Cellular diagnostics and health of juvenile green turtles (*Chelonia mydas*): Identifying environmental cofactors of fibropapillomatosis

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Abstract

Fibropapillomatosis (FP) is a tumor-causing disease that is becoming increasingly common in marine turtles worldwide. In Florida, FP is common in juvenile green turtles (*Chelonia mydas*) foraging in the eutrophic Indian River Lagoon (IRL) and increasing in frequency on a near-shore sabellarid worm rock reef (SWRR), but rare in a Trident Submarine Basin (TSB). In 2004-2005, we collected blood samples from juvenile *C. mydas* and evaluated turtle health in these three sites. Compared to TSB turtles, IRL turtles had elevated levels of cytochrome P450's, glutathione-S-transferase, multidrug resistance protein, heat shock protein 70, and other enzymes indicative of exposure to environmental pollutants. Elevated levels of heat shock protein 70 were significantly correlated with the presence of FP tumors. Mononuclear cells incubated in vitro with malathion or endosulfan confirmed that cellular responses of IRL turtles were consistent with pesticide stress. These results identify likely environmental cofactors of FP and link anthropogenic pollutants with declining health in an endangered marine turtle.

Keywords: Biomarker; Cellular diagnostics; *Chelonia mydas*; Cytochrome P450; Fibropapillomatosis; Florida; Turtle; Xenobiotics

1. Introduction

Fibropapillomatosis (FP) is an infectious disease threatening populations of green turtles (*Chelonia mydas*) in Florida, the Caribbean, Hawaii and Australia (Jacobson et al., 1989; Balazs, & Pooley, 1991; Aguirre, Limpus, Spraker, & Balazs, 2000). It is a tumor-forming disease characterized by multiple, small to medium-sized (1-25 cm) cutaneous masses on the flippers, eyes, neck or tail (Balazs, Aguirre, & Murakawa, 1997). Green turtles with large numbers of tumors often are immunosuppressed and have difficulty swimming, breathing and feeding. The disease is associated with a herpesvirus (Herbst, 1994; Herbst et al., 1995; Quackenbush et al. 1998) and is most common in marine environments degraded by agricultural, industrial and urban development (Adnyana, Ladds, & Blair, 1997; Herbst, Garber, Lockwood, & Klein, 1998). Despite evidence of an environmental component to FP, at present no association has been established with any anthropogenic or natural stressor (Herbst, & Klein, 1995; Aguirre, & Lutz, 2004).

Here, we employ a novel biotechnology - a cellular-diagnostic system (CDS) - to identify environmental stressors associated with FP prevalence in central Florida populations of green turtles. The CDS includes immunoassays that reflect protein metabolic condition, metabolic homeostasis, oxidative stress and response, and xenobiotic response and damage. The CDS is designed to identify sources of stress, the cellular-physiological defenses mounted against them, and accumulation of damage products that indicate defenses are overwhelmed (Downs et al., 2005). Thus, it provides evidence of the type of stress affecting an organism and its physiological consequences, information vital for juvenile green turtles that spend long periods in degraded and potentially stressful ecosystems such as Indian River Lagoon, Florida.

Cellular diagnostic systems are used for health surveillance in a manner similar to medical diagnostics (Downs et al. 2001ab, and Fauth et al., 2003). Health surveillance in medical studies typically uses a defined suite of biomarkers to identify individuals or populations at risk of adverse health conditions so preventative measures can be taken. The best example of this form of monitoring is a standard blood "work-up." Between 17 and 25 molecular biomarkers are assayed, each indicative of a specific physiological condition (Porth, 1994). No single biomarker is used as a definitive benchmark of health - changes in each biomarker are considered in association with all others in evaluating an individual's condition. If some biomarkers differ significantly from nominal levels, additional assays often are conducted to accurately diagnose the health condition. Once the underlying medical problem is identified, physicians tell patients about the expected outcome of ignoring the problem, based on large-scale clinical studies. Physicians then counsel patients about changes they can make to alleviate symptoms (e.g., modify diet, exercise), prescribe medicine to restore the desired physiological condition or intervene surgically. The same is true in the marine environment; once the source of stress is identified, resource managers can mitigate it through regulatory action or restoration. For example, the antifouling paint Irgarol 1051 was banned in Bermuda when CDS data linked it to coral declines (Downs et al., in press).

Here, we compare CDS parameters of three populations of green turtles with differing incidence of FP to identify potential stressors associated with disease prevalence. We also compare CDS parameters of green turtle mononuclear cells exposed *in vitro* to malathion and endosulfan to assess the similarity of responses to wild-caught turtles. Our goal is to identify likely environmental co-factors of FP, thereby providing resource managers with information

useful for reducing the threat this disease poses to marine turtles.

2. Materials and methods

2.1 Study sites

We compared CDS parameters and ecological measures of turtle condition among three green turtle populations with differing incidence of FP (Fig. 1). Indian River Lagoon (IRL) is a degraded estuary and up to 70% of green turtles captured there have FP (Ehrhart, 1991; Ehrhart, Redfoot, & Bagley, 2000). Dissolved nitrogen and phosphorus levels in IRL are high, with run-off from citrus groves, cattle ranches and storm sewers implicated as sources of eutrophication (Trocine, & Trefry, 1993). In addition, the pesticides dicofol, diuron, ethion, malathion, and p,p-DDE were detected in drainage canals emptying into IRL. Diuron and ethion concentrations were at levels likely to be acutely toxic to aquatic organisms and state water quality standards for malathion and ethion were violated 12 times in 1992-1997 (MacDonald, Carr, Calder, Long, & Ingersol, 1996) . Indian River Lagoon also has "hot spots" of metal contamination: local areas with concentrations of cadmium, lead, zinc and copper up to ten times greater than natural levels (Trocine & Trefry, 1993). Sebellarid Worm Rock Reef (SWRR) is a nearshore habitat formed by polychaete worms (*Phragmatopoma lapidosa*). The site is continuously flushed by open ocean currents but occasionally fresh water from IRL flows over this site, which is east of IRL and separated from it by a narrow barrier island. Juvenile green turtle populations at these two sites are distinct in terms of movements and foraging preferences (Holloway-Adkins, 2001; L. Ehrhart, pers. obs.), and <32% of turtles captured at SWRR have FP. Trident Submarine Basin (TSB) is an artificial basin at the mouth of Cocoa Beach inlet and

is continually flushed with oceanic water. Fibropapillomatosis has only been observed in a single TSB turtle, which was captured in 2005. This individual was not vigorous enough to swim into TSB, and may have drifted into it from IRL via the haulover canal.

2.2. Sampling protocol

Turtles were captured using large-mesh tangle nets or by dipnetting (TSB only) from small motorboats. Blood samples were taken from the dorsal cervical sinus (Owens, & Ruiz, 1980) using a syringe and glass vacutainer. Samples were taken within 1-5 minutes of capture to avoid evidence of stress in CDS assays due to capture. Samples were stored on ice until they were centrifuged, usually within a few hours of collection; optimal results were obtained when samples were processed within 30 min. Samples were centrifuged for 10 min at 3,000 x g to separate white blood cells, red blood cells, and plasma. We transferred the latter two components into separate plastic cryovials using a disposable plastic pipette and performed additional rinses with 1X PBS, followed by centrifugation and removal of supernatant. Rinsed samples were immediately placed in liquid nitrogen. We then suspended the buffy coat layer, which contains white blood cells, in 1X PBS solution and centrifuged them at 3,000 x g for 10 minutes. Optimal results were obtained when we repeated this process 2-3 times, then froze the resulting pellet in a plastic cryovial. All blood components were stored at -80°C until CDS analyses were performed.

We recorded several morphological parameters of each turtle, including straight carapace length (SCL), mass and FP severity score: 0 = no FP, 1 = mild FP, 2 = moderate FP, 3 = severe FP. All FP lesions were photographed with a digital camera and measured. Presence of adult

ozobranchus leeches or their eggs also was recorded. At the end of each sampling period, standard oceanographic variables (e.g., salinity, water temperature, turbidity) were recorded and a 5 L water sample and a 250 mL sediment sample were taken in U.S. EPA-certified clean containers and frozen for future analyses.

2.3 Reagents

Chemicals for buffer solutions were obtained from Sigma Chemicals Co. (St. Louis, Missouri, USA) or Haereticus Environmental Laboratory (Amherst, Virginia, USA). PVDF membrane was obtained from Millipore Corp. (Bedford, Massachusetts, USA). Calibrant standards and antibodies against all cellular parameters were obtained from Haereticus Environmental Laboratory. Antibodies were raised against an 8–12 residue polypeptide conjugated to oval albumin. Antigens were designed based on unique, conserved domains within the target protein. Rabbits were immunized with the antigen with a Ribi-adjuvant carrier. All antibodies used in this study were immuno-purified with a Pierce SulfoLink Kit (cat.# 44895) using the original unconjugated peptide as the affinity binding agent. Anti-rabbit conjugated horseradish peroxidase antibodies were obtained from Jackson Immunoresearch (West Grove, PA, USA).

2.4. Cellular-diagnostic system parameters and their biological significance

Cellular diagnostics systems (theory reviewed by Downs, 2005) use an integrated array of biomarkers of exposure, effect, and susceptibility, which are organized based on their cellular functions.

Protein metabolic condition - We evaluated protein metabolic condition using three heat shock proteins (Hsp's: Hsp 60, Hsp 70 and Hsp 90) and glucose-related protein (Grp). Heat shock proteins (Hsp's) are molecular chaperones universal to all eukaryotic cells. During stress, Hsp's protect cells from elevated temperature and are important in repairing cellular damage (Near et al., 1990, and Welch, 1993). Heat shock protein 60 and Hsp70 levels increase in response to stress, specifically in response to increased protein synthesis and denaturation. Heat shock protein 90 has two independent physiological roles: maintaining "wild-type" structure and function, and supporting and protecting spindle fibers during mitosis and meiosis (Holt et al. 1998, and Rutherford and Lindquist, 1998). Glucose regulated protein is a mitochondrial matrix protein related to the Hsp 70 family and is induced under conditions of low glucose and other environmental stresses. It also is involved in various chaperoning functions and possibly in antigen recognition, cell proliferation and senescence (Pockley, 2001). Concentrations of Grp often are used as indicators of nutritional stress.

Oxidative stress and response - We assayed five parameters indicative of cellular stress induced by reactive oxygen species: copper-zinc superoxide dismutase (Cu/Zn SOD), multidrug resistance protein (MDR), heme oxygenase (HO), protoporphyrin oxidase (PPO), and porphyrin. Superoxide dismutases are enzymes that catalyze superoxide radicals to molecular oxygen and hydrogen peroxide, and comprise a main antioxidant defense pathway (Wu et al., 1999). Increased SOD levels have been linked to increased longevity and tolerance to ischemic/reperfusion events and oxidative stress (Fridovich, 1995). Multidrug resistance protein protects against toxicity of 4-hydroxynonenal, a major product of lipid peroxidation that can inhibit DNA, RNA, and protein synthesis, stop the cell cycle, disrupt mitochondrial functions,

and produce pathological disorders (Renes et al., 2000). Elevated MDR accumulations indicate defenses were mobilized against cellular stress. Heme oxygenase and PPO are involved in degradation and biosynthesis of heme, respectively (King, 2005). Heme oxidase degrades heme to biliverdin, which a NADPH-dependent catalytic reaction converts to the powerful antioxidant, bilirubin (Smith et al., 1994). Porphyrins are the major precursors of heme, which is an essential component of hemoglobin, myoglobin and cytochromes. High concentrations of porphyrins are toxic to tissues, and porphyria can be induced by drugs and chemicals (Kauppinen 2005).

Metabolic homeostasis - We assayed three parameters indicative of protein oxidation: ferrochelatase (FC), peripheral benzodiazepine receptors (PBRP), and aconitase. Ferrochelatase is an indicator of changes in metabolic state, usually in response to stress. Ferrochelatase inserts ferrous iron into protoporphyrin IX to form heme, which is essential for cellular metabolism and detoxification (Ferreira, 1999). For example, cytochrome *c* requires a form of heme to become an active electron carrier and cytochrome P450 requires heme to function. In addition, breakdown of heme leads to formation of billirubin and biliverdin (Smith et al. 1994); the former is a very effective anti-oxidant, rivaling Vitamin E as a scavenger of lipid hydroperoxyls. Peripheral benzodiazepine receptors (PBRP) are involved in cytokine-induced cell death and repair of tissue damage (Trincavelli et al. 2002). Aconitase isomerizes citrate to isocitrate, which is a key intermediate in the citric acid cycle. Aconitase is an important parameter of cellular energy production (Beinert and Kennedy, 1993); decreased aconitase activity is associated with oxidative damage caused by aging, Parkinson's disease and other illnesses.

Xenobiotic and heavy metal exposure - We assayed glutathione-S-transferase (Gst) and

cytochrome P450's, which are involved in detoxifying carcinogens, drugs, and environmental pollutants, and therefore reflect xenobiotic stress (Xinhua et al. 1997, Tom et al. 2002). Glutathione-S-transferase detoxifies genotoxic and cytotoxic xenobiotic electrophiles by conjugating them to glutathione (Ketterer et al. 1988). It also can repair DNA by detoxifying DNA hydroperoxides (Tan et al. 1988) and is a main defense for detoxifying 4-hydroxynonenal, an extremely reactive product of lipid peroxidation that cross-links proteins and forms adducts with DNA (de Zwart et al. 1999). We assayed three classes of cytochrome P450: 1-class, 2-class and 3-class. Cytochrome P450-1 class is involved in detoxifying polycyclic aromatic hydrocarbons, CYP 2 mainly detoxifies electrophilic carcinogens, drugs, and environmental pollutants, while CYP 3 detoxifies steroid-based compounds such as endocrine-disrupting chemicals (Danielson, 2002). We also assayed metallothioneins, which are cystein-rich, low-molecular weight proteins that bind and detoxify a variety of metals (Klaassen et al. 1999, Van Cleef-Toedt et al. 2001).

2.5 Sample preparation, ELISA validation, and ELISA

Blood samples were ground to a powder using a ceramic mortar and pestle chilled with liquid nitrogen. Frozen blood samples were placed in 1.8 ml microcentrifuge tubes containing 1400 L of a denaturing buffer comprised of 2% SDS, 50 mM Tris–HCl (pH 6.8), 25 mM dithiothreitol, 10 mM EDTA, 0.001 mM sorbitol, 7% polyvinylpolypyrrolidone (wt/vol), 0.1% polyvinylpyrrolidone (wt/vol), 0.01mM alpha-tocopherol, 0.005 mM salicylic acid, 0.01mM AEBSF, 0.04 mM Bestatin, 0.001E-64, 2 mM phenylmethylsulfonylfluoride, 2 mM benzamidine, 0.01mM apoprotin, 5 lM a-amino-caproic acid, and 1 lg/100 ul pepstatin A.

Samples were heated at 95°C for 3 min, vortexed for 20 s, incubated at 95° C for another 3 min, and then incubated at 25°C for 5 min. Samples were centrifuged at 10,000 X g for 10 min. Supernatant was transferred to a new tube, centrifuged at 10,000 X g for 5 min and again transferred to a new tube and subjected to a protein concentration assay (Ghosh et al., 1988). To ensure equal sample loading, 40 :g of total soluble protein of samples from one site and time point were loaded onto a 12.5% SDS-PAGE gel (16 cm), the gel was run until the bromophenol blue dye front was near the bottom of the gel, stained with a Coomassie blue solution (BB R-250) overnight, and then destained for 4 h with multiple washes of destaining solution. Equal loading was determined by visualization and optical density using a Canonscan scanner and analysis performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). This method visually validated the protein concentration assay and ensured that sample artifact had not occurred between the time of sample homogenization and analysis.

One-dimensional SDS-PAGE and western blotting validated the legitimacy of using enzyme linked immunosorbent assays (ELISA's) on this species of turtle using our specific antibodies (Downs, 2005). Five to 15 micrograms of total soluble protein was loaded onto a 20-, 16-, or 8-cm SDS-polyacrylamide gel with various concentrations of bis/acrylamide. Gels were blotted onto PVDF membrane using a wet transfer system. Membrane was blocked in 7% non-fat dry milk, and incubated with the primary anti-body for 1 h. Blots were washed in tris-buffered saline (TBS)-0.05% Tween (v/v) four times, and incubated in a horseradish peroxidase-conjugated secondary antibody solution for 1 h. Blots were washed four times in

TBS, and developed using a chemiluminescent reporter system.

Once validated, antibodies and samples were optimized for ELISA using an 8 X 6 X 4 factorial design (Crowther 2001). Every ELISA assay was optimized for proper concentration of protein loading, antibody titer, sample-to-standard calibration and handling procedures as a measure of quality control and quality assurance (Downs 2005). A Beckman-Coulter Biomek 2000 using 384-well microplates was used to conduct the ELISA assays. Samples were assayed in triplicate with intra-specific variation of less than 6% for the whole plate. An eight-point calibrant curve using a calibrant relevant to each antibody was plated in triplicate for each plate.

2.6 In vitro *experiment*

To test whether CDS responses of wild-caught turtles were consistent with exposure to xenobiotics, we conducted an *in vitro* experiment using buffy coats (combination of platelets and white blood cells plus small amounts of red blood cells and plasma) isolated from a juvenile green turtle sampled at the entrance to the canal supplying the St. Lucie Nuclear Power Plant, Martin County, Florida. Turtles enter this site from the open ocean and are considered to be minimally affected by anthropogenic contaminants (e.g., Jacobsen et al., 2005).

We obtained blood samples from a single juveile turtle as described in section *2.2* above. We aspirated the plasma to the mononuclear cell band, then pipetted out the mononuclear cells and placed them into a 15 mL Falcon tube. We added PBS to bring the volume to 15 mL, mixed cells by gently inverting the tube five times, and centrifuged them for 15 minutes at 300 RCF. We then aspirated as much supernatant as possible without disturbing the cell pellet and immediately added 10 mL of cell culture media, composed of 950 mL of Cell

Culture Media Base plus 100 mL of CO_2 buffering solution (= C-media). Cell culture media base contained RPMI-1640 (Sigma-Aldrich), 10% (v/v) fetal bovine serum, 1% v/v L-glutamine, 25 mM HEPES, Gibco antibiotic mix, and CO_2 buffering solution (250 mM HEPES, 120 mM sodium bicarbonate, and 1 mM sodium pyruvate).

We added 40 mL of C-media to twelve labeled, 50 mL Falcon tubes containing either 0.5 nM malathion, 100 nM malathion, 0.5 nM endosulfan, 100 nM endosulfan, or solvent controls: xylene for malathion and acetone for endosulfan. Each of these six treatments was replicated four times. We resuspended the white blood cell pellet with 2.5 mL PBS and placed equal aliquots into each tube, then incubated them either for 8 hours at 22ºC. After the incubation period, we centrifuged tubes at 300 RCF for 15 min, then aspirated as much supernatant as possible without disturbing the cell pellet. We added 10 mL PBS, mixed cells by inversion five times, and centrifuged again for at 300 RCF 15 min. We aspirated as much supernatant as possible without disturbing the cell pellet, then resuspend it with 100 uL Denaturing Buffer 107. We then froze samples in liquid nitrogen and transferred them to a -80ºC freezer until CDS analyses were performed, as described in *2.5* above.

2.7 Diagnostic strategy and statistical analyses

We used multivariate analysis of variance (MANOVA) to test the null hypothesis

H_0: the vector of mean CDS parameters did not vary among sites,

versus

H_A: the vector of mean CDS parameters varied among sites.

A priori, we expected IRL turtles to have higher mean CDS responses than turtles at

SWRR and TSB if the former mounted cellular defenses against chronic stress, and lower mean CDS responses if they were stress-compromised or otherwise debilitated. We adhered to model assumptions of multivariate random, normally-distributed and independent residuals by transforming variates as $\log_{10}(x + 1)$. We used analysis of covariance to determine whether CDS parameters varied with turtle size (SCL); they did not, so we removed this covariate from subsequent analyses. We used separate univariate tests to interpret significant MANOVA results and Tukey's Honestly Significant Difference method to separate univariate means. This procedure limited the probability of committing a Type I error even when responses were correlated.

We quantified the relative body condition of juvenile green turtles using residuals from an allometric regression of mass on straight-line carapace length, similar to the "body condition index" of Jessop et al. (2004). Individuals with positive residuals were heavier than expected for a turtle of their length and individuals with negative residuals were lighter than expected. We then used backward stepwise linear regression to identify CDS parameters that explained significant deviance in relative condition. We used nominal logistic regression to identify CDS parameters associated with FP status, defined as either tumors absent (FP category 0) or present (FP categories 1-3).

We used stepwise discriminant function analysis (DFA) to identify CDS parameters useful for assigning turtles to their particular population, and canonical correspondence analysis (CCA) to determine which parameters best revealed stressors at each site. Both techniques are eigenanalysis methods that reveal the basic relationships between two matrices (Gauch 1985), in our case sites and cellular parameters. Stepwise DFA identified the CDS parameters that

independently contributed most to predicting to which population each juvenile turtle belonged, while CCA provided an objective statistical tool for determining which cellular parameter (or suite of parameters) best indicated the presence of a particular environmental stressor (Downs 2005). All statistical analyses were performed using JMP V. 4.0.4 (SAS Institute, Inc., Cary, NC, USA), with " = 0.05 for all hypothesis tests.

3. Results

3.1 Antibody validation

Antibodies against cellular parameters of green turtles did not exhibit significant non-specific cross-reactivity (data not shown) and therefore were valid for use in ELISA.

3.2 Responses of wild-caught turtles

Our samples from November and December, 2004, were representative of the long-term incidence of FP in juvenile green turtles at TSB and IRL (Ehrhart et al., 2000). Included were 22 TSB turtles and fifteen IRL turtles without visible symptoms of FP, ten IRL turtles with external tumors, and one recaptured IRL turtle whose tumors had disappeared since its initial capture. Mean CDS responses of juvenile green turtles in IRL and TSB differed significantly (MANOVA: $F_{10,37} = 24.77$, $P < 0.0001$). All CDS parameters except Hsp 60 and FC were significantly higher in IRL compared to TSB turtles (Fig. 2) although the difference in mean FC accumulation also approached statistical significance (ANOVA: $F_{1,46} = 3.97$, $P = 0.0523$). Parameters indicative of protein metabolic condition (Hsp 70, Hsp 90), oxidative stress and response (Cu/Zn SOD, MDR) and xenobiotic exposure (CYP's 1-3, MDR, GST) were 2-4 times

higher in IRL than TSB turtles (Fig. 2).

Stepwise discriminant function analysis revealed that only two CDS parameters (Cu/Zn SOD and GST) were required to correctly assign most turtles (46 of 48; 96%) to their capture site. Two turtles were misclassified as being from IRL when they were sampled in TSB. It is possible that green turtles could emigrate from the large IRL feeding aggregation into the much smaller TSB. However, to date no turtle tagged in IRL has been recaptured in TSB.

Including individuals from both sites, turtle relative condition varied significantly with their accumulations of CYP 2 and Hsp 70 (Table 1). Turtle condition improved with increasing CYP 2 accumulation and declined with increasing Hsp 70 accumulation. The FP status of individuals did not affect this result and the full model presented in Table 1 explained 22% of the total variation in turtle relative condition.

Nominal logistic regression showed that four CDS parameters were indicative of FP status (Table 2). Turtles with greater accumulations of MDR and Hsp 70 and lesser accumulations of FC and Hsp 60 were more likely to have FP. The best single predictor of FP status was Hsp 70 (Fig. 3). Turtles with more than 3.552 ng Hsp 70/g total soluble protein were more likely to have visible FP tumors than to be scored as FP-free. This test has a false positive rate of about 20% (Table 2).

Juvenile green turtles were significantly more likely to have adult leeches or their eggs at IRL compared to TSB (G-test of independence, $G = 12.79$, $P < 0.0003$). Nine of the 26 turtles sampled at IRL had leeches or their eggs compared to none of the 22 TSB turtles. However, FP status was independent of the presence or absence of leeches and their eggs (G-test of independence, $G = 3.275$, $P < 0.0704$). Leeches also had no effect on the vector of CDS

responses.

3.3 In vitro *dose-response experiment*

Mean CDS responses of buffy coats incubated with malathion and endosulfan differed significantly from both the solvent controls and responses from wild-caught turtles at SWRR and IRL (Fig. 4). Buffy coat profiles of SWRR turtles were most similar to controls (Fig. 4), which is consistent with the hypothesis that turtles foraging on offshore reefs are less exposed to anthropogenic contaminants than juvenile turtles foraging in IRL (Ehrhart et al., 2000). In contrast, profiles of IRL turtles were most similar to those of buffy coats incubated with 100 nM malathion; 95% confidence regions of these two treatments overlapped in canonical space defined by accumulations of Hsp 60, Grp, aconitase and metallothionein (Fig. 4B). In addition, total porphyrin accumulations in white blood cells isolated from IRL turtles were significantly higher than those of white blood cells incubated with 100 nM malathion (Fig. 4A). Combined, these results are consistent with the hypothesis that juvenile turtles were exposed to multiple chemical contaminants in IRL.

4. Discussion

Fibropapillomatosis is an emerging infectious disease that is threatening populations of green turtles worldwide. Fibropapillomatosis is most common in degraded habitats (Adnyana et al. 1997, and Herbst et al., 1998), which led several authors (e.g., Herbst and Klein 1995, and Aguirre and Lutz, 2004) to suggest that FP may have an environmental component. The goal of our study was to use a cellular-diagnostic system (CDS) to identify possible environmental

cofactors of FP. Our approach was four-fold. 1. We compared CDS parameters of juvenile green turtles from highly degraded IRL, where FP has been common since the 1980's, with those of turtles from TSB, where FP has been recorded only once. 2. We compared CDS parameters of turtles with differing FP histories (tumors absent, present, or regressed) to identify potential indicators of disease. 3. We compared CDS profiles of turtles with different relative body conditions to assess the fitness consequences of stress. 4. To test our interpretation of CDS responses, we compared CDS profiles of buffy coats experimentally exposed *in vitro* to two common pesticides with those isolated from wild-caught juvenile green turtles from IRL and SWRR. Results of all four approaches concur: IRL turtles experienced xenobiotic stress and the resulting cellular damage was significantly associated with FP tumors.

Cellular-diagnostic profiles of juvenile green turtles from IRL were consistent with a diagnosis of xenobiotic stress. Compared to individuals from TSB, IRL turtles had very high accumulations of CYP 2, CYP 3, GST and MDR, which are important components of a three-phase xenobiotic detoxification pathway (Fig. 5). In Phase I, polar groups are adducted to xenobiotics by enzymes in the cytochrome P450 superfamily, flavin-containing monooxygenases, and various esterases. In Phase II, these new polar metabolites are conjugated with endogenous substrates (e.g., glutathione, sulfates, acetates, and glucuronides) by enzymes such as GST, sulfotransferases, and UDP-glucuronosyltransferases (Negishi et al., 2001). In Phase III, these water-soluble products are transported to lysosomes for further metabolism, sequestered in lysosome-like structures or excreted from the cell through active diffusion transporters, such as the ATP-binding cassette transporters (e.g., MDR; Borst and Elferink, 2002),

Increased accumulations of CYP 2, CYP 3, and (to a lesser extent) CYP 1 indicate that IRL turtles were exposed to multiple xenobiotics. Cytochrome P450 enzymes are a superfamily of membrane-bound hemoproteins that localize to microsomes, endoplasmic reticulum, and mitochondria, and catalyze the oxidation of diverse substrates (Guengerich, 2004). Animals have >50 cytochrome P450 isoforms, which in addition to their role in Phase I detoxification are crucial components in the metabolism of steroid hormones and lipid biofactors (Omura, 1999). The CYP 2 class is induced by electrophilic carcinogens, pharmaceutical agents and environmental pollutants (Snyder, 2000), and the 3.5-fold increase of CYP 2 in IRL turtles compared to TSB turtles indicates exposure to these xenobiotic substrates. The CYP 3 class is induced by xenobiotics and steroid-based compounds, and the 3.8-fold increase of CYP 3 in IRL turtles relative to TSB turtles indicates exposure to these compounds, which include endocrine-disrupting chemicals (Lamba et al., 2002). The CYP 1 class is induced by crude oils, polyaromatic hydrocarbons (PAH's), polychlorinated biphenols (PCB's), dioxins, and other substrates (Arinç et al. 2000). Its relatively modest (47%) increase in IRL compared to TSB turtles likely caused additional stress because turtles from both sites were in better condition (i.e., they were heavier than predicted for an individual of their carapace length) when levels of CYP 1 were high and Hsp 70 was low. Higher levels of GST and MDR in IRL compared to TSB turtles is further evidence that cellular defenses were mounted against xenobiotic stress.

Cellular-diagnostic profiles also indicated that IRL turtles experienced oxidative stress, which in turn affected protein-metabolic condition. Compared to TSB turtles, individuals from IRL accumulated three times more Cu/Zn SOD and Hsp 90, and twice as much Hsp 70. Metabolism of xenobiotic compounds by microsomal cytochrome P450 generates reactive

oxygen species, which are cleared from the cell by SOD's, catalase and glutathione peroxidase. Higher levels of Cu/Zn SOD in IRL compared to TSB turtles are consistent with xenobiotic detoxification via cytochrome P450's. Furthermore, Hsp 90 is essential for regulating CYP 2 via the ubiquitin/proteosome protein degradation pathway (Goasduff and Cederbaum, 2000) and the level of this molecular chaperone also was higher in IRL than in TSB turtles. Cytosolic Hsp70 primarily renatures denatured proteins back to an active state and assists in degrading proteins that are too damaged to be repaired (Hartl, 1996, and Ellis, 1996). Hsp70 was significantly elevated in IRL compared to TSB turtles, supporting the interpretation that protein complexes were being damaged in the cytosol and cells were compensating for increased protein turnover.

Examining CDS profiles of juvenile green turtles from two sites with differing histories of FP permits diagnosis of potential stressors, as described above. Prognosis, or forecasting of disease (e.g., Fauth et al. 2003), requires linking CDS responses to FP status. Logistic regression identified five CDS parameters suitable for prognosis: CYP 2, CYP 3, GST, MDR and Hsp 70. The first four parameters are involved in Phases I, II, and III of the xenobiotic detoxification pathway, and Hsp 70 indicates that protein turnover occurred. If distress at the cellular level increases susceptibility to FP, increases in all five parameters should be associated with a higher probability of an individual turtle having tumors. This was exactly the pattern exhibited by IRL green turtles. The best single predictor of FP status in juvenile green turtles was Hsp 70 because it had the lowest rate of false positives.

One of the central tenets of cellular diagnostics is that when stressors overwhelm cellular defenses, the condition of tissues, organs, organ systems, and eventually whole organisms deteriorates (Adams, 1990, Adams and Ryon, 1994, Downs, 2005). In our study, juvenile green

turtles with elevated levels of Hsp 70 were in poorer relative condition than turtles with low levels of these parameters. Diminished body condition also has been noted in green turtles experiencing other stressful situations (e.g., breeding: Jessop et al., 2002, handling stress: Jessop et al., 2004) and Hsp 70 has been suggested as a potential early-warning marker of xenobiotics in other taxa (e.g., Nadeau et al., 2001). Further testing of Hsp70 as an indicator of stress exposure and FP susceptibility in green turtles is warranted.

Many studies of environmental stress and response are limited to using a weight-of-evidence or ecoepidemiological approach to infer causality (Adams, 2003). To strengthen the inferences drawn from our field study, we used an *in vitro* experiment on buffy coat cells isolated from juvenile green turtles to test whether CDS responses of IRL turtles could be produced by chemical contaminants. Buffy coat cells incubated with 100 nM malathion had levels of Hsp 60, aconitase, metallothionein, BDRP, and porphyrin similar to accumulations recorded from IRL turtles. Increased accumulation of porphyria species in IRL turtles indicates that they were exposed to a xenobiotic such as malathion, or another pesticide with a similar mode of action. However, upregulation of CYP 1, CYP 2 and CYP 3 in IRL turtles compared to TSB turtles strongly suggests that multiple contaminants are stressing turtles in Indian River Lagoon.

Herbst & Klein (1995) cautioned that environmental contaminants are not the only potential contributors to increased prevalence of FP in green turtles. We agree with their assessment; our study has identified environmental contamination from multiple xenobiotics as causing stress in IRL green turtles, and linked that stress to diminished physical condition and increased probability of having external FP tumors. However, our study does not exclude other

potential mechanisms that could cause increase FP in IRL relative to the other two sites. Leeches of the genus *Ozobranchus*, which are the only known mechanical vector of FP (Greenblatt et al., 2004), are common on IRL green turtles, uncommon on SWRR turtles, and absent from TSB turtles. This pattern also is concordant with the pattern of FP incidence and therefore is a (non-mutually exclusive) hypothesis that warrants immediate investigation.

5. Conclusions

Fibropapillomatosis has been more common in juvenile green turtles (*Chelonia mydas*) in the eutrophied Indian River Lagoon (IRL) than in a nearby Trident Submarine Basin and a Sabellarid Worm Rock Reef off the east-central Florida coast. Buffy coat fractions isolated from IRL turtles had greater accumulations of biomarkers of protein metabolic condition, oxidative stress and response, metabolic homeostasis, and xenobiotic stress and response compared to wild-caught turtles from the other two sites. Turtles with above-average accumulations of multidrug resistance protein and heat shock protein 70 and lesser accumulations of ferrochelatase and heat shock protein 60 were the most likely to have fibropapillomatosis. Cellular-diagnostic profiles of IRL turtles were consistent with increased exposure and stress caused by xenobiotics. Buffy coat fractions exposed *in vitro* to high concentrations of malathion and endosulfan had cellular-diagnostic profiles similar to those obtained from IRL turtles. In contrast, control samples overlapped those of SWRR turtles, corroborating the hypothesis of a xenobiotic exposure. Likely contaminants include porphyria-causing compounds, malathion and other pesticides with similar modes of action, and endocrine-disrupting compounds, but not polyaromatic hydrocarbons (PAH's). Ongoing research is comparing pesticide levels in turtle

blood samples collected from IRL and SWRR in the 1990's-present, to further identify contaminants likely to be cofactors of fibropapillomatosis.

Acknowledgments

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Table 1. Result of backward stepwise regression on turtle relative condition, defined as the residual from the allometric regression of log(mass) on log(straight-line carapace length).[1] Whole model $F_{4,42} = 2.88$, $P < 0.034$, $R^2 = 0.215$. P-values significant at " = 0.05 are in bold.

Source of variation	df	SS	F	P	parameter estimate ± 1 S.E.
Site	1	0.00625	3.66	0.0625	0.0189 ± 0.009894
Log_{10}(CYP 2 + 1)	1	0.00714	4.19	**0.0470**	0.0431 ± 0.02106
Log_{10}(GST + 1)	1	0.00558	3.27	0.0777	-0.0286 ± 0.01583
Log_{10}(Hsp 70 + 1)	1	0.00993	5.83	**0.0202**	-0.0670 ± 0.02776
Error	42	0.07161			
Total	46	0.09126			

[1]Allometric equation: log $mass_{10}$ (kg) = -3.88 + 3.01 log_{10}(straight-line carapace length in cm); $F_{1,45} = 2220.1$, $P < 0.0001$, $R^2 < 0.98$.

Table 2. Results of nominal logistic regression of fibropapillomatosis status (tumors present or absent) versus cellular-diagnostic system (CDS) responses of juvenile green turtles (*Chelonia mydas*) sampled at Indian River Lagoon and the Trident Submarine Basin. Table entries are cellular-diagnostic parameters, $\mathbf{P}^2$ statistics, and P-values. Area under the receiver operating characteristic (ROC) curve is provided for statistically significant regressors. A perfectly sensitive test gives no false positives and has an ROC area of 1.00. Abbreviations of CDS parameters are given in section *2.4* of the text. P-values significant at **"** = 0.05 are in bold.

CDS parameter	X^2	P	Area under the ROC curve
Hsp 60	0.001	0.97	
Hsp 70	7.90	**0.005**	0.792
Hsp 90	2.30	0.13	
Cu/Zn SOD	1.83	0.17	
MDR	3.93	**0.047**	0.750
FC	1.91	0.17	
GST	5.35	**0.021**	0.763
CYP 1	0.19	0.66	
CYP 2	4.00	**0.045**	0.753
CYP 3	5.64	**0.018**	0.730

Figure legends

Figure 1. Location of the three study sites on Florida's central Atlantic coast. Indian River Lagoon (IRL); Trident Submarine Basin (TSB); Sabellarid Worm Rock Reef (SWRR).

Figure 2. Mean (± 1 S.E.) cellular-diagnostic system responses of wild-caught green turtles (*Chelonia mydas*) sampled at Indian River Lagoon and Trident Submarine Basin. Biomarker abbreviations are as in section 2.4 of the text.

Figure 3. Cumulative logistic plot of the probability that a juvenile green turtle had FP as a function of its Hsp 70 accumulation. The smooth curve partitions the probability axis between FP absent at lower left and present at upper right. For example, a turtle with 2000 pg Hsp 70/g total soluble protein has a 78.2% probability of not exhibiting FP and a 21.8% chance of having FP. Gray circles, IRL; black circles, TSB. Model $X^2 = 7.91$, $P < 0.005$, $R^2 = 0.16$.

Figure 4. Canonical plot of cellular-diagnostic system (CDS) responses of green turtle (*Chelonia mydas*) mononuclear cells exposed *in vitro* to different concentrations of endosulfan (gray ellipses), malathion (black ellipses), or requisite solvent controls. Centroids are means of four replicates of each experimental treatment. Also shown are responses of wild-caught turtles sampled at Sabellariid Worm Rock Reef (SWRR; N = 5) and Indian River Lagoon (IRL; N = 3). Canonical 1 is the linear combination of CDS responses that maximizes dispersion of these eight groups. Canonical 2 is the linear combination of CDS responses that maximizes dispersion

along an axis independent of Canonical 1. Canonical 2 necessarily provides less separation than Canonical 1. Within the space defined by the two canonical axes, ellipses show 95% confidence intervals of each group and biplot rays show directions of CDS parameters within canonical space. Biplot rays diverge from a common origin, which is the grand mean of the CDS responses (abbreviations are as in section *2.4* of the text). Longer rays identify CDS responses most closely related to the stressor gradient, while shorter rays indicate responses with little predictive value. Generally, the weighted value of a particular stressor on each CDS parameter is inferred as being higher than average if its projection point is on the same side as the ray, and is lower than average if its projection point is opposite the origin. A) Six parameters of protein metabolic condition, oxidative stress and response, and metabolic homeostasis. B) Four parameters of protein metabolic condition, metabolic homeostasis, and heavy-metal exposure.

Figure 5. General degradation pathway of xenobiotics in vertebrate cells.

Fig. 1

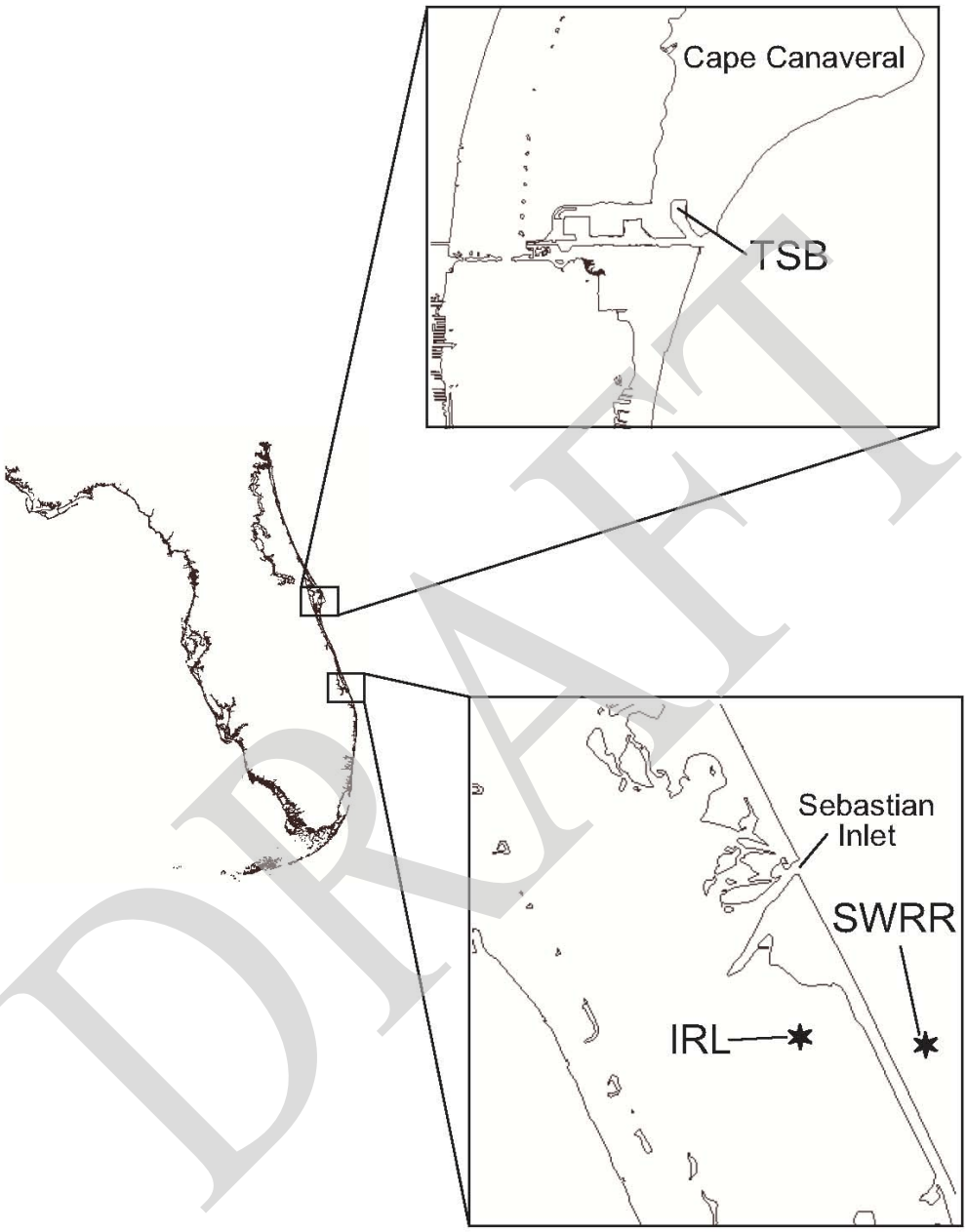


Fig. 2

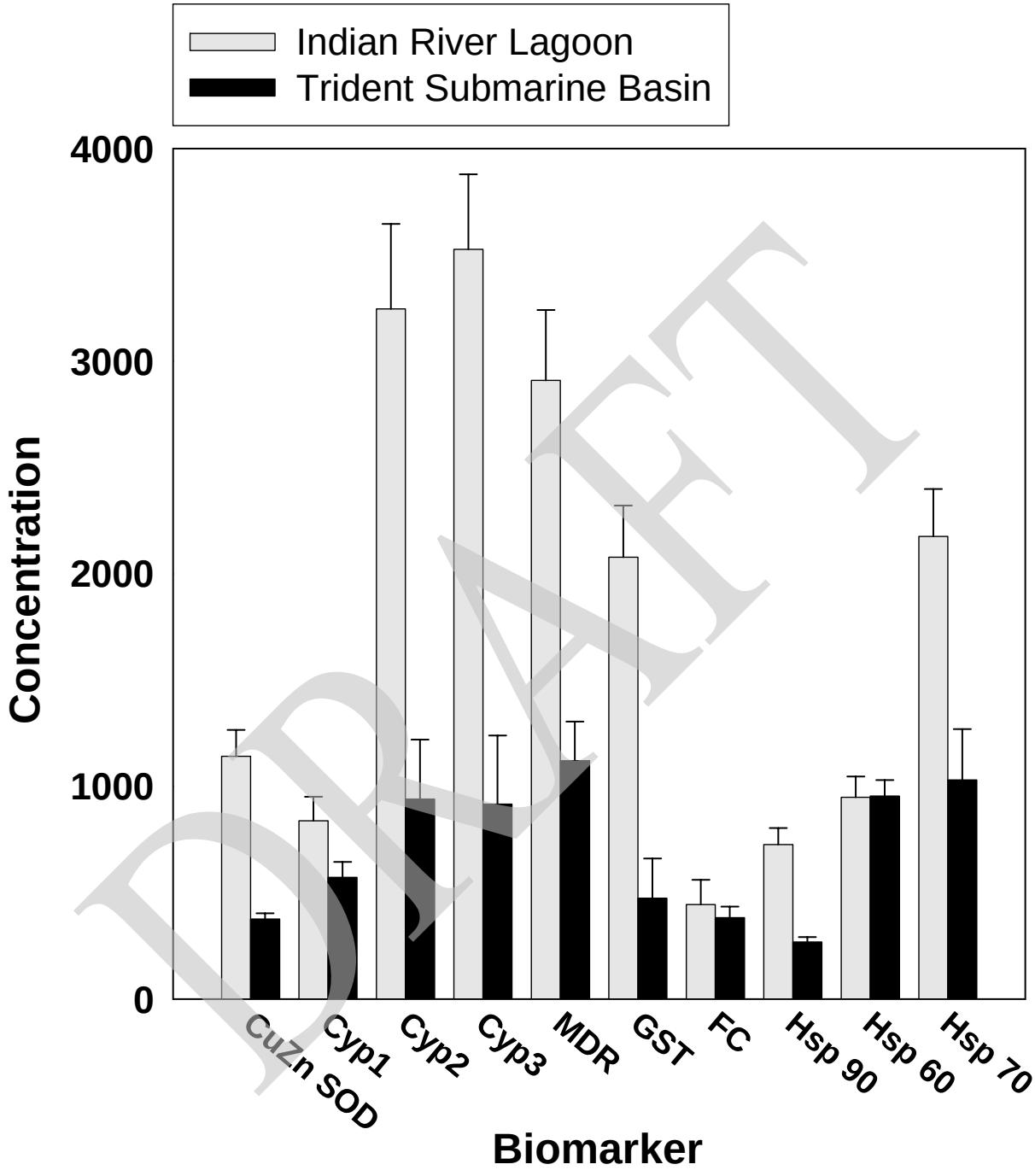

Indian River Lagoon
Trident Submarine Basin
4000
3000
2000
1000
0
Concentration
CuZn SOD
Cyp1
Cyp2
Cyp3
MDR
GST
FC
Hsp 90
Hsp 60
Hsp 70
Biomarker

Fig. 3

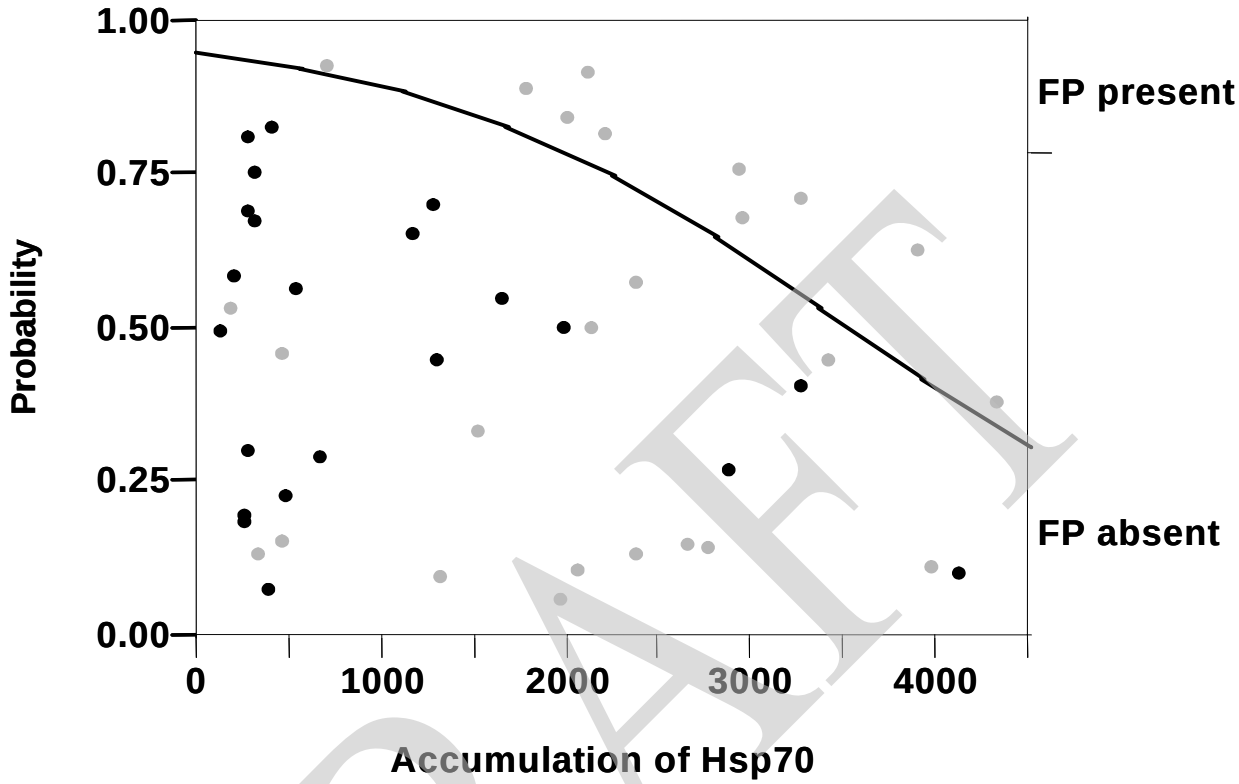

1.00
0.75
0.50
0.25
0.00
Probability
0
1000
2000
3000
4000
Accumulation of Hsp70
FP present
FP absent

Fig. 4A

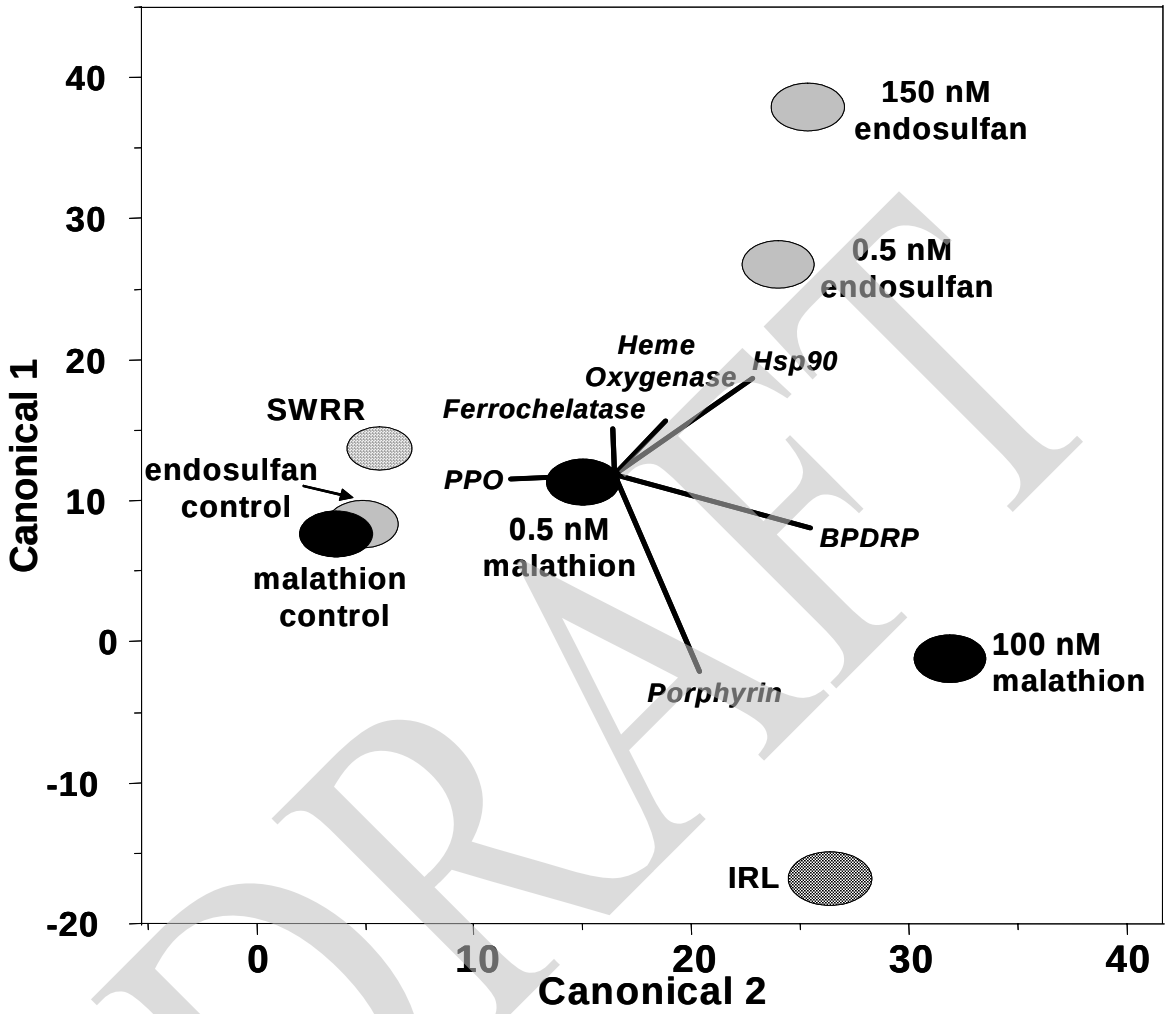

40
30
20
10
0
-10
-20
Canonical 1
0
10
20
30
40
Canonical 2
150 nM endosulfan
0.5 nM endosulfan
Heme Oxygenase
Hsp90
Ferrochelatase
SWRR
endosulfan control
PPO
0.5 nM malathion
BPDRP
malathion control
100 nM malathion
Porphyrin
IRL

Fig. 4B

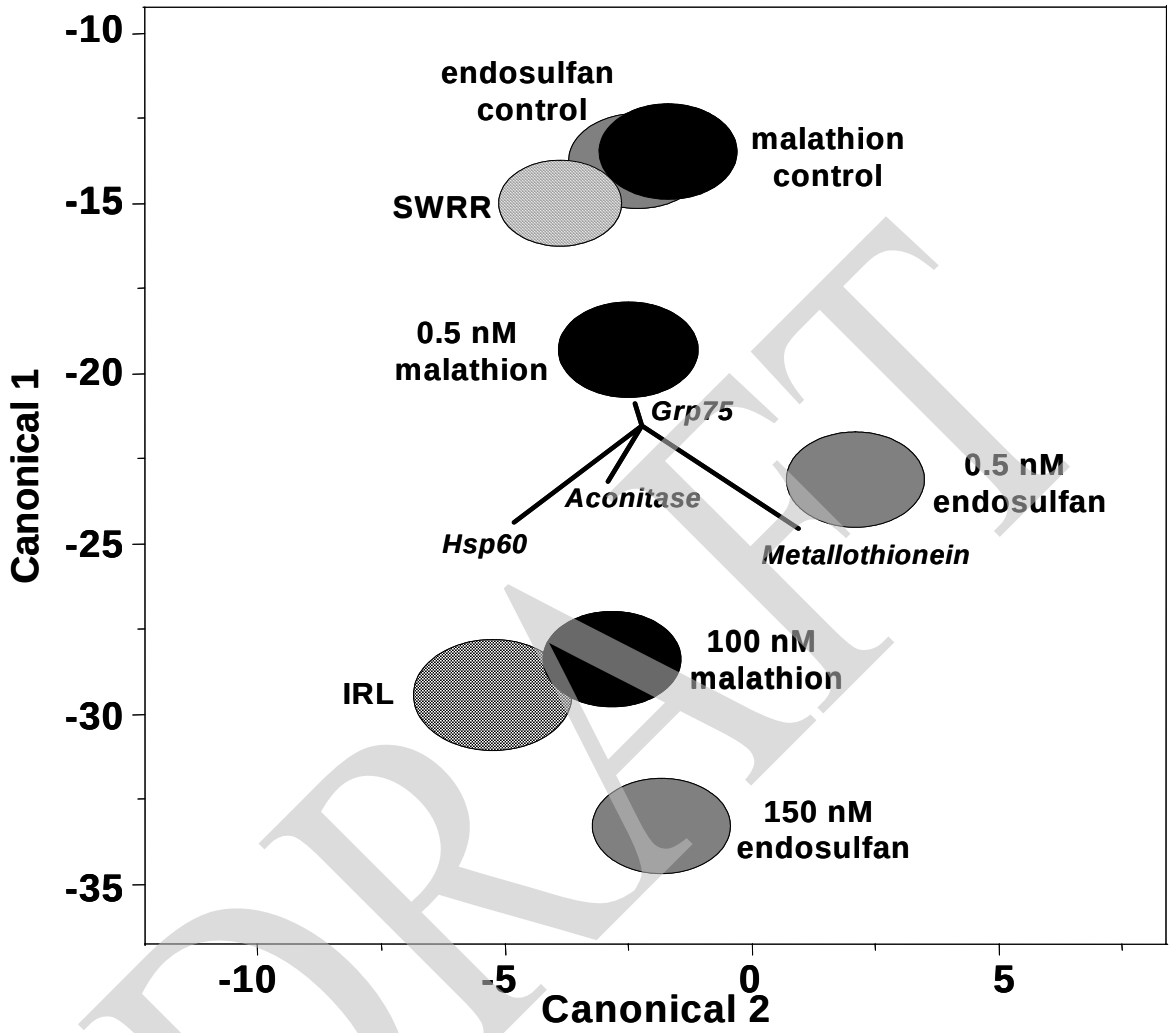
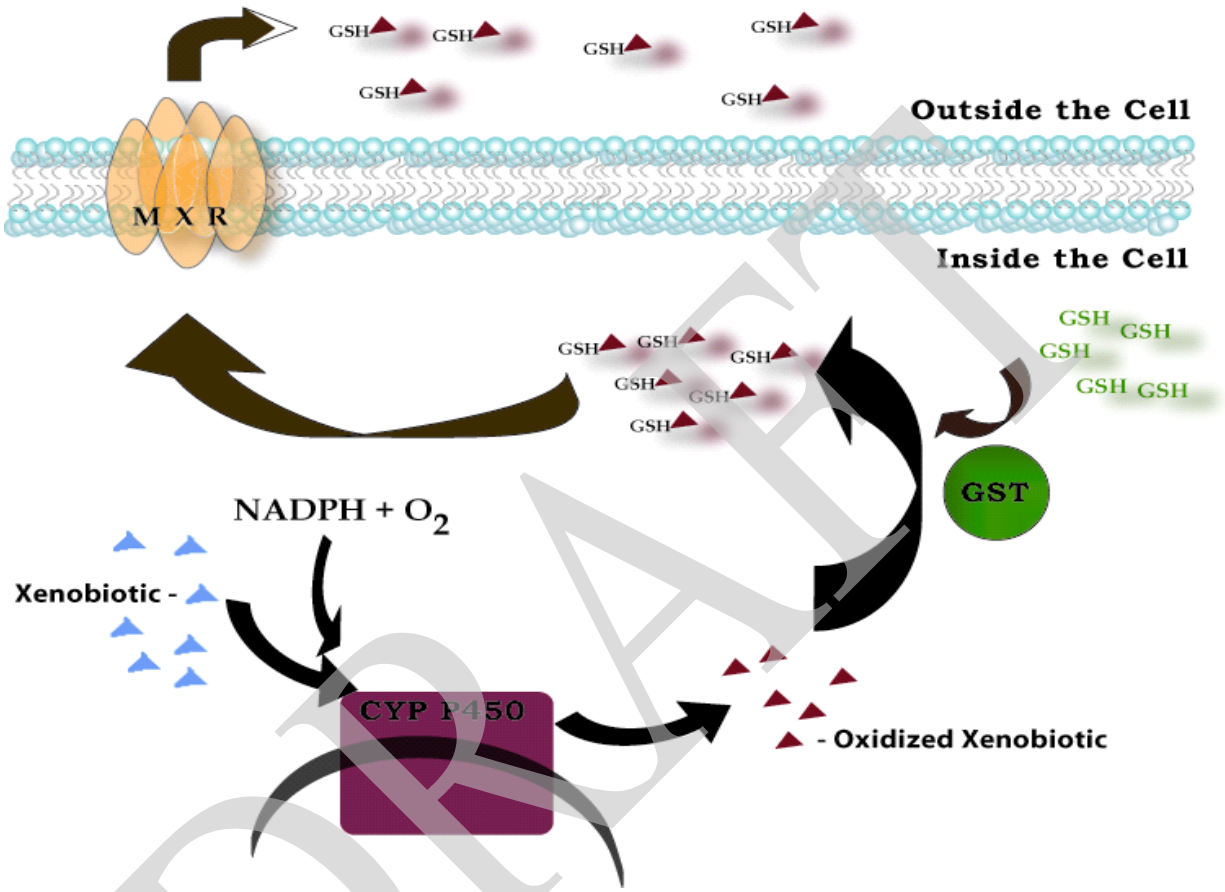

-10
-15
-20
-25
-30
-35
Canonical 1
-10
-5
0
5
Canonical 2
endosulfan control
malathion control
SWRR
0.5 nM malathion
Grp75
Aconitase
Hsp60
Metallothionein
0.5 nM endosulfan
100 nM malathion
IRL
150 nM endosulfan

Fig. 5


GSH
Outside the Cell
M X R
Inside the Cell
GST
NADPH + O_2
Xenobiotic -
CYP P450
- Oxidized Xenobiotic