopodia per Site (2001 and 2003)

H_{01}: There is no difference in *Gambusia affinis* gonopodia lengths among the sites.

Gonopodia lengths from five sites in 2001 and from three sites in 2003 were obtained. From 2001, sites included Bruchard Drain, Hazard Pond 6, No Name Drain, Rock Hill Pond, and Trifolium Drain 19. From 2003, sites included Hazard Pond 6, Trifolium Drain 19, and Thousand Palms.

An analysis of covariance was performed (Toft and others, 2003), whereby gonopodia length was corrected for body length (covariate), and estimated marginal means (SE) from the LSMEANS statement (e.g. adjusted means) were plotted. Based on tests of normality and plots of residuals, however, log transformations of gonopodia length did not improve normality or homogeneity of gonopodia length. Body length was already normally distributed and did not require transformation. In order to reconstruct the analyses and plots as per Toft and others (2003), if log transformations were used on gonopodia length, then the LSMEANS or adjusted means (SED) for the covariate would be back-transformed to enable plotting in the original units.

For the 2001 data, preliminary correlations indicated a significant positive correlation of gonopodia length with body length, and so an analysis of covariance was performed by using log (10) transformation for both gonopodia and body lengths. There

were significant differences among the sites (p=0.0203) with covariate body length (p < 0.0001), where alpha=0.05/10=0.005 (fig. 17).

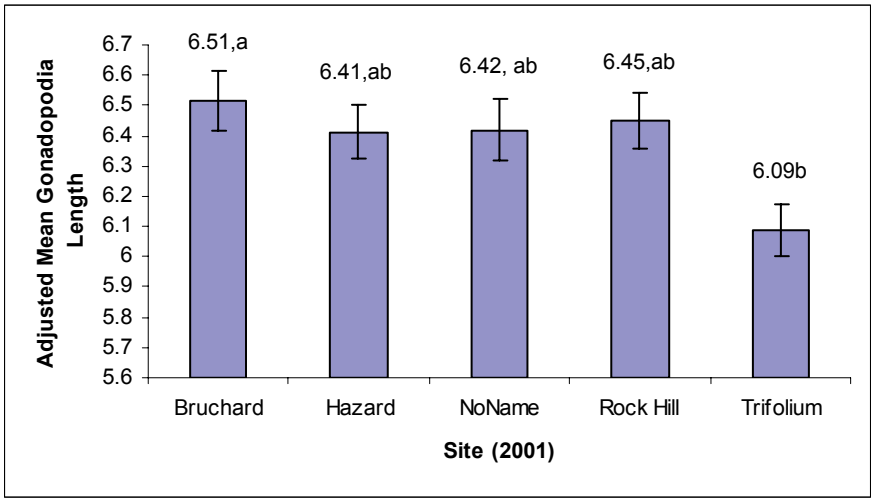


Figure 17. Average adjusted mean gonopodia lengths from mosquitofish (*Gambusia affinis*) collected per site in 2001, where dissimilar letters indicate significant differences between sites (*P*=0.0203).

For the 2003 data, an analysis of covariance was applied for correlated body length by using log (10) transformation. Again, significant differences were found between sites (p < 0.0001) with covariate length p < 0.0001, where alpha=0.05/3=0.0167 (fig. 18).

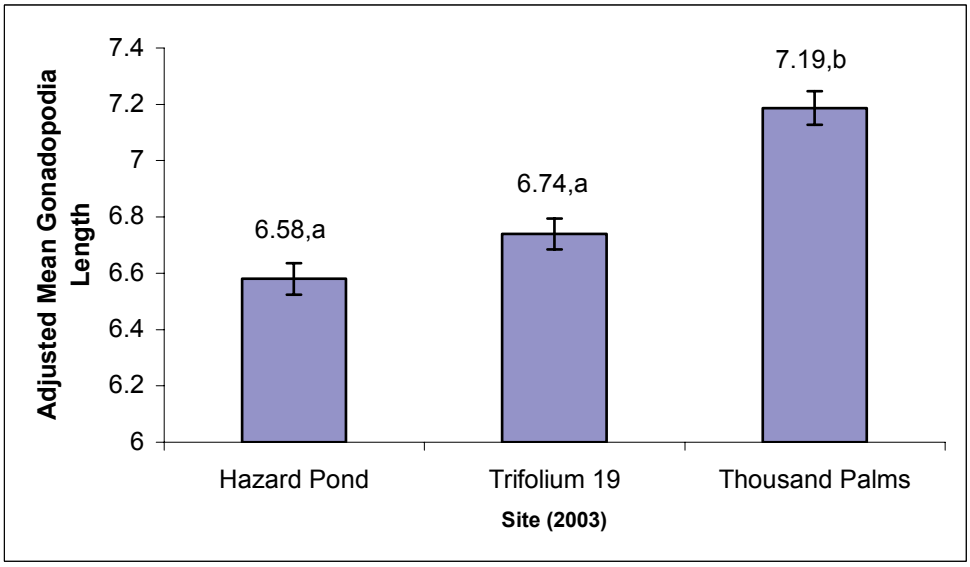


Figure 18. Average adjusted mean gonopodia lengths from mosquitofish (*Gambusia affinis*) collected per site in 2003, where dissimilar letters indicate significant differences between sites (*P*<0.0001).

7. Creation of GI Index (Gonopodia Length/Total Body Length)

H_{01}: A new index can be applied to fish per site with 2001 and 2003 data to test potential differences between sites, separately by year.

In 2001 (June 11-13), fish were collected for analyses from Rock Hill Trail Pond, Hazard Pond 6, No Name Drain, Trifolium 19 Drain, Bruchard Drain, and the reference site San Felipe Creek. At San Felipe Creek, no phenotypic males were available for collection.

In 2003, (July 15-17), males were sampled from Trifolium 19 Drain and Hazard Pond 6, and from Thousand Palms, another reference site where male mosquitofish were more abundant.

Based on the significant differences among sites for 2001 ($p=0.0203$) and 2003 ($p < 0.0001$), whereby ANCOVAs using body length as a covariate in the analyses of gonopodia differences between sites, a new index was applied where GI = gonopodium length/total body length.

Based on tests of normality and plots of residuals, GI was normally distributed for Bruchard, Hazard Pond 6, and Trifolium Drain 19 for 2001 and for all three sites sampled in 2003. Log transformations only slightly improved normality or homogeneity of GI for 2001 in Hazard and Rock Hill sites, and worsened normality for data from No Name, Trifolium (2001), and Hazard Pond 6 (2003). Because data from most sites were already normally distributed before applying a log transformation, no transformations were applied in the analyses. Results of ANOVA are fairly robust in spite of some irregularities.

For 2001 data, GI data from sites were significantly different, $F_{(4,91)} = 3.63$, $P = 0.0087$. Looking at pairwise comparisons with Duncan's multiple range test, where $\forall = 0.05/10 = 0.005$, Bruchard > Trifolium ($P = 0.0042$), and No Name > Trifolium ($P = 0.0006$). All other pairwise comparisons were not significant (fig. 19).

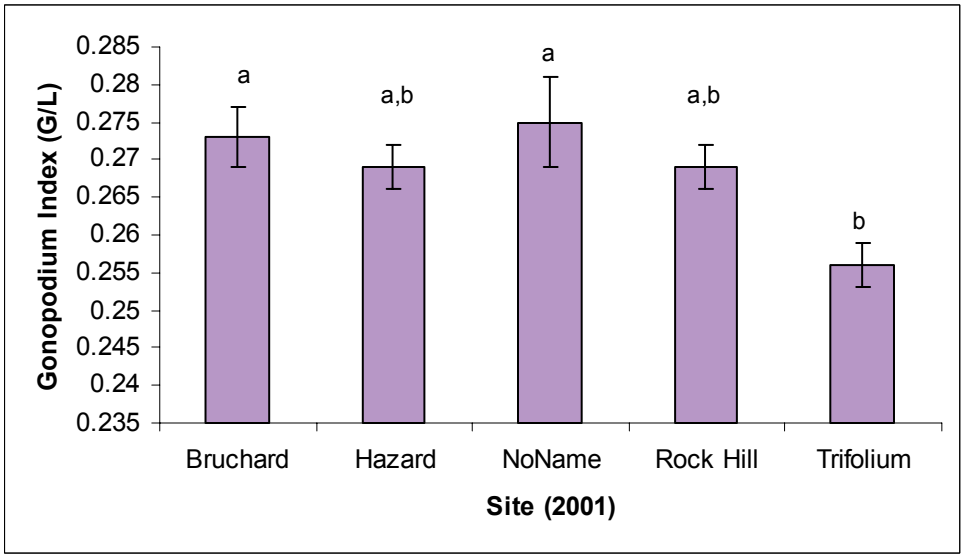


Figure 19. Average gonopodia indices from mosquitofish (*Gambusia affinis*) collected per site in 2001, where dissimilar letters indicate significant differences between sites ($P = 0.0087$).

For 2003 data, GI data from sites were significantly different, $F_{(2,136)} = 39.36$, $P < 0.0001$. Looking at pairwise comparisons with Duncan's multiple range test, where $\forall = 0.05/3 = 0.0167$, Thousand Palms > Hazard and Trifolium, with both $P < 0.0001$, and Hazard =Trifolium, $P = 0.5168$ (fig. 20).

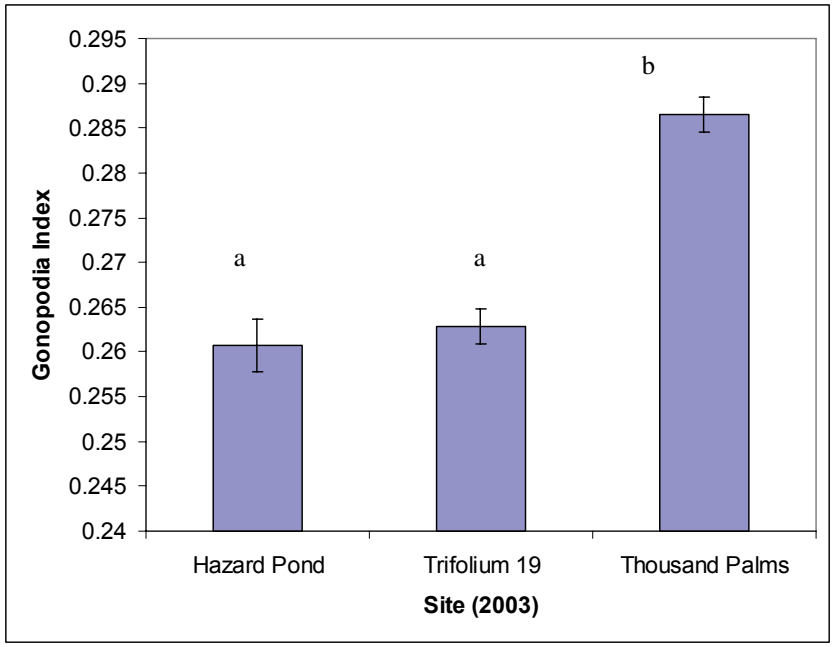


Figure 20. Average gonopodia indices from mosquitofish (*Gambusia affinis*) collected per site in 2003, where dissimilar letters indicate significant differences between sites ($P < 0.0001$).

In order to test the appropriateness of pooling data from 2001 and 2003 for the Trifolium and Hazard Pond 6 sites, an ANOVA using year effect by both sites and per sites showed nonsignificance (P = 0.7917), and by least squares means comparisons, where $\forall$ = 0.05/2 = 0.025, for Hazard 2001 and 2003, P = 0.0475 and for Trifolium 2001 and 2003, P = 0.1007. Therefore, years were pooled and an ANOVA for the three sites was performed. Sites were significantly different, F(2,175) = 44.56, P < 0.0001. By Duncan's, where $\forall$ = 0.05/3 = 0.0167, Thousand Palms > Hazard and Trifolium (both *P* < 0.0001) and Hazard = Trifolium (P = 0.3420) (fig. 21).

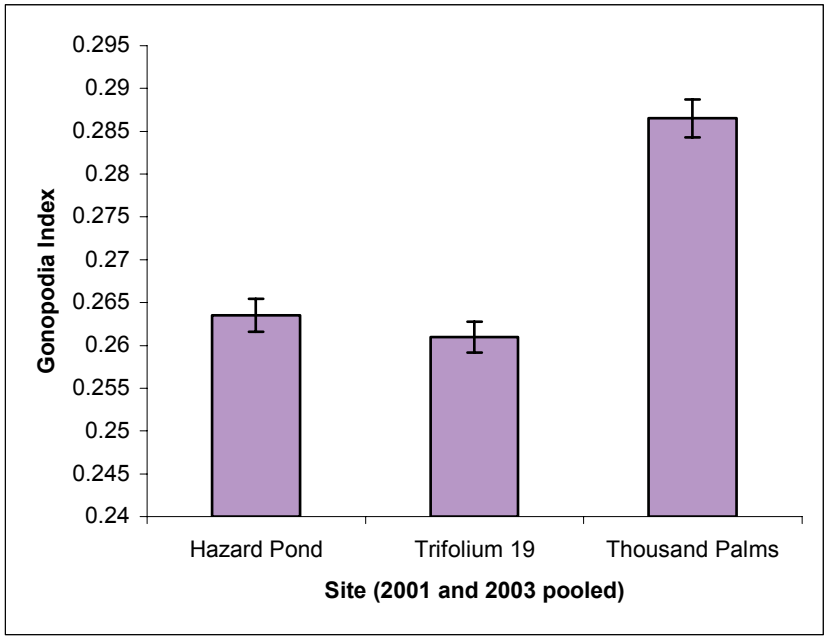


Figure 21. Average gonopodia indices (gonopodia length/body length) from mosquitofish (*Gambusia affinis*) collected per site for pooled years 2001 and 2003, where dissimilar letters indicate significant differences between sites.

In testing whether GI could correctly classify individuals into appropriate sites by using all three sites or two sites recategorized as experimental or reference, recategorization improved classification (Table 7).

Table 7. Discriminant analysis results showing rates of misclassification using GI

Site	Hazard Pond 6	Trifolium Drain 19	Thousand Palms OR	Experimental	Reference
Rate	0.689	0.429	0.255	0.191	0.255
Probability	0.33	0.33	0.33	0.50	0.50

When three sites were used in the discriminant analysis, many Hazard individuals were misclassified as Trifolium and vice versa. Overall, GI (untransformed) is a good indicator for distinguishing between contaminated and uncontaminated sites.

8. Contaminants Analyses (2001, 2003)

H_{01}: There is no difference in contaminants among sites sampled in 2001 using fish tissue data and 2003 using a polar organic compound integrated sampler.

Data were not normalized for lipids for the 2001 data. Further analyses may be needed for better interpretation of these data from these two years.

In 2001, contaminants data were obtained from male and female fish tissue, whereby trifluralin, chlorthal-dimethyl (DCPA), chlorpyrifos (phosphorthioic acid 0, 0-diethyl O-[3,5,6-trichloro-2-pyridinyl] ester), trans-nonachlordane, cis-nonachlordane, dichlorodiphenyldichloroethylene (DDE), dieldrin, endosulfan, pp,'-DDE (from bed sediment), total organochlorines, and percent lipid were variables in the analyses. In 2003, contaminants data from an integrated sampling device included trifluralin, DCPA, fipronil, fipronil sulfide, fipronil sulfone, trans-chlordane, endosulfan I, endosulfan II, endosulfan sulfate, dieldrin, and total organochlorines (fig. 22).

Differences between sites for all years could not be tested because only one contaminant value was provided per site. Standard errors could not be computed either.

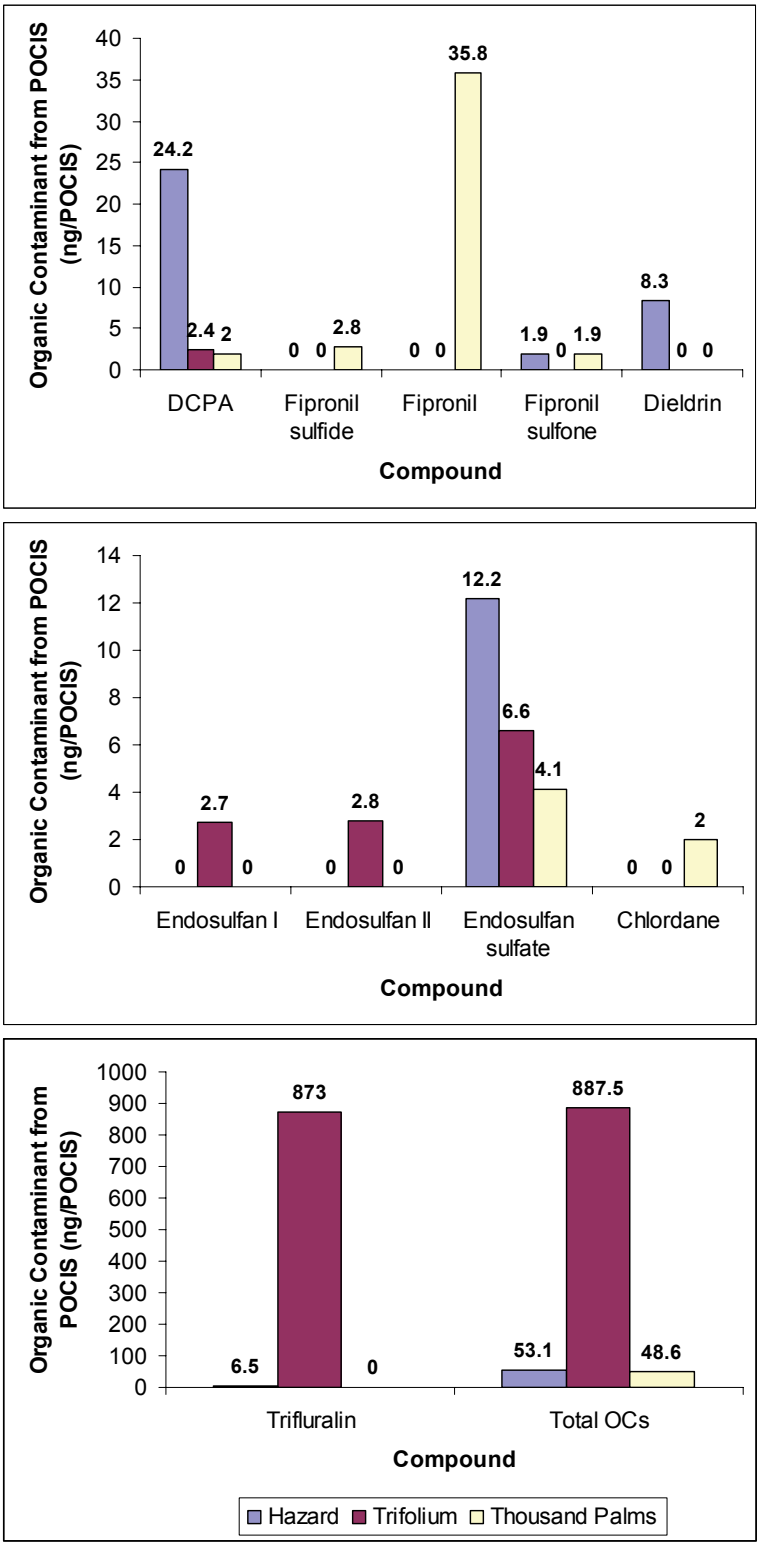


Figure 22. Organic contaminants sequestered by the polar organic compound integrated samplers (POCIS) in 2003.

Discussion

Because aquatic animals are generally more sensitive than humans are to environmental stressors (Adams and others, 2003), they can act as sentinels for resource degradation. Species occupying particular trophic levels or having particular life histories may preferentially reflect environmental conditions. Sublethal stress is generally manifested first at the suborganismal level where immune systems are compromised, reproductive success is reduced, and genetic integrity is altered. Because molecular and biochemical responses of cells are preceded by chemical changes in nuclei, cytoplasm, membranes, and extracellular fluids, these responses can be diagnostic and contribute to an assessment of ecosystem health. *Gambusia affinis* are good sentinels, as they are ubiquitously distributed and have readily measurable sexual characteristics that are affected by exposure to chemicals with estrogenic or antiandrogenic activity (Toft and others, 2003). Male mosquitofish have mature sperm cells in their testes year-round (Fraile and others, 1992).

In this study, combinations of biomarkers indicative of whole animal and reproductive conditions were employed. A special focus was on sperm, the cells of which consist of several membrane compartments, and cell competency requires that each of these membrane compartments be intact. Such biomarker data integrate well contaminants data, may reflect transgenerational effects, and can be interpreted in terms of long-term hazards to a community or population.

Pollutants may disrupt reproductive endocrine function by acting at the hypothalamus, pituitary, gonad, or liver (Kime and Nash, 1999), whereby gonadal development or viability of gametes is impacted. Strategies for coping with stress affect reproductive fitness by gamete or progeny quality (Schreck and others, 2001). Sperm viability has been most rigorously investigated with mammalian sperm in cryopreservation studies. Although the conditions of cryopreservation experiments are not directly correlated to environmental studies, many of the molecular mechanisms behind integrity of cellular structures and in vitro conditions are related. Flow cytometry technology can quickly provide an estimation of sperm cell membrane integrity and has been used with terrestrial species including horse (*Equus caballus*) (Merkies and others, 2000; Graham, 2001), turkey (*Meleagris* spp.) (Donoghue and others, 1995), wild boar (*Sus scrota*), ram (*Apistogramma ramirezi*), dog (*Canis* spp.), mouse (*Amphipyra*

tragopoginis), human (*Homo sapiens*), bull (*Bos taurus*) (Garner and others, 1986; Ericsson and others, 1990), and with some aquatic species including tilapia (*Oreochromis niloticus*) (Segovia and others, 2000), trout (*Salmonidae* spp.) (Cabrita and others, 2001), eastern oyster (*Crassostrea virginica*) (Paniagua-Chavez and others, in press), red abalone (*Haliotis rufescens)* (Salinas-Flores and others, 2005), whiteleg shrimp (*Litopenaeus vannamei*) (Lezcano and others, 2004), and other marine invertebrates (Adams and others, 2003). Gamete viability has been suggested as an indicator of reproductive endocrine disruption in fish caused by exposure to xenobiotics (Kime and Nash, 1999), and this report addresses several sperm quality parameters.

In this study, a proportions (arcsin[sqrt]) transformed) one-way ANCOVA was run for detecting sperm viability differences between sites and employed covariates fish length and weight. Site remained significant in the presence of these covariates, whereby the fish from the experimental sites had sperm viabilities significantly higher than those from the reference site (fig. 11). Chemicals from the POCIS that were highest at Thousand Palms included fipronil and its metabolites and chlordane. Chemicals do not have a single mode of action in the organism, and they affect a variety of physiological factors (Heath, 1987). That relatively fewer contaminated sites had fish with lower sperm viabilities and mitochondrial function may reflect fewer nutrition resources at those sites.

Gonadal development and fecundity are affected by certain essential dietary nutrients, especially in continuous spawners (Izquierdo and others, 2001). Lipid and fatty acid compositions have been identified as major dietary factors that determine successful reproduction and survival of offspring. The most common reaction that occurs between toxicants and lipids is lipid peroxidation (Schlenk, 2001). Dietary polyunsaturated fatty acids in sperm membrane phospholipids have been manipulated in sea bass (*Dicentrarchus labrax*) (Bell and others, 1996) to influence sperm motility and fertility in domestic chicken (*Gallus spp.*) (Cerolini and others, 2005) and sperm membrane integrity in rainbow trout (*Oncorhynchus mykiss)* (Pustowka and others, 2000). Highly unsaturated fatty acids with 20 or more carbon atoms, either directly or through their metabolites, affect fish maturation and steroidogenesis (Izquierdo and others, 2001). Sperm plasma membrane organization, fluidity, permeability, and lipid composition are parameters important to membrane integrity. Sperm integrity has been

interrupted by membrane peroxidation (Donoghue and Donoghue, 1997) whereby reactive oxygen species induce tissue damage and motility can be lowered. Nutrients that add to the protection of reactive oxygen species contribute to the protection of membrane integrity, such as the selenium-dependent enzyme glutathione peroxidase (Wu and others, 1979). Dietary intake of antioxidants such as vitamins C and E, and micronutrients such as folate and zinc are critical for normal semen quality in animals and humans (Eskenazai and others, 2005).

Evaluating milt characters directly reflects on fertilization potential, where several quality bioindicators, in addition to sperm viability, are associated with sperm physiology. These include mitochondrial membrane potential, sperm count, seminal plasma components, sperm cell maturity, and sperm function. If changes caused by external chemicals are severe enough, histological changes may be seen. Because organs are composed of many cell types, effects on one or more of these cell types will be reflected in changes in organ function. For example, cellular composition of testes is reflective of endocrine impacts, whereby the larval stages are most sensitive (Heath, 1987). Fish testes are composed of cellular cysts containing Sertoli cells, and all cells with a cyst and cysts with a lobule are normally at the same stage of spermatogenesis (Dawson, 1998). Therefore, assessing ploidy of single cell suspensions stained with PI from mosquitofish testes is indicative of spermatogenic stage, whereby diploid stem cells (spermatogonia) either undergo mitotic divisions to reproduce themselves or differentiate into primary spermatocytes (4N, containing twice as much DNA per cell). These cells undergo meiosis, followed by two cell divisions to produce four haploid cells (1N, spermatids) (Levek-Motola and others, 2005). In this study, the ploidy of cells from testes were determined and categorized into tetraploid or primary spermatocytes (least mature) and other, lesser ploidy levels (fig. 12). When the spermatogenesis pathway is disrupted, there is a reduction in the production of mature sperm forms. In a histological study of sperm relative to other germ cell stages of feral common carp (*Cyprinus carpio*) from contaminated and reference sites, fewer sperm were found at the contaminated site and GSI values were lower (Patino and others, 2003). In this study, there were significantly higher numbers of immature sperm at the Hazard Pond 6 site than at Thousand Palms (fig. 13), where DCPA, endosulfan sulfate, dieldrin, and total organochlorines were more numerous by POCIS.

Reproductive performance depends on metabolic processes (Zrimsek and others, 2004). Damage to sperm mitochondria causes a decrease in ATP production and thus in sperm motility, although this association is sometimes not made because of the nature of visual imprecise assessments (Thomas and others, 1998; Garner and others, 1997). The most widely used mitochondrial-specific probe, R123, is a cationic compound that excites at 488 nm and emits at 515-575 nm (green fluorescence). It accumulates in the mitochondria as a function of transmembrane potential, of R123 concentration, and of sperm numbers. Mitochondrial membrane function in sperm is sometimes correlated with membrane integrity and with motility (Garner and others, 1997; O'Connell and others, 2002; Paniagua-Chavez and others, in press). Estradiol administration in vivo is known to affect sperm metabolism and motility (Saberwal and others, 2003), whereby patients with asthenozoospermia (reduced sperm motility) show improvement in sperm motility with estradiol, indicating that it is involved in an aspect of sperm metabolism that is associated with motility (Saberwal and others, 2003). Tamoxifen, a nonsteroidal estrogen antagonist, was shown to increase R123 uptake in rat (*Murida* spp.*)* sperm, yet membrane integrity was not impacted, suggesting disturbance of transmembrane ion pumps and increased calcium levels which alters sperm motility (Saberwal and others, 2003). In this study, mitochondrial function at the reference site was significantly lower than at each of the experimental sites (fig. 10), where more endocrine disruptive chemicals were detected (fig. 22).

The ratio of liver to body weight differs greatly between species of fish, and their nutritional state has considerable influence on liver size (Heath, 1987). In this study, the HSI values at the experimental sites were higher than those at the reference site, thereby lending additional support toward there being a difference in the nutritional resources available to the fish. The liver is responsible for enzymatic decontamination processes, vitellogenin production, and stores glycogen as energy reserves. The largest store of carbohydrate in the fish body is in the liver. Stressors raise serum glucose, and liver glycogen stores can be depleted in adverse conditions. The HSI values have been shown to increase after exposure to certain types of contaminants, particularly petroleum hydrocarbons (Fletcher and others, 1982; Fabacher and Baumann, 1985; Baumann and others, 1991), perhaps because of herplasia and hypertrophy caused by the induction of the missed-function-oxidase system in the liver to detoxify the contaminants (Poels and

others, 1980). In this study, the HSI was significantly greater at the experimental sites than that at the reference site (fig. 15).

Mature male mosquitofish posses a highly modified anal fin called a gonopodium that develops under the influence of endogenous androgens at sexual maturity, and this structure is a useful marker for quantifying the effects of known or suspected environmental androgens (Angus and others, 2001). In this study, analyzing gonopodia adjusted for fish length showed that those at the reference site were significantly longer than those at the experimental sites (fig. 18). In this study the new index, GI, was developed for comparing differences between sites. The untransformed GI for a single year (fig. 20) and pooled over years (fig. 21) indicated significantly greater indices at the reference site and nonsignificance between the experimental sites in each case.

Gametogenesis is largely under the control of steroid hormones in vertebrates (Song and Gutzeit, 2003), whereby xenobiotics may interfere with hormone action and consequent impacts. Although it is suggested that the molecular and cellular effects of hormonally active compounds are best studied under well-defined conditions in vitro (Song and Gutzeit, 2003), observations from sperm cell maturity levels and sperm cell counts indicated that the animals at the experimental sites were less sexually advanced than those at the reference site. Sperm count is a very common biomarker of testis function in humans, and the use of flow cytometry in fertility assays has been helpful, and it allows for other analysis such as DNA content and integrity (Eustache and others, 2001). Dibromochloropropane has been found to cause sperm count depression in male humans (Glass and others, 1979). Appropriate levels of zinc are crucial for proper sperm physiology, counts, and motility (Turgut and others, 2003), and pp-DDE, vinclozolin, and flutamide were found to inhibit guppy (*Poecilia reticulata*) gonopodia development and sperm count (Bayley and others, 2002). In this study, a flow cytometric technique for counting *Gambusia affinis* sperm was verified (fig. 8), and the results from examining samples from 2003 established that there were significantly higher numbers of sperm from the reference site than from each of the experimental sites.

Overall, there are several lines of evidence pointing toward hormonal disturbance in male *Gambusia affinis* at experimental sites in the Imperial Valley. A new index for communicating endocrine impacts on morphology, GI, is recognized, and a method for

counting fish sperm was verified. There is room for further development of ideas relative to the parameters studied.

Acknowledgments

The authors thank Steve Goodbred from USGS and Scott Sobiech from FWS for the opportunity to contribute to these studies, B. Vairin and J. Davis at NWRC for editorial support, D. Johnson at NWRC for statistical review, and E. Theall and J. Shin at NWRC for technical support. The concept of the gonopodia indices (gonopodia length/body length) is attributed to Steve Goodbred. The authors thank Dr. A. Rocha from the University of Portugal, T. Campbell from LSU, and R.G. Thomas from the LSU AgCenter for critical reviews.

References Cited

Adams, S.L., Hessian, P.A., and Mladenov, P.V., 2003, Flow cytometric evaluation of mitochondrial function and membrane integrity of marine invertebrate sperm: Invertebrate Reproduction and Development, v. 44, p. 45-51.

Alvarez, D.A., Petty, J.D., Huckins, J.N., Jones-Lepp, T.L., Getting, D.T., and Manahan, D.T., 2004, Development of a passive, in situ, integrative sampler for hydrophilic organic contaminants in aquatic environments: Environmental Science and Technology, v. 23, p.1640-1648.

Angus, R.A., McNatt, H.B., Howell, W.M., Peoples, S.D., 2001, Gonopodium development in normal meal and 11-ketotestosterone-treated female mosquitofish (*Gambusia affinis*): A quantitative study using computer image analysis: General and Comparative Endocrinology, v. 123, p. 222-234.

Baumann, P.C., Mac, M.J., Smith, S.B., and Harshbarger, J.C., 1991, Tumor frequencies in walleye (*Stizostedion vitreum*) and brown bullhead (*Ameiurus nebulosus*) and sediment contaminants in tributaries of the Laurentian Great Lakes: Canadian Journal of Fisheries and Aquatic Sciences, v. 48, p. 1804-1810.

Bayley, M., Junge, M., Baatrup, E., 2002, Exposure of juvenile guppies to three antiandrogens causes demasulinization and a reduced sperm count in adult males: Aquatic Toxicology, v. 56, p.227-239.

Bell, M.V., Dick, J.R., Thrush, M., and Navarro, J.C., 1996, Decreased 20:4n-6/20:5n-3 ratio in sperm from cultured sea bass, *Dicentrarchus labrax*, broodstock compared with wild fish: Aquaculture, v. 144, p. 189-199.

Cabrita, E., Martinez, F., Real, M., Alvarez, R., and Herraez, M.P., 2001, The use of flow cytometry to assess membrane stability in fresh and cryopreserved trout spermatozoa: CryoLetters, v. 22, p. 263-272.

Carey, C., and Bryant, C.J., 1995, Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environmental Health Perspectives, v. 103, p. 13-17.

Cerolini, S., Pizzi, F., Gliozzi, T., Maldjiani, T., Zaniboni, L., and Parodi, L., 2005, Lipid manipulation of chicken semen by dietary means and its relation to fertility: a review: Worlds Poultry Science Journal, v. 59, p. 65-75.

Colborn, T., and Clement, C., 1992, Chemically Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection: Princeton, Princeton Scientific Publishing Co., 403 p.

Dawson, A., 1998, Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment. Comparative reproductive physiology of nonmammalian species: Pure and Applied Chemistry, v. 70, p. 1657-1669.

Donoghue, A.M., and Donoghue, D.J., 1997, Effects of water- and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage: Poultry Science, v. 76, no. 10, p. 1440-1445.

Donoghue, A.M., Garner, D.L., Donoghue, D.J., and Johnson, L.A., 1995, Viability assessment of turkey sperm using fluorescent staining and flow cytometry: Poultry Science, v. 74, p. 1191-1200.

Ericsson, S.A., Garner, D.L., Johnson, L.A., Redelman, D., and Ahmad, K., 1990, Flow cytometric evaluation of cryopreserved bovine spermatozoa processed using a new antibiotic combination: Theriogenology, v. 33, p. 1211-1220.

Eskenazi, B., Kidd, S.A., Marks, A.R., Sloter, E., Block, G., and Wyrobek, A.J., 2005, Antioxidant intake is associated with semen quality in healthy men: Human Reproduction, v. 20, p. 1006-1012.

Eustache, F., Jouannet, and Auger, J., 2001, Evaluation of flow cytometric methods to measure human sperm concentration: Journal of Andrology v. 22, p. 558-567.

Fabacher, D.L., and Baumann, P.C., 1985, Enlarged livers and hepatic microsomal mixed function oxidase components in tumor-bearng brown bullheads from a chemically contaminated river: Environmental Toxicology and Chemistry, v. 4, p. 703-710.

Fletcher, G.L., King, M.J., Kiceniuk, J.W., and Addison, R.F., 1982, Liver hypertrophy in winter flounder following exposure to experimentally oiled sediments: Comparative Biochemistry and Physiology, Part C, v. 73, p. 457-462.

Foreman W.T., Connor, B.F., Furlong, E.T., Vaught, D.G., and Merten, L.M., 1995, Methods of analysis by the U.S. Geological Survey National Water Quality Laboratory—determination of organochlorine pesticides and polychlorinated biphenyls in bottom sediment by dual capillary-column gas chromatography with electron-capture detection. U.S. Geological Survey Open-File Report 95-140, Denver, 78p.

Fraile, B., Saez, F.J., Vicentini, C.A., De Miguel, M.P. Paniagua R., 1992, The testicular cycle of *Gambusia affinis holbrooki* (Teleostei: Poeciliidae): Journal of Zoology, v. 228, p. 115-126.

Garner, D.L., and Johnson, L.A., 1995, Viability assessment of mammalian sperm using SYBR-14 and propidium iodide: Biology of Reproduction, v. 53, p. 276-284.

Garner, D.L., Pinkel, D., Johnson, L.A., and Pace, M.M., 1986, Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses: Biology of Reproduction, v. 34, p. 127-138.

Garner, D.L., Thomas, C.A., Joerg, H.W., DeJarnette, J.M., and Marshall, C.E., 1997, Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa: Biology of Reproduction, v. 57, p. 1401-1406.

Glass, R.I., Lyness, R.N., Mengle, D.C., Powell, K.E., Kahn, E., 1979, Sperm count depression in pesticide applicators exposed to dibromochloropropane: American Journal of Epidemiology, v.109, p.346-351.

Glenn, W.D., III, 1998, Effect of osmolality, extender and temperature on gamete storage of koi carp (*Cyprinus carpio*): Baton Rouge, Louisiana State University, 202p.

Goodbred, S.L., Gillion, R.J., Gross, T.S., Denslow, N.P., Bryant, W.L., and Schoeb, T.R., 1997, Reconnaissance of 17beta-estradiol, 11-ketotestosterone, vitellogenin, and gonad histopathology in common carp of United States streams: potential for

contaminant-induced endocrine disruption: U.S. Geological Survey Open File Report 96-627, p. 1-47.

Graham, J.K., 2001, Assessment of sperm quality: a flow cytometric approach: Animal Reproduction Science, v. 68, p. 239-247.

Graham, J.K., Kunze, E., and Hammerstedt, R.H., 1990, Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry: Biology of Reproduction, v. 43, p. 55-64.

Gross, T.S., Arnold, B.S., Sepulveda, M.S., and McDonald, K., 2003, Endocrine disrupting chemicals and endocrine active agents, *in* Hoffman, D.J., Rattner, B.A., Burton, G.A. Jr., and Cairns, J.Jr., eds., Handbook of Ecotoxicology: Boca Raton, Lewis Publishers, p. 1033-1098.

Heath, A.G., 1987, Water Pollution and Fish Physiology: Boca Raton, CRC Press, 245 p.

Hudson, J.L., Duque, R.E., and Lovett, E.J., 1985, Applications of flow cytometry in immunotoxicology, *in* Dean, J.H., Luster, M.I., Munson, A.E., Amos, H., eds., Immunotoxicology and Immunopharmacology: New York, Raven Press, p. 159-177.

Izquierdo, M.S., Fernandez-Palacios, H., and Tacon, A.G.J., 2001, Effect of broodstock nutrition on reproductive performance of fish: Aquaculture, v. 197, p. 25-42.

Jenkins, J.A., 2000, Infectious disease and quality assurance considerations for the transfer of cryopreserved fish gametes, *in* Tiersch, T.R, and Mazik, P.M., eds., Cryopreservation in Aquatic Species: Baton Rouge, World Aquaculture Society, p. 343-363.

Kime, D.E., and Nash, J.P., 1999, Gamete viability as an indicator of reproductive endocrine disruption in fish: Science of the Total Environment, v. 233, p.123-129.

Leiker T.J., Madsen, J.E., Deacon, J.R., and Foreman, W.T., 1996, Methods of analysis by the U.S. Geological Survey National Water Quality Laboratory-determination of chlorinated pesticides in aquatic tissue by capillary-column gas chromatography with electron-capture detection. U.S. Geological Survey Open-File Report 94-710, Denver, 42 p.

Levek-Motola, N., Soffer, Y., Shochat, L., Raziel, A., Lewin, L.M., and Golan, R., 2005, Flow cytometry of human semen: a preliminary study of a non-invasive method for the detection of spermatogenic defects: Human Reproduction, v. 20, p. 3469-3475.

Lezcano, M., Granja, C., and Salazar, M., 2004, The use of flow cytometry in the evaluation of cell viability of cryopreserved sperm of the marine shrimp: Cryobiology, v. 48, p. 349-356.

McNiven, M.A., Gallant, R.K., and Richardson, G.F., 1992, In vitro methods of assessing the viability of rainbow trout spermatozoa: Theriogenology, v. 38, p. 679-686.

Merkies, K., Chenier, T., Plante, C., and Buhr, M.M., 2000, Assessment of stallion spermatozoa viability by flow cytometry and light microscope analysis: Theriogenology, v. 54, p. 1215-1224.

Nickum, J.G., Bart, H. L. Jr., Bowser, P.R., Greer, I.E., Hubbs, C., Jenkins, J.A, MacMillan, J.R., Rachlin, J.W., Rose, J.D., Sorensen, P.W., and Tomasso, J.R., 2004, Guidelines for the use of fishes in research: Bethesda, The American Fisheries Society, http://www.fisheries.org/html/Public_Affairs/Sound_Science/Guidelines2004.shtml

O'Connell, M., McClure, N., and Lewis, S.E.M., 2002, The effects of cryopreservation on sperm morphology, motility and mitochondrial function: Human Reproduction, v. 17, p. 704-709.

Paniagua-Chavez, C.G., Jenkins, J., Segovia, M., and Tiersch, T.R. (in press), Assessment of gamete quality for the eastern oyster (*Crassostrea virginica*) by use of fluorescent dyes: Cryobiology.

Patino, R., Goodbred, S., Draugelis-Dale, R., Barry, C.E., Foott, J.S., Wainscott, M.R., Gross, T.S., and Covay, K.J., 2003, Morphometric and histopathological parameters of gonadal development in adult common carp from contaminated and reference sites in Lake Mead, Nevada: Journal of Aquatic Animal Health, v. 15, p. 55-68.

Petty, J.D., Huckins, J.N., Alvarez, D.A., Brumbaugh, W.G., Cranor, W.L., Gale, R.W., Rastall, A.C., Jones-Lepp, T.L., Leiker, T.J., Rostad, C.E., and Furlong, E.T., 2004, A holistic passive integrative sampling approach for assessing the presence and potential impacts of waterborne environmental contaminants: Chemosphere, v. 54, p.695-705.

Poels, C.L.M., van der Gaag, M.A., and van der Kerkhoff, J.F.J., 1980, An investigation into the longterm effects of Rhine water on rainbow trout: Water Research, v. 14, p. 1029-1035.

Pustowka, C., McNiven, M.A., Richardson, G.F., and Lall, S.P., 2000, Source of dietary lipid affects sperm plasma membrane integrity and fertility in rainbow trout *Oncorhynchus mykiss* (Walbaum) after cryopreservation: Aquaculture Research, v. 31, p. 297-305.

Saberwal, G.S., Gill-Sharma, M.K., Balasinor, N., Choudhary, J., Padwal, V., and Juneja, H.S., 2003, Tamoxifen, protein kinase C and rat sperm mitochondria: Cell Biology International, v. 27, p. 761-768.

Salinas-Flores, L., Paniagua-Chavez, C.G., Jenkins, J.A., and Tiersch, T.R, 2005, Cryopreservation of sperm of red abalone (Haliotis rufescens): Journal of Shellfish Research, v. 24, p. 415-420.

SAS Institute Inc., 1999, SAS/STAT® User's Guide, Version 8, Fourth Edition, Volume 2, Cary, NC: SAS Institute Inc., p 3569-3590.

Schlenk, D., 2001, General Mechanisms of Toxicity, *in* Schlenk, D., and Benson, W. H., eds., Target Organ Toxicity in Marine and Freshwater Teleosts: New York, Taylor and Francis, p. 1-25.

Schreck, C.B., Contreras-Sanchez, W., and Fitzpatrick, M.S., 2001, Effects of stress on fish reporduction, gamete quality, and progeny: Aquaculture, v. 197, p. 2-24.

Segovia, M., Jenkins, J.A., Paniagua-Chavez, C., and Tiersch, T.R., 2000, Flow cytometric evaluation of antibiotic effects on viability and mitochondrial function of stored sperm of Nile tilapia: Theriogenology, v.53, p.1489-1499.

Shelton L.R., and Capel, P.D., 1994, Guidelines for collecting and processing samples of stream bed sediment for analysis of trace elements and organic contaminants for the National Water-Quality Assessment Program. U.S. Geological Survey Open-File Report 94-458, Sacramento, 20 p.

Song, M., and Gutzeit, H.O., 2003, Effect of 17-alpha-ethynylestradiol on germ cell proliferation in organ and primary culture of nedaka (*Oryzias latipes*) testis: Developmental Growth Differentiation, v. 45, p. 327-337.

Thomas, C.A., Garner, D.L., DeJarnette, J.M., and Marshall, C.E., 1998, Effect of cryopreservation on bovine sperm organelle function and viability as detemrined by flow cytometry: Biology of Reproduction, v. 58, p. 786-793.

Toft, G., Edwards, T.M., Baatrup, E., and Guillette, L.J., 2003, Disturbed sexual characteristics in male mosquitofish (*Gambusia holbrooki*) from a lake contaminated with endocrine disruptors: Environmental Health Perspectives, v.111, p.695-701.

Tuschl, H., and Schwab, C.E., 2004, Flow cytometric methods used as screening tests for basal toxicity of chemicals: Toxicology in Vitro, v. 18, p. 483-491.

Turgut, G., Abban, G., Turgut, S., Take, G., 2003, Effect of overdoes zinc on mouse testis and its relation with sperm count and motility: Biological Trace Element Research, v.96, p.271-279.

Wu, A.S.H., Oldfield, J.E., Shull, L.R., and Cheeke, P.R., 1979, Specific effect of selenium deficiency on rat sperm: Biology of Reproduction, v. 20, p. 793-798.

Xu, L., Wang, S., Yang, X., and Wang, X., 2001, Effects of cadmium on rat sperm motility evaluated with computer assisted sperm analysis: Biomedical and Environmental Sciences, v. 14, p. 312-317.

Zar, J.H., 1984, Data Transformations, *in* Biostatistical Analysis (2nd ed.): Englewood Cliffs, Prentice-Hall, Inc., p. 236-243.

Zrimsek, P., Kunc, J., Kosec, M., and Mrkun, J., 2004, Spectrophotometric application of resazurin reduction assay to evaluate boar semen quality: International Journal of Andrology, v. 27, p. 57-62.