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Non Invasive High Resolution In Vivo Imaging of α -naphthylisothiocyanate (ANIT) Induced Hepatobiliary Toxicity in STII Medaka

Ron Hardman, Seth Kullman, Bonny Yuen, and David E. Hinton

Duke University, Nicholas School of the Environment and Earth Sciences, Durham NC, 27708, United States

Abstract

A novel transparent stock of medaka (*Oryzias latipes*; STII), homozygous recessive for all four pigments (iridophores, xanthophores, leucophores, melanophores), permits transcutaneous, high resolution ($< 1\mu\text{m}$) imaging of internal organs and tissues in living individuals. We applied this model to in vivo investigation of α -naphthylisothiocyanate (ANIT) induced hepatobiliary toxicity. Distinct phenotypic responses to ANIT involving all aspects of intrahepatic biliary passageways (IHBP), particularly bile preductular epithelial cells (BPDECs), associated with transitional passageways between canaliculi and bile ductules, were observed. Alterations included: attenuation/dilation of bile canaliculi, bile preductular lesions, hydropic vacuolation of hepatocytes and BPDECs, mild BPDEC hypertrophy, and biliary epithelial cell (BEC) hyperplasia. Ex vivo histological, immunohistochemical, and ultrastructural studies were employed to aid in interpretation of, and verify, in vivo findings. 3D reconstructions from in vivo investigations provided quantitative morphometric and volumetric evaluation of ANIT exposed and untreated livers. The findings presented show for the first time in vivo evaluation of toxicity in the STII medaka hepatobiliary system, and, in conjunction with prior in vivo work characterizing normalcy, advance our comparative understanding of this lower vertebrate hepatobiliary system and its response to toxic insult.

Keywords

Fish; Toxicology; Hepatobiliary; Liver; Toxicity; Medaka; ANIT; Biliary; Biliary Toxicity; α -naphthylisothiocyanate; Hepatotoxicity; Piscine Liver

1. Introduction

Bile synthesis and transport, performed by the hepatobiliary system, are essential life functions; fundamental to the elimination of metabolic byproducts, and vital to the assimilation of lipid soluble nutrients (e.g. vitamins A, K, E, triacylglycerols) (Arias 1988; Boyer 1996a; Trauner and Boyer 2003; and others). Impairment or inhibition of bile synthesis & transport (cholestasis), a common response of the mammalian hepatobiliary system to xenobiotic insult,

Correspondence to: Ron Hardman, Environmental Sciences and Policy Division, Nicholas School of the Environment and Earth Sciences, LSRC A333, Box 90328 Durham, NC 27708-0328; ron.hardman@duke.edu.

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results in morbidity and mortality; the result of systemic accumulation of endogenous & exogenous compounds and their metabolites (Alpini et al. 2002b; Arias 1988; Arrese et al. 1998; Boyer 1996b; Groothuis and Meijer 1996; Trauner et al. 2000; Trauner et al. 1998; Wolkoff and Cohen 2003).

The majority of our understanding of hepatobiliary transport, and vertebrate biliary disease and toxicity, has been derived from mammalian liver studies (Alpini et al. 2002a; Bove et al. 2000; Boyer 1996a; Boyer 1996b; Chignard et al. 2001; and others). We know comparatively less about the piscine biliary system, though are we gaining greater insight into piscine hepatobiliary structure/function relationships (Ballatori et al. 1999; Ballatori et al. 2000; Boyer et al. 1976a; Boyer et al. 1976b; Hampton et al. 1989; Hampton JA 1988; Hardman et al. 2007b; Hinton et al. 1987; Hinton et al. 2001; Rocha et al. 2001; Rocha et al. 1997). Because our understanding of the piscine biliary system has lagged, particularly in a comparative sense, our ability to interpret and communicate biliary disease and toxicity in piscine species has been limited. By example, cholestasis (impaired/inhibited bile transport) has never been described in fish, a fact more representative of our lack of understanding (investigation), as opposed to the lack of occurrence of this response in piscine systems.

Relevant to the findings presented here is a brief synopsis of what is known about the piscine biliary system. Previous studies from this laboratory have shown the hepatobiliary systems of channel catfish (*Ictalurus punctatus*), trout (*Oncorhynchus mykiss*), and medaka (*Oryzias latipes*) to exhibit numerous transitional biliary passageways, termed bile preductules, between hepatocellular canaliculi and biliary epithelial cell (BEC) delimited bile ductules (Hampton JA 1988; Hardman et al. 2007b; Okihira and Hinton 2000). These transitional biliary passageways, first described in the mammalian liver by Steiner and Carruthers (1961), are anatomically associated with peri-portal canals of Hering and oval cells in the mammalian liver (Fausto 2000; Fausto and Campbell 2003; Golding et al. 1996; Theise et al. 1999).

More recent in vivo investigations in STII medaka that elucidated structure/function relationships in both 2 and 3 dimensional contexts revealed medaka livers to be replete with bile preductular epithelial cells (BPDECs), and the transitional biliary passageways (bile preductules, BPDs) associated with them (Hardman et al. 2007a; Hardman et al. 2007b). These investigations revealed that the intrahepatic biliary system in medaka is largely an interconnected network of equidiameter (1–2 μm) canaliculi and bile preductules, organized through a polyhedral (hexagonal) structural motif, that occupies the majority of the liver corpus (~95%) uniformly. Larger bile ductules and ducts were predominantly found in the hilar and peri-hilar region of the liver, and it follows, an arborizing biliary tree (as described in mammals) was largely absent, seen only in the rudimentary branching of intrahepatic ducts from the hilar hepatic duct). From prior investigations we recognized injury to BPDECs may serve to distort bile preductular lumina and result in transient or longer alterations to intrahepatic bile flow, and that attention to BPDECs/BPDs, and their relationship to the interconnected intrahepatic biliary network, is essential to understanding the spectrum of responses of the piscine hepatobiliary system to xenobiotics that target this organ system.

With a better comparative understanding of the medaka hepatobiliary system established in prior studies, and normalcy characterized, we were then able to investigate response of the hepatobiliary system to xenobiotics in vivo. To do so we used α -naphthylisothiocyanate (ANIT), a well described hepatotoxicant that induces hallmark responses in the mammalian biliary system, namely: cytotoxicity in biliary epithelium of bile ductules and ducts (e.g. impaired mitochondrial function, necrosis), cholestasis (Hill and Roth 1998; Orsler et al. 1999; Waters et al. 2002; Woolley et al. 1979), and biliary tree arborization (biliary epithelial cell hyperplasia) (Alpini et al. 1992; Connolly et al. 1988; Masyuk et al. 2003). Because biliary toxicity and cholestasis are poorly understood in piscine species, and because we now

understand the medaka hepatobiliary system to be more similar to mammalian liver architecture than previously considered (Hardman et al. 2007b), we considered ANIT good toxicant for investigation of comparative hepatology. The goals of these investigations were to determine the cellular targets of ANIT in medaka and to characterize toxic response, relative to what is known regarding ANIT induced hepatotoxicity in mammalian liver.

2. Materials and Methods

2.1 STII Medaka

For decades various color mutant strains of medaka (*Oryzias latipes*), acquired from natural and commercially available populations, have been maintained in the Laboratory of Freshwater Fish Stocks at Nagoya University, Japan. Cross breeding from these stocks was used to produce a stable “transparent” strain of medaka. See-through (STII) medaka are homozygous recessive for all four pigments (iridophores, leucophores, xanthophores, melanophores). Exhibiting no expression of leucophores and melanophores, and minimal expression of xanthophores and iridiophores, STII medaka are essentially transparent throughout their life cycle (Wakamatsu et al. 2001), and allow high resolution (< 1µm) non invasive in vivo imaging of internal organs and tissues at the subcellular level (Figure 1) (Hardman et al. 2007a; Hardman et al. 2007b; Hinton et al. 2004). Our STII medaka colony, maintained at Duke University since 2002, was first cultured with stock obtained from Prof. Y. Wakamatsu (Nagoya University). Medaka were housed in a charcoal filtrated, UV treated re-circulating system (City of Durham, NC water) maintained at 25±0.5° C. Water chemistries were maintained at: pH (7.0–7.4), dissolved oxygen (6–7 ppm), ammonia (0–0.5 ppm), nitrite (0–0.5 ppm) and nitrate (0–10 ppm). A diel cycle of 16:8 hr light:dark was employed. Medaka larvae were fed ground (pressed through a 60 µm sieve) Otohime β diet (Ashby Aquatics, West Chester, PA) via an automatic feeder 7 times per day. Because Otohime β has been shown to be free of estrogenic complications (Inudo et al. 2004), we considered it an optimal fish food. In addition, all brood stock fish diets were supplemented daily with Artemia nauplia (hatched brine shrimp). Egg clusters, collected daily, were cleaned in embryo rearing medium (ERM), and individual fertilized eggs were separated and maintained in ERM at 25°C. Unconsumed diet, detritus and associated algal material were removed from rearing and brood stock tanks daily. Care and maintenance of medaka were in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC; A117-07-04; A141-06-04; A173-03-05).

2.2 Xenobiotic Exposures

Studies were designed to evaluate hepatobiliary structure/function during the onset, progression, and recovery from ANIT exposure. Multiple cohorts of STII medaka (10 to 30 fish) were exposed to the reference toxicant α-naphthyl isothiocyanate (ANIT) to target hepatocytes and biliary epithelia for toxic response. Acute and chronic aqueous bath exposures were carried out from 3 to 60 days post fertilization (dpf). Controls consisted of untreated medaka, and medaka treated with dimethyl sulfoxide (DMSO; ANIT solvent). All exposures were carried out in 750 ml wide-bottom glass rearing beakers at 25° C, using a 16 hr light/8 hr dark cycle. Aqueous bath exposure medium consisted of ERM:de-ionized water (1:3). Acute exposures: medaka cohorts were reared in aqueous baths that were given a single aliquot of ANIT to achieve exposure concentrations ranging from 0.25 µM to 10 µM ANIT. Chronic exposures: medaka were serially exposed every 3 days or once weekly (static renewal) for the duration of study using the same concentrations given for acute exposure. At given time points during exposure regimes (e.g. at 5 min, 15 min, 3, 6, 12, 24, 48, 72 and 96 hrs, and day 7, 10, 20, 30, 40 and 60 post exposure) subpopulations of medaka were removed from a cohort for in vivo and ex vivo studies (histological, immunohistochemical, and transmission electron microscopy) of the hepatobiliary system.

Adult STII medaka (> 90dpf) were anesthetized with 0.1 g/L tricane methanesulfonate (MS-222) and twice injected (intraperitoneal; IP) with 200 mg/kg ANIT, with a 7 day depuration period between injections. Livers were removed from anesthetized fish 7 days after the 2nd ANIT injection, placed in 4% paraformaldehyde overnight at 4°C, dehydrated in graded ethanol solutions, cleared with xylene, and paraffin embedded at 60°C. A 200 mg/kg ANIT dose was employed because: our preliminary studies in medaka showed 200 mg/kg ANIT to be the LD50 (96 hr) for IP injection; an LD50 of 200 mg/kg of ANIT has been reported in rats following oral exposure (McLean and Rees 1958); and biliary epithelial cell proliferation has been observed in rats treated with a single oral dose of 150 mg/kg of ANIT (Kossor et al. 1995). While embryo, larval and juvenile medaka were subjected to waterborne ANIT exposures, IP injections were employed in adult fish for comparison to published ANIT studies in rodent animal models, and to investigate the sensitivity of medaka to both routes of exposure.

2.3 In Vivo Imaging

Prior studies (Figure 1) described the utility of fluorophores such as β -Bodipy C5 phosphocholine and fluorescein isothiocyanate in elucidating the intra- and extrahepatic biliary system of STII medaka, and the application of exogenous fluorophores for in vivo evaluation of blood to bile transport. For detailed methods see (Hardman et al. 2007a;2007b). Briefly; fluorescent probes were administered to control and ANIT treated STII medaka, via aqueous bath, prior to in vivo imaging to aid in elucidation of biological structure/function and interpretation of normalcy and toxic response. Time points for in vivo investigations varied from 10 mins to 2 hrs post fluorophore administration, dependent on the fluorescent probe employed, and the portion of the hepatobiliary system being studied. By example; Bodipy C5 HPC and fluorescein isothiocyanate accumulation in the hepatic parenchyma was first observed at ~10 minutes post fluorophore exposure, with saturation (peak fluorescence; proxy for equilibrium of fluorophore uptake and excretion) of the fluorophores in the hepatic parenchyma occurring at ~40 minutes. In contrast, Bodipy C5 ceramide saturation was commonly observed at ~70 minutes. The majority of in vivo observations were made between 15 and 50 minutes post fluorophore exposure. In addition to the use of exogenous fluorophores, autofluorescence was also employed for in vivo elucidation of cell and tissue morphology, and xenobiotic response.

After fluorophore exposure medaka embryos, larvae and juveniles (treated and untreated), at various stages of development, and at the time-points described, were sedated with 10 μ M tricaine-methane sulfonate (MS-222) in accordance with IACUC approved animal protocols. Once sedated, medaka were mounted in a solution of de-ionized water:ERM (3:1) on depression well glass slides, oriented in the desired anatomical position, and glass slides sealed with a cover slip. Medaka were then imaged live with brightfield, widefield and/or laser scanning confocal fluorescence microscopy (LSCM). With widefield and LSCM salient features of the organ system such as canaliculi, space of Disse, endothelial cells, biliary epithelial cells, red blood cells, and hepatocytes and their nuclei, were clearly resolved. Confocal stacks from in vivo imaging (LSCM) of the hepatobiliary system were used for 3D reconstructions, and from these, architectural, morphometric and volumetric analyses were made.

2.4 Imaging Systems

Confocal fluorescence microscopy was performed on a Zeiss 510 Meta system with Zeiss LSM 5 Axiovision image acquisition software, Argon and HeNe laser, Carl Zeiss C-apochromatic 40x/1.2, and C-apochromatic 10x/0.45. Widefield fluorescence microscopy was performed on a Zeiss Axioskop with DAPI/TRITC/FITC filter cube set. Excitation/emission parameters for widefield microscopy were: DAPI/UV (Ex 360–380nm/Em All Vis >400nm); FITC (Ex 450–490nm/Em 515–565nm); TRITC (Ex 528–552nm/Em 578–632nm). For brightfield

microscopy a Nikon SZM 1500 dissecting microscope with a Nikon DXM 1200 digital capture system was employed. Software used: EclipseNet (Nikon, USA), Adobe Photoshop (Adobe, Inc.), Amira 3D (Mercury Computer Systems, Berlin), ImageJ (V1.32j), IP Lab software (Scanalytics, Inc., version 3.55), and Zeiss Image Browser (Carl Zeiss). All transmission electron microscopy (TEM) was performed at the Laboratory for Advanced Electron and Light Optical Methods (LAELOM), College of Veterinary Medicine, North Carolina State University. For TEM investigations individual medaka were anesthetized and fixed in 4F:1G fixative (4% formaldehyde and 1% glutaraldehyde in a monobasic phosphate buffer with a final pH of 7.2–7.4 and a final osmolality of 176 mosmol). Following processing and embedment thin sections (Spurr resin embedded) were made and examined using a FEI/Philips EM 208S Transmission Electron Microscope.

2.5 Fluorescent probes

Fluorescent probes employed are listed in Table 1. Fluorophores were acquired through Invitrogen/Molecular Probes (Carlsbad, CA). All fluorescent probes were administered to STII medaka via aqueous bath at the exposure concentration ranges given, under dark conditions, at room temperature.

2.6 Chemicals

α -naphthyl iosthiocyanate (Sigma, N4525), tricaine-methane sulfonate (Sigma, E10521), dimethyl sulfoxide (DMSO)(Sigma, 276855), and Pronase (streptococcal protease, Sigma).

2.7 Immunohistochemistry

Cytokeratins were localized on paraffin sections of medaka liver using mouse pan-cytokeratin (AE1/AE3, 1:200 dilution) antibody (Zymed, CA, USA) and visualized with the DAKO EnvisionTM+System (Dako, CA, USA). In brief; after de-waxing and rehydration of paraffin sections, heat induced epitope retrieval was carried out prior to immuno-labeling. Endogenous peroxidase activity was blocked by incubating sections with 0.03% hydrogen peroxide and non-specific labeling was reduced by blocking sections with 5% normal goat serum for 20 mins. Sections were incubated with AE1/AE3 (0.01M phosphate buffer saline at pH 7.4, 0.1% Tween 20, 0.1% sodium azide and 1% BSA) at 4°C overnight. After rinsing in PBS sections were incubated with secondary antibody (peroxidase labeled polymer conjugated goat anti-mouse immunoglobulin (IgG)) at room temperature for 30 mins. Sections were rinsed a second time with PBS, visualized with diaminobenzidine (DAB), counterstained with Harris' hematoxylin and observed/imaged with brightfield microscopy. Negative controls were prepared by substituting primary antibody with non immune serum.

After fixation of anaesthetized medaka in 10% formalin for 24 hrs whole mount paraffin sections were prepared and assayed with proliferating cell nuclear antigen (PCNA; Biogenics, San Ramon, CA) by the histopathology laboratory in the College of Veterinary Medicine at North Carolina State University. As a positive control, PCNA labeling of the gut was evaluated.

2.8 Statistics

Differences in fluorescence intensity in digital image captures were analyzed statistically using ImageJ (NIH, (V1.32j)) and Statview software (SAS institute, Cary, NC). Two way ANOVA with Fisher's T-test was employed to assess statistically significant differences in fluorescence intensity. Background fluorescence and autofluorescence were accounted for in statistical analyses. Descriptive statistics were used for volumetric and morphometric analyses. Pearson's Correlation Coefficient was used for comparison of calculated versus measured morphometric values in vivo. Equality of Variance F-test was used for assessment of blood to bile transport; temporal evaluation of fluorescence intensities across sinusoid, hepatocytic cytosol and

canalicular spaces. All quantitative analyses were performed on unaltered (no deconvolution), or normalized, single optical sections from in vivo confocal image captures.

3. RESULTS

3.1 Overview

Exposure of medaka to ANIT resulted in distinct concentration dependent responses, summarized in Figure 2. These included: (1) canalicular attenuation and dilation in response to 1 – 3 μM acute aqueous ANIT exposure; (2) bile preductular lesions in response to 2 – 5 μM chronic ANIT exposure; (3) hydropic vacuolation, at ANIT concentrations of 2 – 8 μM ANIT, which resulted in a distinct “pebbling” of the liver when evaluated in vivo; and (4) chronic passive hepatic congestion, an end stage response of the liver associated with high mortality, at 6 – 8 μM ANIT. In vivo observations were correlated with ex vivo histological and electron microscopic studies to aid in interpretation of in vivo findings and to verify affected cell types. These responses are described in detail in the following sections.

3.2 Canalicular Attenuation and Dilation and Bile Preductular Lesions

Medaka cohorts exposed to 1 – 5 μM aqueous ANIT exhibited distinct alterations to the intrahepatic biliary system, to include canalicular attenuation and dilation (acute exposure), and bile preductular lesions (chronic exposure). In vivo investigations in acutely exposed medaka revealed a simultaneous attenuation and dilation of bile canaliculi, first observed 4 hrs post ANIT exposure (1 – 3 μM , Figure 3). Canalicular attenuation/dilation appeared relatively uniformly throughout the parenchyma, and persisted for 96 to 120 hrs post acute exposure. Both attenuated and dilated canaliculi occurred in close spatial proximity (e.g. within 20 μm); where one bile segment, or canaliculus, was observed to be attenuated, the adjacent connected branching bile segment was observed to be dilated. Dilated canaliculi, which ranged between ~3 and 4 μm , were found to be up to ~3 times normal diameter (1.3 $\pm$ 0.4 μm). Attenuated canaliculi were distinct, appearing as fine sinuous passageways 0.4 μm to 0.8 μm in diameter (Figure 3A, B).

Where acute exposure to 1 – 3 μM aqueous ANIT resulted in canalicular dilation/attenuation, chronic exposures to 2 – 5 μM aqueous ANIT (> 3 days) resulted in foci of alteration that appeared more consistent with changes to bile preductule (BPD) structural integrity. Where normal (control) canaliculi and bile preductules appeared as equidiameter tubular passageways (1.3 $\pm$ 0.4 μm), altered bile preductules exhibited marked irregularities in lumen morphology and increased lumen diameter (Figure 4B, C, E, F), suggesting loss of integrity of hepatocellular/bile preductular epithelial cell junctions. Foci of alteration were frequently associated with changes to bile preductular epithelia cell (BPDEC) morphology. Transmission electron micrographs (TEM) of livers from medaka cohorts exposed to 2 – 5 μM aqueous ANIT typically showed BPDECs with an increased cytosolic area, cytosolic vacuolation, and altered cell membrane integrity (Figure 4E, F). When lesions were reconstructed and examined in 3 dimensions (Figure 4D, E) altered BPDs were often found to terminate in blind ends, unconnected to surrounding canaliculi/bile preductules. It follows that the majority of lesions appeared to be localized to BPDEC/hepatocellular junctional complexes (bile preductules); unique morphological complexes described by Hampton et al. (1988) and Hardman et al (2007b).

BPD lesions were observed in all fish chronically exposed to 2 – 5 μM aqueous ANIT. Onset of lesions appeared approximately 48 hrs post ANIT exposure, and a higher incidence of lesions was associated with increased exposure duration. By example; at 10 days of chronic ANIT exposure approximately 10% of bile preductular complexes (transitional biliary passageways) appeared to be affected. BPD lesions persisted for the duration of chronic exposure studies

(e.g. out to 60 days), and foci of alteration appeared randomly distributed in the livers examined. BPD lesions, like canalicular attenuation/dilation, were observed to be a reversible form of injury, as medaka withdrawn from chronic exposures (2 – 5 μ M aqueous ANIT) exhibited no such phenotype when allowed to recover for 7 days in an ANIT free bath.

3.3 Hydropic Vacuolation

Acute and chronic aqueous exposures to 2 – 8 μ M ANIT resulted in a distinct “pebbling” of the liver, a phenotypic response consistently evident by 24 hrs post ANIT exposure (Figure 5), which was not observed in DMSO or untreated medaka, nor readily apparent at lower or higher ANIT exposure concentrations. Non invasive in vivo imaging (Figure 5C, E) revealed intracellular ovate structures within hepatocytes and biliary epithelia, which, when viewed at the organ level of organization (Figure 5A, B), manifested as a “pebbling” of the hepatic parenchyma. This phenotype was observed with the aid of autofluorescence (Figure 5A, B) and/or differential interference microscopy (DIC) alone (Figure 5E). The application of exogenous fluorophores was not necessary for elucidating/imaging this phenotypic response (Figure 5A, B).

ANIT exposed medaka exhibiting a “pebbling” phenotype were treated with the nuclear stain DAPI (via aqueous bath) to investigate whether intracellular ovate structures were associated with nuclei. Ovate structures did not label with DAPI, were distinguishable from hepatocyte and biliary epithelial cell nuclei, and were localized to the cytosol of affected cell types (Figure 5C).

Ultrastructural investigations (TEM) revealed the alterations to be membrane-less cytosolic structures ranging in diameter from 2 – 10 μ m, which contained moderate to dense granular infiltrates of low electron density (Figure 5D); features consistent with hydropic vacuolation (symptomatic cell injury and swelling, the result of the intracellular accumulation of water). Hydropic vacuoles (HV) occurred in both hepatocytes and BPDECs (Figure 5D, 5E), and were observed to displace, and in some instances wrap themselves around, cell nuclei. Vacuoles appeared as early as 6 hrs post ANIT exposure, and were consistently marked by 24 hrs post exposure.

Formation of HVs was concentration dependent, with increasing prevalence of vacuolation associated with increasing aqueous ANIT concentrations from 1 – 6 μ M. HV was observed to be most prevalent in the livers of medaka exposed to 4 – 6 μ M ANIT, and occurred throughout the hepatic parenchyma, affecting ~ 95% of the area of livers examined (Figure 5B). Vacuoles were first observed in response to 1 μ M ANIT, and of low prevalence, affecting ~ 10% of observed areas of the liver. Vacuoles were also of lower prevalence at ANIT concentrations exceeding 6 μ M (ranging from 5% – 20% of the area of the liver examined). Hence, a concentration dependent increase in HV was observed from 1– 6 μ M ANIT, and decrease observed from 6 – 10 μ M. Medaka exhibiting this phenotypic response showed no overt signs of impaired health, exhibiting normal swimming and feeding behavior. HV was not observed in the livers of DMSO control medaka.

In the majority of livers studied hepatocytes and biliary epithelia affected by HV appeared throughout the liver corpus, with no apparent zonation; an observation enabled by the ability to observe/image internal liver structure at various depths (e.g. up to 200 μ m from the liver surface with confocal microscopy). Hydropic vacuoles were found to be a reversible form of ANIT induced cell injury; not observed in any cell type following recovery from ANIT exposure. By example, medaka exposed to 3 – 6 μ M ANIT for 3 days, and subsequently reared in an ANIT free bath for 7 days, showed no signs of HV in vivo or in ultrastructure (TEM) studies.

3.4 In Vivo Investigation of Apoptosis

Preliminary studies suggest it is possible to detect cells with compromised cell membranes in vivo. While these observations are not conclusive, they merit mention. Putative apoptotic, or necrotic cells with compromised cell membranes were detected/imaged in vivo using confocal microscopy and the fluorescent probes SYTO® 16 green and YO-PRO-1, both of which are established in vitro nucleic acid stains used for detection of apoptosis (the cationic fluorophores only enter cells with compromised cell membrane integrity) (Al-Gubory 2005; Santos et al. 2006; Wlodkowic et al. 2007). Of these, YO-PRO-1 was the fluorophore of choice for in vivo studies (Figure 5E). Cells incorporating YO-PRO-1 were observed in the livers of medaka exposed to 3 – 6 μ M ANIT as early as 6 hrs post exposure. In the fields of liver observed in vivo, ~ 3% of cells fluoresced as a result of YO-PRO-1 incorporation, indicative of cells with compromised membrane integrity. YO-PRO-1 fluorescent cells were not observed in DMSO controls, and were only observed in tandem with the development of hydropic vacuoles, typically within 24 to 72 hrs post ANIT exposure. Due to the staining characteristics of YO-PRO-1, which primarily labeled nuclei, and the fact that labeled cells also exhibited loss of normal cell morphology, it was difficult to differentiate affected cell types.

3.5 Biliary Epithelial Cell Proliferation

In vivo and immunohistochemical analyses (AE1/AE3 and PCNA), used to assess proliferation of BPDECs, BECs (cells lining bile ductules and ducts) and hepatocytes in response to ANIT, suggest biliary epithelial cells are early responders to ANIT, with hepatocytic changes occurring subsequently (Figure 6). BEC hyperplasia was first observed at 5 days of chronic exposure to 2 – 6 μ M ANIT, and remained apparent out to 60 days during chronic exposure studies. The majority of proliferating cell nuclear antigen (PCNA) positive cells were localized to the hilar and peri-hilar region of the livers examined, suggesting BECs were the most responsive (hyperplastic) cell type (Figure 6D, D1). PCNA staining was occasionally observed in more distal regions of the liver in small cuboidal biliary epithelia of bile ductules/ducts (see Figure 7A for these cell types), though this observation was infrequent, and encountered in livers of mature medaka, older than 120 days (not shown in figures). PCNA positive hepatocytes, BECs, and BPDECs were not evident in acute ANIT exposures, or chronic exposures exceeding 6 μ M ANIT. While PCNA positive BPDECs were not apparent in any exposure regime, it is possible that BPDECs, if labeled, were not clearly resolved in immunohistochemical preparations due to their small size (3 – 8 μ m), and relatively low density compared to hepatocytes and biliary epithelial cells (per unit volume of liver). While BPDEC proliferation was not evidenced by PCNA analyses, qualitative in vivo observations suggested changes to BPDECs consistent with mild BPDEC cytomegaly, and hyperplasia. In chronically exposed medaka BPDECs consistently appeared, in vivo, more numerous, contiguous, and larger, as opposed to control livers (Figure 6C).

Immunohistochemical studies with the pan cytokeratin stain AE1/AE3, used to detect in biliary epithelial and hepatocytic cytokeratins, also suggest BECs to be early responders to ANIT. In adult medaka injected with 200 mg/kg ANIT, BECs exhibited cytomegaly, as compared to DMSO (vehicle/solvent) controls (Figure 6A, A1, A2). BECs lining bile ductules and ducts in ANIT treated livers also appeared to label more heavily with the AE1/AE3 cytokeratin antibodies, suggesting greater density, and perhaps, proliferation of these cell types. Modest vascular endothelial staining with AE1/AE3 was also observed, though with less consistency and intensity.

3.6 ANIT Associated Biliary Cystic Vacuolation

Chronic exposures to 3 μ M ANIT resulted in cystic vacuolation of larger bile ducts of the liver hilus, with accompanied hepatocellular changes; responses consistent with spongiosis hepatitis, a probable degenerative lesion (Brown-Peterson et al. 1999) (Figure 7B). In histological

preparations (H&E) cysts were distinct, ranged from 40 to 100 μm in diameter, and often contained material consistent with inspissated bile or proteinaceous material. Evident in the same livers were smaller hepatocellular vacuoles consistent with hydropic vacuolation (see 3.3). Cystic formations of this nature were encountered only during chronic exposures, and were not observed in livers of acutely exposed medaka (< 3 days), regardless of ANIT concentration.

3.7 Hepatobiliary Transport

STII medaka exposed to 0.25 μM to 8 μM aqueous ANIT were treated with the fluorophores FITC and Bodipy C5 Ceramide and examined for altered hepatobiliary transport at 6 hrs, 8 hrs, 12 hrs, 24 hrs, 48 hrs, and 96 hrs (Figure 1, Figure 8). In vivo confocal image captures (single optical sections) were measured (random repeated measures) for fluorescence intensity in sinusoid, cytosol and canalicular spaces. No impairment of blood to bile transport was detected for either fluorophore in response to 0.25 μM to 6 μM aqueous ANIT exposures (Figure 8). Loss of hepatobiliary transport function (for both FITC and Bodipy C5 Ceramide) was only observed in vivo at ANIT exposure concentrations exceeding 6 μM , ANIT concentrations also associated with vasodilation and decreased cardiac output (discussed following).

3.8 Volumetric Studies on ANIT Induced Biliary Changes

While altered blood to bile transport was not evident via quantitative in vivo studies with FITC and Bodipy C5 Ceramide, volumetric analyses (which can be considered a proxy for intrahepatic bile flow) from 3D in vivo investigations suggest differences in intrahepatic biliary volume in ANIT treated vs. untreated medaka. Quantitative morphometric and volumetric analyses were performed on 3D reconstructions from 2 ANIT treated medaka and 3 controls (Table 2). Volumetric indices of an individual medaka (40dpf) from a cohort exposed to 2.5 μM ANIT for 30 days suggest a reduction in canalicular lumen volume of ~50% (Table 2). A second evaluation of an individual medaka from a cohort exposed to 1 μM ANIT for 48 hrs volumetric indices suggested an increase in intrahepatic biliary volume. Relative to liver volume, biliary volume was found to be 1.18%, versus a mean of 0.95%, (+/- 0.08) in control livers (Table 2). In both cases volumes of vasculature, parenchyma and hepatocellular space, relative to total liver volume examined, remained well conserved across ANIT treated and control fish, from 8 to 40 dpf, indices which serve as controls, and which can be used to assess precision across individual 3D volumetric studies (Table 2).

3.9 Cardiovascular Changes

Aqueous ANIT concentrations of 4 μM to 8 μM resulted in phenotypic changes consistent with passive hepatic congestion. In vivo investigations revealed a marked cardiovascular response, where all liver vasculature, afferent and efferent vessels, showed increasing dilation in response to increasing ANIT concentrations (Figure 9). Dilation of sinusoids, hepatic vein, and hepatic portal vein, were all observed. Sinusoid diameter was observed to increase ~ 2 fold at ANIT concentrations approaching the LC100 (8 μM). Where control sinusoids averaged 7.4 μm in diameter, sinusoid diameter averaged 15.3 μm (+/- 4.1, n = 18) at 8 μM ANIT, 48 hrs post exposure (Figure 9). Vasodilation of hepatic vasculature was observed in tandem with a concentration dependent decrease in heart rate and motility, observed in all medaka (n = 18) exposed to 4 μM to 8 μM ANIT (acute and chronic exposures). By example: where control heart rates averaged 134 (+/- 9) beats per minute (bpm), medaka exposed to 8 μM ANIT exhibited heart rates of 118 (+/- 12) bpm at 6 hrs post exposure, 73 (+/- 13) bpm at 24 hrs post exposure, and 61 (+/- 7) bpm at 48 hrs post exposure. Hence, the magnitude of both the vasodilation and heart rate responses were concentration (ANIT) dependent, and appeared to be coupled.

4. Discussion

Results of this study suggest cells of medaka intrahepatic biliary system respond to ANIT, and that responses, both cellular and system level, are similar to those described in rodents (Alpini et al. 2001; Carpenter-Deyo et al. 1991; Connolly et al. 1988; Hill et al. 1999; Kossor et al. 1993; Lesage et al. 2001; Orsler et al. 1999; Waters et al. 2001). Changes observed in vivo, and confirmed with ex-vivo analyses (histological, immunohistochemical, ultrastructural), were; attenuation and dilation of bile canaliculi, bile preductular lesions, hydropic vacuolation of hepatocytes and BPDECs, BPDEC cytomegaly, and hyperplasia of BECs in the hilar and peri-hilar region of the liver. While in vivo evaluations revealed no alterations to bile transport, volumetric analyses of 3D reconstructions from ANIT treated medaka suggest a reduction in intrahepatic biliary passageway volume at 2.5 μ M ANIT (cholestasis?), and an increase in intrahepatic biliary passageway volume (choleresis?) at 1 μ M ANIT, with no changes to other volumetric liver indices (Table 2).

4.1 Canalicular Attenuation and Dilation

Canalicular dilation/attenuation, frequently observed in response to 1 – 3 μ M aqueous ANIT, and evaluated both in vivo and via 3D reconstructions, appeared to be more consistent with an adaptive response of the intrahepatic biliary system, as no clear alteration to overall canalicular integrity or loss of function was observed in association with this change, nor were mortality or morbidity observed. Apical hepatocyte membrane integrity in dilated canaliculi appeared to be maintained, and canalicular lumens appeared uniform and smooth, equidiameter in dimension, as observed in the hepatocytes (canaliculi) of normal livers. In short, altered canalicular lumen diameter was the only observed change as compared to untreated livers. This type of canalicular response (e.g. mosaic of normal and abnormal canaliculi; either dilated canaliculi, or in some instances, collapsed canaliculi of reduced diameter) is often observed during cholestasis in the mammalian liver (Arias 1988; Thung and Gerber 1992).

Of interest, canalicular dilation/attenuation was only distinct in vivo. Were in vivo observations not made, it is questionable whether this phenotypic response would have been detected simply employing histological or ultrastructural studies alone. Even knowing what to look for from in vivo observations, attenuated/dilated canaliculi could not be clearly discerned in histological preparations. In fixed tissue sections canaliculi were often indistinct. It is also possible that tissue processing may alter canalicular lumen diameter. Hence, even when resolved/identified, histological observations of canaliculi may have proven inaccurate. Likewise, canalicular attenuation/dilation may have gone undetected in TEM investigations were the response not previously well recognized in vivo (Figure 3). Hence, in vivo observations were not only important to elucidation of this phenotypic response, but elucidated this change in ways ex vivo techniques were not, we found, well suited.

What may explain canalicular dilation/attenuation? Bearing in mind that hepatocytes have been observed to contribute to 1 to 3 canaliculi, in both mammals and medaka (Arias 1988; Hardman et al. 2007b; Motta and G. 1975), canalicular attenuation/dilation could result from two sources: (1) a contractile regulatory problem at the pericanalicular region of hepatocytes involving cytoskeletal and tight junctional elements, or (2) it may result from the swelling of hepatocytes (or BPDECs, while attenuation/dilation of intrahepatic biliary passageways appeared to be largely associated with canaliculi, bile preductules cannot be ruled out). In the latter case, hepatocellular swelling could preclude or diminish bile flow via reduction of canalicular lumen diameter. If bile secretory rates remained unchanged, canalicular attenuation would force bile to take alternate routes through the interconnected intrahepatic biliary network, which, in the event bile volume is conserved, would necessitate the occurrence of dilated canaliculi in other portions of the intrahepatic canaliculo-ductular network. Dilated canaliculi may reflect locally redirected bile from attenuated passageways (i.e. alternate routes of flow). This

structural change may comprise an adaptive/functional response of the liver to maintain bile secretory/transport functions in the presence of impaired bile flow, and may illustrate one of the underlying attributes of an interconnected, polyhedral based, canaliculo-bile preductular network; in that it provides alternate routes of bile flow for continuous secretion of hepatocellular bile (Hardman et al. 2007a; Hardman et al. 2007b). Because diminished/precluded canalicular transport of bile may result in hepatocellular toxicity by reducing or inhibiting elimination of potentially toxic cytosolic solutes (from intracellular to canalicular lumen), an interconnected network of canaliculi and preductules would allow alternate/multiple routes of elimination (transport) of bile solutes away from hepatocytes for export to the gall bladder and elimination via the gut, circumventing potential hepatotoxicity (bile retention) in the event of impaired/precluded bile flow in portions of intrahepatic biliary passageways. Hence, because hepatocytes synthesize and secrete bile, and may contribute bile to one to three individual canaliculi, it is possible that simultaneous attenuation/dilation of canaliculi is reflective of an adaptive response of the liver to maintain flow of total bile volume (homeostasis of bile synthesis and secretion).

While attributing canalicular dilation/attenuation to swollen hepatocytes is not definitively supported by the findings presented, it can be hypothesized this is a feasible mechanism by which lumen attenuation/dilation could be occurring. Companion TEMs showing altered hepatocytes, in conjunction with associated dilated/attenuated canaliculi, support this conjecture (Figures 3D, 4F, 5D, 6B, 6B1), and studies in rodents describe ANIT induced cytotoxicity in hepatocytes, and biliary epithelium (Alpini et al. 1992; Connolly et al. 1988; Leonard et al. 1981; Lesage et al. 2001). Because *in vivo* techniques focused on elucidation of IHBP's via application of FITC and Bodipy HPC fluorophores (excellent for elucidation of intra- and extrahepatic bile passageways, not optimal for elucidating cell cytosol or organelles), swollen hepatocytes and BECs could not be consistently evaluated *in vivo* in livers exhibiting dilated/attenuated canaliculi. It is possible to co-administer Bodipy C5 ceramide (good for elucidating intracellular morphology) with either of the aforementioned fluorophores to simultaneously elucidate both intrahepatic bile passageways and cell morphology *in vivo*. However, we were not able to adequately refine this *in vivo* procedure during the course of the ANIT studies. In summary, results from *in vivo* and *ex vivo* analyses suggest hepatocellular swelling may be responsible for the dilated/attenuated canaliculi observed, a phenotypic response to ANIT that merits further study in medaka and other piscine species, particularly in regard to the effect of xenobiotics on bile synthesis and transport.

An alternate hypothesis is that ANIT induced cytoskeletal derangements may be responsible for the altered canalicular morphology (e.g. constriction/dilation regulation). Because canaliculi are contractile structures, the function of which is governed by cytoskeletal proteins and actin filaments in the pericanalicular region of hepatocytes, it is possible the canalicular changes observed may be attributable to ANIT induced (either direct or indirect) cytoskeletal alterations in hepatocytes. Cytoskeletal derangements resulting from ANIT exposure have been observed in isolated rat hepatocyte couplets (Orsler et al. 1999), and *in vivo* (Furuta et al. 2004; Kan and Coleman 1986; Lowe et al. 1985). Hence, mammalian studies suggest ANIT induced cytoskeletal alterations a plausible mechanism by which the observed canalicular attenuation/dilation in medaka may manifest. We have to date not fully explored ANIT associated cytoskeletal changes in medaka, though future studies aim to incorporate assessment of cytoskeletal integrity in medaka hepatocytes and biliary epithelia pre and post ANIT exposure.

While canalicular dilation/attenuation was distinct, there did not appear to be any loss of canalicular (bile) transport of the fluorophores Bodipy FL C5 Ceramide, β -Bodipy C5-HPC or fluorescein isothiocyanate associated with this phenotypic response. The only qualitative and quantitative changes in bile transport observed were, perhaps not surprisingly, a modest

increase in fluorescence in dilated canaliculi (a proxy for increased bile volume), and decrease in fluorescence in attenuated canaliculi (a proxy for decreased bile volume). While these local changes were apparent, overall transport of fluorophores from IHBP to extrahepatic bile ducts and gall bladder did not appear qualitatively or quantitatively different than that observed in control fish. Hence, in vivo investigations into hepatobiliary transport suggest the intrahepatic biliary system, due to its interconnected network of canaliculi and bile preductules, was able to maintain overall bile transport from intrahepatic to extrahepatic bile passageways at the time canalicular attenuation/dilation was observed.

4.2 Bile Preductular Epithelial Cell Toxicity

The cause of bile preductular (BPD) lesions is perhaps more clear. In vivo and ex vivo investigations suggest the observed BPD lesions are likely attributable to BPDEC swelling/cytotoxicity, as opposed to hepatocellular injury. BPD lesions (as opposed to attenuated/dilated canaliculi) were commonly associated with changes in BPDEC morphology, observed in vivo, and in TEM investigations (Figure 4C, 4F, 5D). 3D reconstructions also help affirm the hypothesis that BPDEC cytotoxicity contributed to altered bile preductule morphology (Figure 4D, E), and may in part be responsible for observed alterations to bile flow/transport (discussion follows). Hence, in vivo and ex vivo findings together suggest BPDEC toxicity to be a likely candidate for the BPD lesions observed.

Relevant to BPDEC cytotoxicity discussed here are prior studies investigating carcinogenesis in medaka (Okihira and Hinton 1999), which revealed that larval medaka exposed to diethyl nitrosamine (DEN) exhibited a higher prevalence of biliary tumors (46.4% of all tumors in larval-exposed medaka were biliary versus 8.1% in adult-exposed fish), as opposed to hepatocellular carcinomas, which were the prevalent neoplasm when medaka were exposed as adults (100% of hepatocellular tumors in adult-exposed medaka were malignant, while only 51.5% of larval hepatocellular tumors were malignant). These findings, describing a differential response to DEN, depending on the life stage at which exposure occurred, may reflect differences in cell populations during development (e.g. presence and prevalence of BPDECs), and perhaps the role of these cell types on growth and differentiation of tumors in embryonic and adult livers. Given: (1) the findings discussed here, (2) that previous findings show the livers of early life stage medaka, at least up to 40 days post fertilization, to be replete with BPDECs (Hardman et al. 2007b; Okihira and Hinton 2000), and (3) that these cell types are putative bipotent/pluripotent stem-like cells, akin to mammalian oval cells (Farber 1956; Fausto and Campbell 2003; Golding et al. 1996; Okihira and Hinton 1999; Theise et al. 1999); it is possible that BPDEC toxicity may be responsible for the age dependent differences in the types of liver tumors observed by Okihira and Hinton (1999) (e.g. stem cell hypothesis of carcinogenesis). Although anatomical variations between mammalian and medaka liver exist (e.g. while BPDECs are located throughout the hepatic parenchyma of medaka, mammalian oval cells are localized to the peri-portal canals of Hering) (Hardman et al. 2007b; Hinton et al. 2007), response of BPDECs to ANIT is intriguing in the presence of our current understanding of ANIT induced changes in progenitor cells of the mammalian liver (Alpini et al. 1992; Faa et al. 1998; Roskams et al. 1998; Roskams et al. 2003). It follows that BPDECs may play important roles in further elucidating comparative structure/function relationships, and toxic response, in piscine and mammalian livers.

4.3 Hydropic Vacuolation

Hydropic vacuolation (HV), the result of the intracellular accumulation of water and symptomatic of cell swelling, has been employed as a biomarker of exposure to assess the response of wild fishes to environmental contaminants, particularly polynuclear aromatic hydrocarbons, halogenated aromatic hydrocarbons, and pesticides (Bodammer and Murchelano 1990; Gardner and Pruell 1988; Moore et al. 2005; Moore et al. 1996; Murchelano

and Wolke 1985; Myers et al. 1998a; Myers et al. 1998b). By example, winter flounder (*Pleuronectes americanus*) were annually surveyed for trends in hepatotoxicity and chemical body burden to evaluate water quality and effluent treatment efficacy in Boston Harbor, with HV employed as a key indicator of hepatic injury (Moore et al. 2005). HV as a biomarker of environmental stress has also been employed on the U.S. West coast to monitor contaminated coastal environments (Bodammer and Murchelano 1990; Gardner and Pruell 1988; Moore et al. 2005; Moore et al. 1996), and was most commonly observed in biliary epithelial cells and hepatocytes in starry flounder (*Platichthys stellatus*), white croaker (*Genyonemus lineatus*) and rock sole (*Lepidopsetta bilineata*).

Of interest, Nayak et al. (1996) suggest HV may reflect an adaptive, as opposed to toxic, cellular response. Observations in animal and human livers suggest vacuolated hepatocytes observed during liver injury are cells adaptively altered to resist further insult, as opposed to cells undergoing hydropic degeneration. By example, morphological and biochemical investigations have shown that cytoplasmic vacuolation of hepatocytes following low doses of CCL4 was due to excess accumulation of glycogen, predominantly of the monoparticulate form. Low dose CCL4 exposed cells lacked features of degeneration or regeneration, and were much less susceptible to injury by larger subsequent CCL4 doses, as assessed by structural and serum enzyme analyses (Nayak et al. 1996).

The occurrence of HV in medaka was found to be concentration dependent, with increasing prevalence up to 6 μ M ANIT (LC50 = 5 μ M), beyond which HV prevalence declined. While electron dense particulates, which may represent glycogen deposits, were observed sporadically in HVs, we did not attempt to further characterize this response, and cannot elaborate on whether HV was an adaptive vs. toxic response. The in vivo findings presented do however yield a clearer understanding of the morphology of this response at the cellular and system level of organization.

4.4 Biliary Epithelial Cell Proliferation and Biliary Tree Arborization

While several of the structural and functional changes observed in ANIT treated medaka are consistent with a cholestatic response, relatively well described in rodents and humans, such as a mosaic of either dilated or collapsed canaliculi and BEC toxicity (Anwer 2004; Muller and Jansen 1998; Trauner et al. 2005), arborization of the biliary tree (hyperplasia of biliary epithelia of bile ductules and ducts), a common response observed during chronic cholestasis in the mammalian liver (Alpini et al. 1989; Lesage et al. 2001; Masyuk et al. 2003), was not observed in STII medaka in response to ANIT. While arborization appeared to be absent, examination of the liver hilus revealed BEC proliferation, suggesting a biliary tree “arborization-like” response in medaka liver, as compared with its mammalian counterparts. Lack of biliary tree arborization (as compared to rodent) may be due to at least two important factors: (1) biliary tree arborization described in mammalian livers is a function of proliferation of biliary epithelia of bile ductules and ducts, which are largely localized to portal tracts, and (2) because larger bile ducts in medaka are found predominantly in the hilar/peri-hilar region of liver, arborization of the biliary tree in medaka would be localized primarily to liver hilus. These two points are discussed in the following.

First, in vivo and ex vivo (histological, immunohistochemical, TEM) analyses presented show ANIT induces specific changes in the medaka hepatobiliary system involving hepatocytes (vacuolation, cytomegaly), biliary epithelial cells (hyperplasia, vacuolation, cytomegaly) and BPDECs (cytomegaly, vacuolation). AE1/AE3 staining suggests response BECs of the intrahepatic biliary passageways, and PCNA analyses suggest BEC proliferation in the hilar and peri-hilar region of the liver, in response to chronic ANIT exposure (Figure 4C, 4F, 5D, 5E, 6). These findings, particularly BEC hyperplasia in the hilar/peri-hilar region of the liver, are consistent with prior observations correlating medaka and mammalian hepatobiliary

structure/function relationships (Hardman et al. 2007b), and suggest the hepatobiliary system of medaka responds to ANIT in a manner consistent with that observed in the mammalian liver. Previously investigations suggested that the liver of medaka, in total, can be considered the structural and functional analogue of an individual mammalian lobule, with both livers (medaka and mammalian) sharing a common functional unit. However, in medaka, “arborization” of the biliary tree would be localized to the liver hilus, the sole region of medaka liver distinctly akin to the mammalian portal tract. Because the mammalian liver is comprised of numerous lobules, with portal tracts containing bile ductules and small ducts at the lobule periphery (which, structurally, yields a biliary tree), arborization occurs throughout the mammalian liver. In contrast, bile ductules and ducts of the medaka liver are largely found near or within the liver hilus (single lobule hypothesis). Hence, while arborization of the biliary tree occurs in medaka and mammals, this response, as suggested earlier (Hardman et al. 2007b), and reconfirmed hererin, will be different in pattern, site and amount.

Importantly, because of the comparative anatomical arrangement of mammalian and medaka biliary systems, there will be differences as to the interpretation of responses of these livers to insult, though the response may, fundamentally, be similar. Lack of distinct “biliary tree arborization” throughout the liver corpus of medaka, and localization of BEC proliferation to the hilar region of the liver, is illustrative of this concept and an important comparative finding given arborization of portal tracts (BECs) in rodents is a hallmark response to reduced bile flow and BEC injury. By example, studies by Masyuk et al. (2003) found total biliary tree volume (one measure of arborization) in ANIT treated rats to increase 18 times above controls. In conjunction, hepatic artery volume and portal vein volume increased 4 times and 3 times that of control animals, respectively, while bile duct diameter between ANIT treated and control rodents remained unchanged. Note: arborization of the biliary tree has also recently been described in three dimensions in the human liver (Ludwig et al. 1998).

It follows from the above discussion, and previous findings (Hardman et al. 2007b), that arborization of the biliary “tree” in medaka, in response to proliferative agents, would likely be less pronounced than that observed in the mammalian liver, given medaka can be considered to possess a single portal tract (the liver hilus), as opposed to mammals, which possess myriad portal tracts throughout the liver corpus. It should be noted the findings presented here were be no means all inclusive in terms of investigation of BEC proliferation in response to ANIT, and it is interesting to consider that, upon chronic ANIT exposure extending for months, one may see BEC proliferation infiltrate more distal regions of the liver (relative to hilus), with an increase in intrahepatic ductules and ducts.

4.5 Hepatobiliary Transport

No impairment of blood to bile transport of the fluorophores FITC, Bodipy HPC and Bodipy C5 Ceramide was observed at ANIT concentrations $< 6 \mu\text{M}$. The only observed impairment of hepatobiliary transport function in vivo was at aqueous ANIT concentrations $> 6 \mu\text{M}$, which approached the LC100 ($10 \mu\text{M}$, 48hrs), which cannot be necessarily attributed to altered hepatocellular transport mechanisms (e.g. ATP binding cassette of transmembrane transporters). At $8 \mu\text{M}$ ANIT STII medaka were non-motile by 6 hrs of exposure and exhibited passive hepatic congestion in tandem with decreased heart rate (determined by rate of ventricular contraction). Reduced heart rate was likely associated with reduced cardiac output. Given the apparent systemic toxicity at $6 \mu\text{M}$ ANIT and above, the observed decrease in fluorophore transport could have resulted from; (1) decreased respiratory (branchial) uptake of the fluorophores (which in vivo observations suggest is via gill uptake mechanisms), (2) a decrease in intrahepatic circulation (reduced heart rate, sinusoidal flow rates, reduced fluorophore availability over time) and/or (3) from general systemic toxicity not characterized in these studies. Because impaired transport function was associated with cardiovascular

changes and overall morbidity, it is likely that the loss of transport function observed (in vivo) at ANIT concentrations $> 6 \mu\text{M}$ was symptomatic of general systemic toxicity, rather than ANIT mediated disruption of hepatocellular and biliary epithelial cell transmembrane transporters, which play key roles in bile transport and cholestasis.

In contrast to in vivo transport studies with fluorescent probes, volumetric analyses of 3D reconstructions, while performed in only two ANIT treated medaka and three control livers, suggest modest cholestatic and choleretic responses. The findings are noteworthy given; (1) 3D analyses from 3 control livers at 8, 12, and 30 dpf yielded average intrahepatic biliary (canalicular, bile preductular) volumes of 0.97%, (± 0.09) relative to the volume of liver examined, and (2) in hepatobiliary metrics were consistent across 2D and 3D in vivo analyses, and ex vivo evaluations, revealing accuracy of in vivo and ex vivo quantitative assessments. Where the volumes of sinusoidal, hepatocellular, and parenchymal space, relative to total liver volume examined, remained unchanged in ANIT treated medaka, bile canalicular volume was found to be diminished (cholestasis?) at low ANIT concentrations, and increased (choleresis?) at higher concentrations (Table 2). Assuming 3D reconstructions from in vivo imaging accurately represent physiological change, the results are of interest given prior studies describing an adaptive choleretic response reported at non-toxic doses of ANIT (Alpini et al. 1999), and altered intrahepatic biliary volume (Masyuk et al. 2003), in rodent livers. While statistical analyses are not possible, the changes in biliary volume suggested in volumetric analyses are perhaps representative of real physiological change in response to ANIT, and may illustrate the ability to perform volumetric analyses in vivo in STII medaka.

What can explain the discrepancy between volumetric and in vivo fluorophore transport studies? Foremost, bile transport in mammals is a multi-phasic process (bi-directional apical and basal membrane transport) governed in part by a suite of transmembrane transporters such as; the basolateral Na^+ /taurocholate cotransporter (NTCP), organic anion transporting proteins (OATPs), apical bile salt export pump (BSEP), and the multidrug resistant family of transporters (MDR/MRP) [see reviews by (Boyer 1996a; Boyer 1996b; Trauner and Boyer 2003; Trauner et al. 2000)]. Because fluorophore physico-chemical properties determine transporter substrate specificity, it is possible that the fluorophores employed were not substrates for affected transporters (if affected by ANIT exposure). By example, FITC, an organic anion, is a potential candidate for OATP transport on the basal membrane, and MRP2 on the apical membrane, neither of which may be affected by ANIT (no information on the effects of ANIT on transporter proteins in fish exists). Hence, while no loss of fluorophore transport was observed in vivo, it is important to recognize that; (1) being the first in vivo transport studies of their kind, these studies were of a screening nature, and (2) we did not attempt to elucidate which transporters the employed fluorophores were substrates for, nor did we evaluate other fluorophores that may have proved better substrates for the variety of transporters present (as understood in the mammalian liver). Given these factors discrepancies between volumetric and in vivo fluorophore transport studies are not altogether surprising, and more refined study designs will be required to elucidate the mechanisms of altered fluorophore transport in vivo.

4.6 Cholestasis

While we have been reluctant to use the term cholestasis here, we feel discussion of this response important, given its lack of description in piscine species. Cholestasis, a hallmark response of the mammalian liver to injury and insult, is a complex response that involves not only the hepatobiliary system but many other organ systems (e.g. gastrointestinal tract, kidney, cardiovascular system, endocrine system) as well. Because a variety of pharmaceuticals and environmental contaminants have been shown to alter bile transport in mammals (Chang and Schiano 2007; Mohi-ud-din and Lewis 2004; Sakurai et al. 2007), in vivo models and

methodologies, as described here, may prove valuable for investigation of altered bile transport in piscine species. Evaluation of this response in the piscine liver is important given the relevance of this type of hepatic injury/adaptation to ecotoxicological considerations, for instance; the effects of antibacterial agents, pesticides, and hormones employed in aquaculture, and other environmental contaminants, on fish reproduction, fitness and population health (Alderman and Hastings 1998; Cabello 2006; Goldberg and Naylor 2005; Rhodes et al. 2000). In short, understanding a cholestatic type response in fish is imperative to more fully elucidating the effects of environmental contaminants and prophylactic substances on hepatobiliary metabolic and transport function, and health of the individual.

5. Conclusions

While the responses described are largely morphological in nature, these findings reveal the intrahepatic biliary system of medaka to be targeted by the reference hepatotoxicant, ANIT. The cytological changes (e.g. BEC hyperplasia, cytomegaly, hydropic vacuolation, attenuated/dilated canaliculi), and putative changes in bile volume and transport in ANIT exposed medaka, are consistent with those observed in rodent ANIT studies. Biliary tree “arborization”, or rather, the interpretation of this response, differs, since the vast majority of the liver corpus of medaka is comprised of a canaliculo-bile preductular network, while BECs, and their associated bile ductules and ducts, are largely localized to the liver hilus.

These findings, which describe similarities and differences between mammalian and medaka hepatobiliary systems in response to a reference hepatotoxicant (ANIT), in conjunction with prior *in vivo* work characterizing normalcy (Hampton et al, 1988; Hampton et al, 1989; Hardman et al, 2007b; Hinton et al, 2004, Hinton et al, 2007; and others), illustrate the importance of our comparative understanding of the vertebrate liver, and the significance of this understanding on the interpretation and communication of xenobiotic induced injury in piscine livers. From these and previous findings it is apparent that appreciating the spectrum of responses of the piscine liver to xenobiotics that target the this organ system, particularly in a comparative sense, requires more attention to bile preductular epithelial cells, bile preductules, and their relationship to the interconnected intrahepatic biliary network. This is becoming increasingly important given that toxicity screening in embryos and eleutheroembryos is a key factor in the regulatory evaluation of chemicals of environmental concern (e.g. REACh protocol; regulatory framework for Registration, Evaluation, Authorization and Restriction of Chemicals) (ECHA 2007), and that the liver is a key target organ of toxicity.

The findings presented have also shown for the first time *in vivo* evaluation of toxicity in the STII medaka hepatobiliary system, and demonstrate the ability to study and image, with high resolution, normalcy and toxicity in living medaka; a valuable diagnostic and investigatory tool. Given the described coupling of *in vivo* and *ex vivo* investigations, this suggests the future ability to integrate molecular mechanisms of disease and toxicity to system level phenotypes, a current research aim in this laboratory.

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Abbreviations

ANIT	α -naphthylisothiocyanate
BEC	biliary epithelial cell
BPDEC	bile preductular epithelial cell
BPD	bile preductule
DIC	differential interference microscopy
ERM	embryo rearing medium
DMSO	dimethylsulfoxide
FITC	fluorescein isothiocyanate
HV	hydropic vacuolation
LSCM	laser scanning confocal microscopy
PCNA	Proliferating cell nuclear antigen
TEM	transmission electron microscopy
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals

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Figure 1. Non Invasive In Vivo Imaging of Hepatic Parenchyma and Blood to Bile Transport
 (A) Brightfield microscopy, STII medaka, 30 dpf, left lateral view. The liver (L), gall bladder (GB) and associated organs are observable through the abdominal wall. (A1) Widefield fluorescence microscopy of region of interest (gray square) in frame A, illustrating in vivo imaging of β -Bodipy C5 phosphocholine fluorescence (green) transport through intrahepatic biliary passageways (IHBP) of the liver, and gall bladder. (B1) Confocal DIC image, single optical section. STII medaka, 9 dpf. 2 rows of hepatocytes in longitudinal section characterize parenchymal architecture (muralium). Hepatic nuclei (HN). Red blood cells in circulation through sinusoids (S/r) appear as stacked ovate structures. (B2) Same as B1, illustrating concentrative transport of β -Bodipy C5 phosphocholine (FITC, green fluorescence) from sinusoids (S/r) to IHBP. Imaged acquired in vivo 30 minutes post administration of fluorophore in aqueous bath. (B3) Composite of B1 (DIC) and B2 (FITC) localizing fluorophore transport to the area between apical membranes of adjacent hepatocytes. (C) Surface map of region of interest (white square) in B2 illustrating concentration of the fluorophore in IHBP. (D) Semi-quantitative analysis of fluorescence profile across an 18.3 μ m section (white rectangle in frame C) of the parenchyma from sinusoid (S) to IHBP, showed an increase in fluorescence, from sinusoid to canaliculus (IHBP), of ~ 20 fold.

Figure 2.
 Overview: Responses of the Medaka Hepatobiliary System to Aqueous ANIT

Figure 3. Phenotypic Responses of the Medaka Hepatobiliary System: Canalicular Attenuation and Dilation

(A & B) STII medaka, 24 dpf, 2.5 μM aqueous ANIT (48 hrs, acute exposure): LSCM in vivo imaging illustrating dilated (black arrowheads) and attenuated bile canaliculi (white arrowheads). IHBP (green fluorescence) elucidated with fluorescein isothiocyanate (FITC). Epithelium is largely non-fluorescent, aside from weak fluorescence of surrounding hepatocellular cytosol & nucleus (HN, gray arrowhead). Frame B is same as frame A, in a different plane of section in the liver. Example diameters of IHBP are given. (C) STII medaka control, 30 dpf, showing typical morphological appearance of IHBP in vivo. Normalcy finds canaliculi and bile preductules equidiameter throughout the liver, averaging 1.3 μm (± 0.3 μm), also see blue arrowhead in frame B. (D) TEM, STII medaka, 30 dpf, 48 hrs post exposure

to 1 μ M ANIT. Ultrastructure suggested hepatocellular swelling (HN, hepatocyte nuclei) to be associated with canalicular attenuation/dilation (white & black arrowheads). Normal morphological appearance of a bile preductular epithelial cell indicated by green arrowhead.

Figure 4. Phenotypic Responses of the Medaka Hepatobiliary System: Bile Preductular Lesions
 (A) STII medaka control, 23 dpf: LSCM, in vivo, single optical section. Sinusoids (S), bile preductules (BPD), hepatocellular nuclei (HN). Parenchyma elucidated with Bodipy C5 Ceramide. (B) STII medaka, 30 dpf: LSCM, in vivo, single optical section, 10 days chronic exposure to 2.5 μ M aqueous ANIT illustrating BDP lesions (orange arrowheads). White arrows indicate BPDECs. Sinusoid with red blood cell (S/r). (C) STII medaka, 28 dpf: LSCM, in vivo, single optical section, 3 days chronic exposure to 5 μ M ANIT, illustrating appearance of dilated BPDs (gray arrowheads) and mild BPDEC cytomegaly (white arrows). (D) STII medaka control, 30 dpf: example of 3D reconstruction of IHBP (green) and surrounding sinusoids (S, red). (E) STII medaka, 30 dpf, 10 days chronic exposure to 2.5 μ M ANIT, reconstruction of terminal BPD lesion (IHBP). (F) TEM: STII medaka, 26 dpf, 5 μ M ANIT, illustrating alterations to BPDECs associated with BPD lesions. Inset illustrates normal BPDEC morphology, scale bar = 2 μ m.

Figure 5. Phenotypic Responses of the Medaka Hepatobiliary System: Hydropic Vacuolation
 (A) STII medaka control, 20 dpf, ventral view, widefield fluorescence microscopy, autofluorescence (DAPI/UV excitation). Inset scale bar = 100 μ m. Gill (GI), Ventral Aorta (VA), Heart Atrium (Ha), Heart Ventricle (Hv), Liver (L), Gall Bladder (GB), Gut (Gt). (B) Pebbling phenotype: STII medaka, 20 dpf, ventral view, 4 μ M ANIT, 48 hrs of exposure, widefield fluorescence microscopy (autofluorescence; FITC-DAPI/UV composite). Inset scale bar = 20 μ m. (C) STII medaka, 29 dpf, 4 μ M ANIT, 48 hrs of exposure. DAPI labeling (blue) differentiating intracellular ovate structures from nuclei. (D) TEM: STII medaka, 28 dpf, 4 μ M ANIT, 24 hrs of exposure. Hydropic vacuolation in hepatocytes (black arrowheads) and bile preductular epithelia (inset). (E) STII medaka, 29 dpf, 4 μ M ANIT, 24 hrs of exposure: In vivo confocal image (DIC and TRITC composite) of YO-PRO-1 labeling (green). Hydropic vacuoles (black arrowhead).

Figure 6. Phenotypic Responses of the Medaka Hepatobiliary System: Biliary and Bile Preductular Epithelial Cells

(A & A1) AE1/AE3 immunohistochemistry: STII medaka DMSO control, ~ 9 months of age. (A2) AE1/AE3 immunohistochemistry: STII medaka, ~ 9 months of age, 72 hrs post injection 200 mg/kg ANIT, illustrating denser BEC AE1/AE3 labeling and BEC cytomegaly. (B) TEM: STII medaka, 31 dpf, 5 days post exposure to 1 μ M ANIT, illustrating BPDEC alterations (red arrowheads) and associated BPDs (orange arrows). Inset: normal BPD and BPDEC (arrowhead) morphology, 26 dpf, scale bar = 2 μ m. (B1) TEM: STII medaka, 34 dpf, 5 μ M ANIT, 24 hrs of acute exposure, illustrating BEC cytotoxicity (red arrowhead) and associated dilated bile passageway (black arrowhead). Lipid vesicles (gray arrowhead), hydropic vacuolation (white arrowhead). Inset (scale bar = 5 μ m) illustrates normal appearance of BECs (gray arrowheads) and associated bile passageway. (C) LSCM, in vivo, single optical section, STII medaka, 80 dpf, 60 days 2.5 μ M ANIT exposure. Under chronic exposure BPDECs (red arrowheads) appeared enlarged and more numerous (contiguously) per unit area of liver examined, as compared to controls. (C1) STII medaka control illustrating normal in vivo appearance of BPDs/BPDECs (red arrowheads). Hepatocytes (white arrowhead), sinusoid (gray arrowhead). (D) STII medaka, DMSO control, 55 dpf: 6 of 8 control livers exhibited no

or minimal PCNA staining throughout the liver (L). Gall bladder (GB), Gut (Gt). (D1) STII medaka, 55 dpf, 2.5 μ M ANIT, 30 days of chronic exposure: ANIT treated medaka exhibited stronger and more prevalent PCNA staining in the hilar and peri-hilar regions of the liver (circled areas in D and D1).

Figure 7. Bile Duct Cystic Vacuolation in Response to Aqueous ANIT

(A) STII medaka control, 40 dpf, H&E, showing normal morphology of an intrahepatic bile duct and associated biliary epithelial cells (square and enlarged inset). (B) STII medaka, 40 dpf, H&E, 40 days of chronic exposure to 3 µM ANIT. Cystic formations (black arrowhead) were observed in bile ducts of the liver hilus. Cysts often contained inspissated bile or proteinaceous material. An enlarged vessel filled with red blood cells (gray arrowhead), likely an early branch of the hepatic portal vein, can be seen below and left of the biliary cyst.

Figure 8. In Vivo Evaluations of Altered Hepatobiliary Transport Function in Response to ANIT (A) STII medaka control, 40 dpf, LSCM, single optical section, illustrating normal appearance of FITC (green fluorescence) transport from blood to bile, through IHBP of the liver (see also Figure 1). (B) STII medaka, 40 dpf, LSCM, single optical section of medaka liver 48 hrs post exposure to 6 μ M ANIT, illustrating reduced fluorescence of FITC in IHBP and hepatocellular cytosol. Note increased FITC fluorescence in blood plasma (gray arrowhead), but not in hepatocyte cytosol, and minimal fluorescence in IHBP. Such an altered fluorescence profile would be consistent with decreased hepatocellular uptake of fluorophore from blood plasma. (C) STII medaka, 20 dpf, quantitative analysis of blood to bile transport. The statistical means of fluorescence intensity ($n = 30$) are given. No statistically significant difference in fluorophore transport was observed between controls and ANIT treated animals (in vivo), at ANIT concentrations below 6 μ M.

Figure 9. Phenotypic Responses of the Medaka Hepatobiliary System: Passive Hepatic Congestion (A) STII medaka control (DMSO), 20 dpf, 24 hrs post exposure, widefield fluorescence (DAPI/UV autofluorescence). Vasculature (V) appears non fluorescent (dark), epithelia of hepatic parenchyma appears light gray. Inset scale bar = 100 μ m. Ventral Aorta (Va), Heart Ventricle (Hv), Heart Atrium (Ha), Liver (L). (B & C) STII medaka, 18 dpf, 4 μ M ANIT, 24 hrs post exposure: widefield fluorescence (DAPI/UV autofluorescence) illustrating moderate dilation of hepatic vasculature (V). Inset scale bar = 100 μ m. Gill (Gl), Sinus Venosus (SV), Gall Bladder (GB). (C) Widefield fluorescence (DAPI/TRITC composite) illustrating FITC (white arrows) in transport through the hepatic parenchyma (gray arrowhead), in the presence of ANIT induced vasodilation (white arrowhead, also indicating red cells in circulation (S/r)). (D) STII medaka, 17dpf, 8 μ M ANIT, 24 hrs, widefield microscopy (DAPI/UV). (E) STII medaka, 24 dpf, 8 μ M ANIT, 48 hrs post exposure: In vivo LSCM confirmed dilation of intrahepatic vasculature occurred uniformly throughout the liver. Vasculature lumena (white arrowheads), replete with red blood cells, appears dark gray, parenchyma fluoresces green (Bodipy C5 ceramide). (F) TEM: STII medaka, 8 μ M ANIT, 20 dpf, 48 hrs post exposure: intrahepatic vessel showing abnormal (attenuated) endothelial cell membrane morphology (gray arrowhead), and vasodilation (V). Endothelial cell nucleus (black arrowhead). Graph: ANIT dose-duration relationship of heart rate to sinusoid diameter. Indices are mean, $\pm$ SE.

Table 1
Fluorescent Probes Employed for In Vivo Investigations

Probe	Exposure Concentration
7 BR: 7-benzyloxyresorufin	10 μ M – 50 μ M
β -Bodipy C5-HPC [BODIPY® 581/591 C5-HPC (2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl- sn-glycero-3-phosphocholine)	30 nM - 10 μ M
Bodipy FL C5-ceramide [N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine]	500 nM - 5 μ M
FITC: Fluorescein isothiocyanate	1 nM - 50 μ M
DAPI [4',6-diamidino-2-phenylindole, dihydrochloride] (0.3 – 3.0 μ M); and YO-PRO®-1 iodide (491/509) *1 mM solution in DMSO	1 μ M

Table 2
Volumetric Indices from 3D Reconstructions: ANIT Treated vs. Untreated Medaka

Compartment	Control				ANIT Treated			
	8 dpf	12 dpf	30 dpf	Mean (SD): 8-12 dpf	2.5 μ M ANIT 30 dpf	1 μ M ANIT 30 dpf	1 μ M ANIT 30 dpf	1 μ M ANIT 30 dpf
IHBPs	1.03%	0.86%	1.01%	0.97% (0.09)	0.50%			1.18%
Vasculature	6.33%	8.60%	7.60%	7.51% (1.14)	7.30%			7.92%
Parenchyma	93.67%	91.40%	92.40%	92.49% (1.14)	92.70%			92.08%
Hepatocellular	92.64%	90.54%	91.39%	91.52% (1.06)	92.20%			92.15%

Volumetric comparisons between 2 ANIT treated and 3 untreated medaka. Individual indices, statistical mean and standard deviation (SD) of volumetric analyses from control (untreated) livers at 8, 12, and 30 dpf are given. Indices are % volumes relative to volume of liver examined.