Transcriptomic, lipid, and histological profiles suggest changes in health in fish from a pesticide hot spot

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Abstract

Barramundi (*Lates calcarifer*) were collected at the beginning (1st sampling) and end (2nd sampling) of the wet season from Sandy Creek, an agriculturally impacted catchment in the Mackay Whitsundays region of the Great Barrier Reef catchment area, and from Repulse Creek, located approximately 100 km north in Conway National Park, to assess the impacts of pesticide exposure. Gill and liver histology, lipid class composition in muscle, and the hepatic transcriptome were examined. The first sample of Repulse Creek fish showed little tissue damage and low transcript levels of xenobiotic metabolism enzymes. Sandy Creek fish showed altered transcriptomic patterns, including those that regulate lipid metabolism, xenobiotic metabolism, and immune response; gross histological alterations including lipidosis; and differences in some lipid classes. The second sampling of Repulse Creek fish showed similar alterations in hepatic transcriptome and tissue structure as fish from Sandy Creek. These changes may indicate a decrease in health of pesticide exposed fish.

Keywords: Atrazine; Diuron; Imidacloprid; RNA Seq; Asian sea bass (Barramundi); Agricultural Pollution; Lipids; Pesticides

Highlights: 1. Fish from a pesticide hot spot were compared to those in fish from a national park. (83 characters)

2. Fish from the hotspot had alterations in histology, transcriptome and some lipid classes. (88 characters)

3. Fish from the second collection from the national park showed similar alterations. (82 characters)

4. The physiological alterations may coincide with pesticide exposure or health declines. (85 characters)

Abbreviations:

BHMT = betaine--homocysteine S-methyltransferase 1; CYP = Cytochrome p450; Cep85 = Centrosomal protein of 85 kDa; UDPGT = uridine diphosphate glucuronosyltransferase; GCS = Glutamate--cysteine ligase catalytic subunit; PAPSS 2= functional 3'-phosphoadenosine 5'-phosphosulfate synthase 2; LDM = Lanosterol 14-alpha demethylase; RARRE = Retinoic acid receptor responder protein; MAT 2= Methionine adenosyltransferase 2 TAT= Tyrosine aminotransferase; CAT = catalase; GST = Glutathione S Transferase; m GST = microsomal Glutathione S Transferase; zDJ-1 = Protein deglycase DJ-1zDJ-1; GPx-1= Glutathione peroxidase 1; PHGPx = Phospholipid hydroperoxide glutathione peroxidase, mitochondrial; C1-B17.2= NADH-ubiquinone oxidoreductase subunit B17.2; FGH = formylglutathione hydrolase; FALDH = Glutathione-dependent formaldehyde dehydrogenase; GCS = Glutamate--cysteine ligase catalytic subunit; DHCR24 = Delta(24)-sterol reductase; Glx II =Glyoxalase II; ndufa6 = NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 6; GRB2 = Growth factor receptor-bound protein 2; park2= E3 ubiquitin-protein ligase; tmem161a =transmembrane protein 161A; ZnF = Zinc finger like protein; FASN = Fatty acid synthase; CF = Complement factor; MyD88 = Myeloid differentiation primary response protein; CCL= CC- chemokine; NF-κ = Nuclear factor NF-kappa; IgE Fc rγ = High affinity immunoglobulin epsilon receptor subunit gamma; RP = RNA polymerase; pS100B = S100 calcium-binding protein B; RSAD = Radical S-adenosyl methionine domain-containing protein; p53Lyn = Tyrosine-protein kinase Lyn; SPRK1 = Serine/arginine-rich protein-specific kinase 1; THBS-1 = Thrombospondin-1; Apo = apoptosis-inducing ligand; GPBP = Goodpasture antigen-binding protein; CAR = Coxsackievirus and adenovirus receptor homolog; endoU = Poly(U)-specific endoribonuclease; C1 Inh = Plasma protease C1 inhibitor; CEPS= Ubiquitin-60S ribosomal protein L40; SPT = Serine--pyruvate aminotransferase; CAT = catalase; CRBP-II =

Cellular retinol-binding protein II; DECR1 = 2,4-dienoyl-CoA reductase; FABP = Fatty Acid Binding Protein; EL = Endothelial lipase; PAF-AH = Platelet-activating factor acetylhydrolase; GT = Gastrotropin; LBP = Lipopolysaccharide-binding protein; AOX = Peroxisomal acyl-coenzyme A oxidase; LPIN1 = Lipin-1; ACSBG2= Long-chain-fatty-acid--CoA ligase; FERMT2 = Fermitin family homolog 2; PLC = Phospholipase C; gdpd 2 = Glycerophosphodiester phosphodiesterase 2; LK4 = Lipid kinase 4; StARD11 =StAR-related lipid transfer protein 11; INP54 = Phosphatidylinositol 4,5-bisphosphate 5-phosphatase; VLACS = Very long-chain acyl-CoA synthetase; SCD = Acyl-CoA desaturase GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; PP1 = Protein phosphatase 1; PGM 1 = Phosphoglucomutase-1; PGK1 = Phosphoglycerate kinase 1; TIM-B = Triosephosphate isomerase B; PGAM1 = Phosphoglycerate mutase 1; ATF = cAMP-dependent transcription factor; G6PD = Glucose-6-phosphate 1-dehydrogenase; GYS = Glycogen synthase; MDH = Malate dehydrogenase; EMAP-2 = Endothelial monocyte-activating polypeptide 2; MAX = Myc-associated factor X; EGR-1 = Early growth response protein 1; PKM = Pyruvate kinase; AKR1A1 = Aldo-keto reductase; GTase = glucanotransferase; HPX = Hemopexin; CaM = Calmodulin; PI3K = Phosphatidylinositol 3-kinase; PKB = Protein kinase B beta; p53Lyn = Tyrosine-protein kinase Lyn; GSK = Glycogen synthase kinase; PCB = Pyruvic carboxylase; GNMT = Glycine N-methyltransferase

1. Introduction

Poor water quality from agricultural runoff is a concern for the Great Barrier Reef and the adjacent catchment area in northern and central Queensland, Australia (Brodie et al., 2012; Kroon et al., 2012; Smith et al., 2012). Elevated levels of sediment, nutrient, and pesticides are transported from agricultural land to freshwater and estuarine ecosystems, and subsequently discharged to the Great Barrier Reef (GBR) lagoon (Devlin and Schaffelke, 2009; Kroon et al., 2012; Smith et al., 2012). The presence of these agricultural contaminants in the GBR has been linked to loss of coral cover and species from this iconic ecosystem (e.g. Brodie and Pearson, 2016). Notably, catchments within the Mackay Whitsunday region have been recognised as ecosystems with a high risk from pesticides (Brodie et al., 2013 a,b), with elevated levels of photosystem II inhibiting herbicides, such as atrazine, diuron and hexazinone, as well as the neonicotinoid imidacloprid (e.g., Garzon-Garcia et al., 2015; Wallace et al., 2016). In particular, Sandy Creek in the Plane basin, has recorded some of the highest pesticide concentrations of any monitored catchment that discharge to the GBR (Smith et al., 2012; Brodie et al., 2013; Garzon-Garcia et al., 2015; Smith et al., 2015; Wallace et al., 2016).

The economic and ecological importance of the GBR to Queensland and Australia, as well as the threats of climate change to reef ecosystems, has driven most research on the GBR to focus on the marine ecosystems of the GBR (Schaffelke et al., 2012; Brodie and Pearson, 2016). Far less emphasis has been directed towards evaluating the impacts from poor water quality on ecosystem health within catchments that discharge to the GBR (Kroon et al., 2015). Concerningly, pesticide concentrations, and therefore risk, are higher in freshwater and estuarine ecosystems as these ecosystems are closer to the pesticide source (Devlin et al., 2015; Waterhouse et al., 2017). Much of the contemporary research concerning pesticide risks in the region has focussed on impacts to photosynthetic species, such as algae and seagrass (e.g., Magnusson et al., 2010; Magnusson et al., 2012; Flores et al., 2013) due to the proliferation of photosystem II (PSII) inhibiting herbicides in aquatic ecosystems (Lewis et al., 2009). However, there are also concerns relating to the impact of poor water quality on the health of local fish populations (e.g. Kroon et al., 2015; Hook et al., 2017a, b). The herbicides measured at elevated concentrations in GBR catchments have been shown to cause changes in fish health in laboratory studies. For example, exposure to atrazine caused decreased fecundity in fish continuously exposed to environmentally realistic (0.5 µg/L) concentrations of atrazine in laboratory studies (Rohr and McCoy, 2010; Tillitt et al., 2010), although the mechanism by which this occurs has not been established. However, the sublethal impacts on fish from exposure to many of the compounds present in the GBR catchments have not been determined (Kroon et al., 2015). Moreover, impacts on fish health from exposures to complex mixtures of herbicides, their break down products, and the adjuvants present in commercial pesticide products, that exist in the GBR catchments, also have not been elucidated.

Global gene expression, is increasingly analysed via RNA Seq (Mehinto et al., 2012). Analysing global gene expression has the benefit of being able to identify changes in transcript levels, which suggest changes at the physiological level, without an *a priori* hypothesis as to which pathways are altered by changes in water quality (e.g., Hook et al., 2017a,b). The ability to measure physiological changes without a pre-identified mode of toxic action is an advantage when working with pesticides, many of which have not been studied at the molecular level in fish, and in field studies, where contaminants exist in complex mixtures and may interact (e.g., Gustavsson et al., 2017; Scott et al., 2018). In theory, changes in transcript abundance can also be used as evidence of altered signalling, a cellular response in adverse outcome pathways (e.g. Ankley et al., 2010), and be used to predict potential higher organism responses (Villeneuve et al., 2014). However, to be ecologically relevant, changes in

the transcriptome need to be linked to a "higher level" change – such as increased incidence of disease, as changes in transcript abundance do not necessarily reflect changes in gene expression due to post transcriptional modification, altered rates of transcript degradation, and other regulatory processes (Waters and Fostel, 2004; Lorenzetti et al., 2008, Taylor et al., 2013). Although our previous studies (Hook et al., 2017a; b) have identified changes in the transcriptome in fish in the GBR region, associations between these measures and other higher level physiological changes have not yet been confirmed.

The goals of this study were to determine whether changes in the hepatic transcriptome of a resident fish species reflected differences in water quality, and to determine whether the changes observed in the transcriptome are reflected in measurable changes at the whole organism level. To do this, Barramundi, *Lates calcarifer,* a perciforme fish with regional, cultural, commercial and recreational importance, was selected. Barramundi were collected from two catchments in the Mackay Whitsunday region: Repulse Creek, a catchment dominated by conservation land use (Conway National Park) with no known routine pesticide spraying, and from Sandy Creek, a catchment dominated by intensive agricultural land use (DSITI, 2016). Fish health status was evaluated by examining histological alteration in the liver and gill, differences in lipid metabolism were evaluated from lipid and fatty acid class composition in muscle tissue, and RNA-Seq based transcriptome profiles were generated from liver tissue. In addition, pesticide concentrations were measured in water samples from both creeks.

2. Materials and Methods

2.1 Study organism

Barramundi, also known as Asian Sea Bass, *(Lates calcarifer)* (Bloch) (Family Latidae), is a perciforme teleost fish. The species is a protandrous hermaphrotide, with juveniles maturing as males >250 mm total length (TL) in the first 2–4 years, and beginning the transition into females anywhere in the 680–900 mm TL size range. The median size for sexually mature males is 895 mm and for females 1015 mm (Moore, 1979). It is a common aquaculture species, is fished extensively recreationally and commercially, and has cultural importance to Australia's aboriginal communities (Kailola et al., 1993). Barramundi are found in river and estuarine habitats in Northern Austraila. Adults spawn near the mouths of estuaries in the period leading up to and the start of the wet season, (typically between November and January in Northern Queensland). Larval stage fish reside in coastal wetlands after December, and some individuals may migrate towards freshwater habitats later in the year.

2.2 Site description

The two sampling sites, Sandy and Repulse creeks (Figure 1), lie within the Mackay Whitsunday Natural Resource Management (NRM) region in central Queensland, Australia. It is a wet tropical and highly productive region that has been substantially modified primarily for agricultural purposes, with low industrial activity and a low population density (on a global scale) (Brodie et al., 2013). Of note, the small coastal region (~9000 km^2, 2% of the total area of all GBR catchment regions), is the largest sugarcane region in Australia, producing approximately one third of Australia's sugar. Sandy Creek catchment is in the Plane Basin located in the central part of the Mackay Whitsunday region (Figure 1), which flows into Sandringham Bay. It is a small catchment (326 km^2) dominated by sugar cane land use (48%) with only small areas classified as having high ecological value (HEV) and these are located in the headwaters of the catchment (Folkers et al., 2014; Wallace et al., 2016).

Very few catchments in the region are entirely classified as a HEV area, limiting the choice of reference sites. The Repulse Creek catchment is in the Conway National Park, therefore it is classified as a HEV area (Folkers et al., 2014) and selected as the reference site for this study. Repulse Creek catchment is in the Proserpine Basin in the northern part of the region and drains directly to Repulse Bay (Figure 1). The mouth of Repulse Creek is adjacent to the mouth of the Proserpine River, a catchment also within the Proserpine basin and dominated by grazing and sugar cane land use (Supplementary Figure S1). Repulse Creek is quite remote and could only be accessed during certain tidal cycles. Elevated levels of polycyclic aromatic hydrocarbons (PAHs), metals or personal care products have not been identified in previous water quality monitoring programs of Repulse Creek. Pesticides have frequently been detected in catchments and coastal ecosystems of the Mackay Whitsunday region (e.g. Smith et al., 2012; Folkers et al., 2014; Devlin et al., 2015; Garzon-Garcia et al., 2015; Wallace et al., 2016;Huggins et al., 2018) at concentrations above the Australian and New Zealand water quality guidelines (ANZECC/ARMCANZ, 2000).

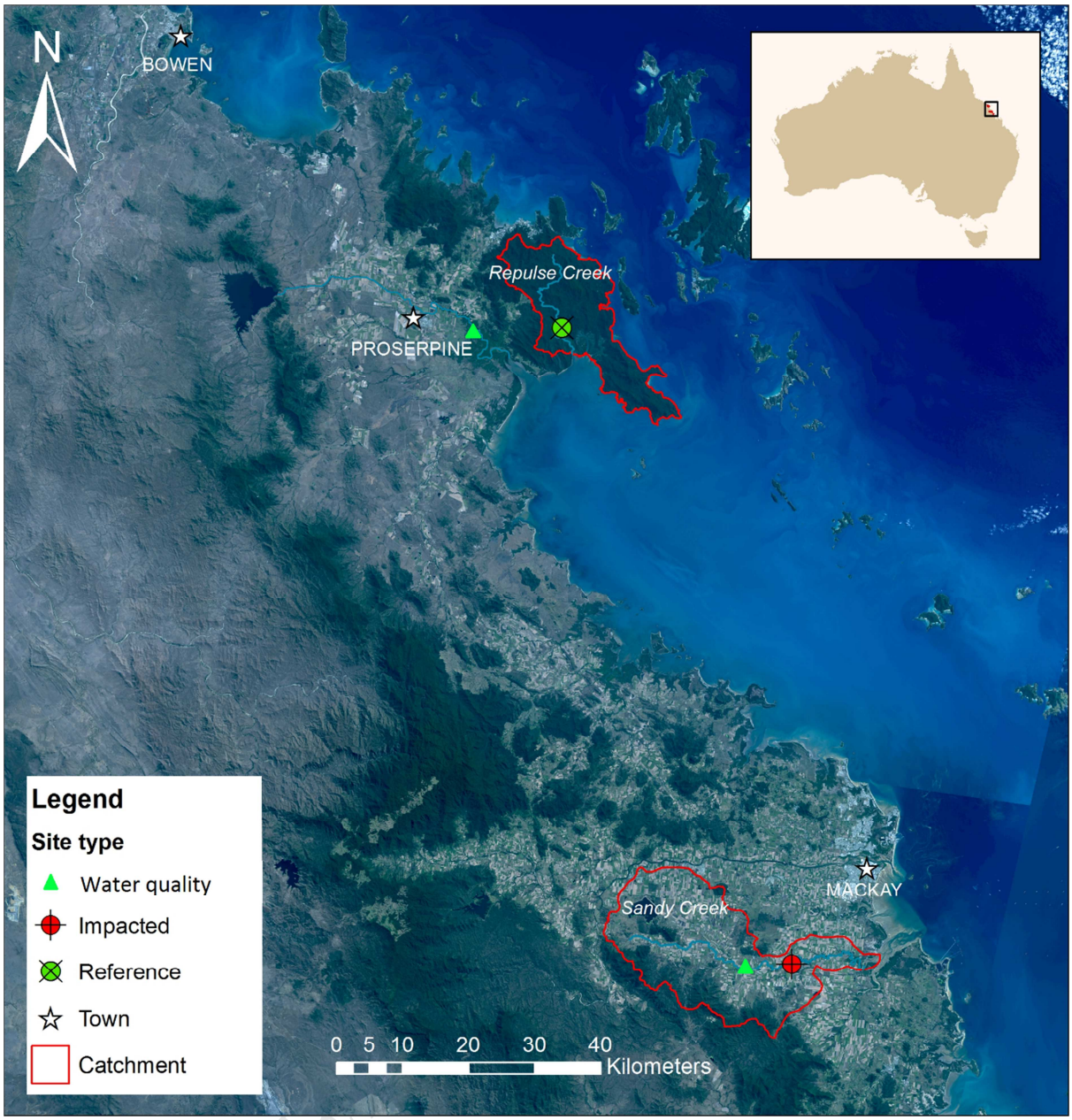


Figure 1 Study site locations. Catchments are outlined in red. The impacted and reference sites show where fish and concurrent water samples were collected, the water quality sites are where routine monitoring is carried out by the state of Queensland, as described in section 2.3.

This project was conducted with the approval of the CSIRO Brisbane Animal Ethics Committee, under permit number A11/2015. Juvenile Barramundi were collected by electrofishing twice at each site: at the start of the wet season – October in Repulse Creek and December in Sandy Creek; and at the end of the wet season (March for both sites). Five fish were collected at each sampling time, except for the Sandy Creek collection in March, when only three individuals could be collected. Fish were collected in the estuarine zone of both catchments (see Table 1 for the latitude and longitude of the sampling sites).

While stunned, fish were placed in a water bath with 100 mg/L clove oil to euthanise them. As soon as gill movement had stopped, fish weight and length were recorded, then the liver, gill, and muscle from the same fish specimen were immediately excised. The time lapsed from collection of the specimen to harvesting the tissues was typically 10 minutes. A subsection of liver for transcriptomic

analysis was submerged in RNAlater and stored on ice initially, and at -20 ° C for transfer to the laboratory. Another subsection of liver from the same fish and a subsection of the gill were placed in tissue cassettes and fixed in 70% ethanol and stored at ambient temperature for histological analysis. The section of muscle to be used in lipid analysis collected from the same fish used in previous analyses was placed in aluminium foil and stored under the same conditions as the liver for transcriptome analysis. Condition index was calculated using weight and length as per Frose (2006).

2.3 Collection of water samples

Time-matched sampling

The aim of the sampling design for this study was to collect time-matched samples (fish and water) during peak pesticide concentration periods at the beginning of the wet season, and again when pesticide concentrations had dissipated at the end of the wet season (as shown in Figure 2). Manual grab water samples were collected with fish at the initial two sampling periods (October and December 2015) of the 2015–2016 wet season. Samples were collected into 200 ml solvent rinsed glass bottles, stored at 4° C in the dark until and transported on ice for pesticide analysis. Due to collection issues, water samples could not be analysed for pesticides from the second fish collection times. As a substitute for the Sandy Creek water samples, pesticide concentrations collected as part of nearby routine monitoring programs was used.

Routine catchment monitoring

To provide a more detailed view of the temporal patterns in pesticides transported in Sandy Creek, pesticide concentration data from the GBR Catchment Loads Monitoring Program (GBRCLMP) was also examined. The GBRCLMP has been monitoring pesticides in a number of catchments that discharge to the GBR lagoon since 2009 (e.g. Wallace et al., 2016), including Sandy Creek, near the fish sampling location (see Figure 1). Routine automatic water sampling was undertaken between 1st of July 2015 and 30th of June approximately 5 km upstream of the fish collection site, above the tidal zone. Details of the sampling methods are outlined in Wallace et al., (2016). In summary, the sampling program principally targeted runoff events, with high frequency sampling during event conditions (~ 7 samples per event) and lower frequency during ambient conditions (~ 1 sample per month). Samples were collected using the sampling procedures outlined in the Environmental Protection (Water) Policy Monitoring and Sampling Manual (DEHP, 2013).

In the following year (2015–2016) routine pesticide monitoring also began in the Proserpine River (Figure S1) through the GBRCLMP. The monitoring site is located 13.5 km upstream of the river mouth within the estuarine zone of the catchment (Figure 1).

2.4 Pesticide Analysis

Water samples were sent to Queensland Health Forensic and Scientific Services Organics Laboratory (Coopers Plains, Queensland) for pesticide analysis. Samples were first extracted via solid phase extraction and then analysed using liquid chromatography-mass spectrometry (LC-MS) to quantify 55 pesticides and pesticide metabolites (the full list of chemicals in the analysis suite and their detection limits are presented in Table S1). This method extracts and analyses for organic compounds with low octanol-water partition coefficient values.

2.5 RNA extractions

RNA was extracted as described previously (Kroon et al., 2015). Briefly, approximately 10 mg of liver tissue was submerged in TRIzol, and tissue was lysed using MP Biomedical's bead beater and lysing

matrix D. RNA extractions then followed the manufacturer's TRIzol extraction (Invitrogen) protocol through the removal of the aqueous phase. At this stage, RNA was concentrated and purified using the Qiagen RNeasy protocol instead of an isopropanol based precipitation. Concentration and purity of the RNA was ascertained using the nanodrop© spectrophotometer, with a minimum concentration of 100 ng μl^{-1}, and a 260:280 ratio of 2.0. RNA integrity was checked using the nano RNA 6000 protocol (Agilent) on a bioanalyser microfluidic device (Agilent). The RNA obtained from samples r1, r2, and s1 was degraded and could not be included in the study.

2.6 Sequencing

Sequencing was performed at the Australian Genome Research Facility (Parkville, VIC). Libraries (one per individual/sample) were prepared using 1 µg total RNA and Illumina's TruSeq stranded mRNA sample preparation kit and protocol. Briefly, mRNA is concentrated using oligo dT beads, then randomly primed for first and second strand cDNA synthesis. Adapters are ligated to each library, then amplified via PCR. These libraries were sequenced using Illumina's HiSeq HT chemistry on an Illumina HiSeq 2000 sequencer and single direction 100 bp fragments were generated.

2.7 Transcriptomic analysis

Sequence reads were deposited in the NCBI SRA database with the accession numbers SRX2764790-SRX2764804. Data are also available via the CSIRO data access portal at doi: 10.4225/08/59000835b8d40. Sequence reads were analysed for quality using the FAST QC program embedded within the CSIRO instance of Galaxy (Giardine et al., 2005; Goecks et al., 2010). Sequences were then trimmed with Trimmomatic using a sliding window approach, with a step size of one, a minimum quality score of 20 and a window size of 20 (Blankenberg et al., 2010). Reads were mapped to a previously developed barramundi transcriptome (Hook et al., 2017b) using the RSEM algorithm in Trinity (Haas et al., 2013). A matrix of transcript abundance as fragments per kilobyte mapped (FPKM) was uploaded into CLC Genomics workbench version 9.0. Data were filtered to remove transcripts with a median abundance of 0 and a mean abundance less than 5 to avoid false calls of differential abundance resulting from very low read abundances. Differential abundance was calculated using the EDGE-R algorithm within the CLC Genomics workbench, with the total filter cut-off set to 5.0, and estimation pf tagwise dispersion enabled (Zhou et al., 2014). Data were visualised in CLC Genomics workbench. Functional annotation was done by BLASTing (nuceleotideBLAST) against the tilapia (*Oreochromis niloticus*) transcriptome with a minimum e value cut-off of 10^{-5}, and BLASTx against the SwissProt database with a minimum e value cut-off of 10^{-5}. Tilapia was chosen because it is a perciforme fish with a RefSeq transcriptome. Mapping to pathways was performed using BLAST2GO (Conesa et al., 2005). Enrichment analysis of GO terms was carried out using the hyper-geometric test on annotations within CLC Genomics workbench. Enrichment analysis was also carried out using DAVID (Huang et al., 2009), which uses a broader suite of annotation databases than only GO terms. Clustering and PCA were also carried out using tools embedded within CLC Genomics workbench.

2.9 Lipid class analysis

Lipid class composition of muscle tissues was determined using a Iatroscan Mark V TH10 thin layer chromatograph coupled with a flame ionization detector (TLC-FID). For each sample, the total extract was spotted and developed in a polar solvent system (70:10:0.1 v/v/v hexane:diethyl ether: glacial acetic acid). Samples were run with standard solutions, obtained from NU-CHEK-PREP. Inc, which contained known quantities of common lipid classes. After elution, chromarods were oven-dried for 10 min at 100° C and analyzed immediately. Peaks were quantified using the manufacturer

supplied SIC-480 Scientific Software. Differences between lipid classes were analysed via one way analysis of variance, followed by Holm-Sidak's method for pairwise comparison in SigmaPlot version 12.3.

2.10 Fatty Acid analysis

Fatty acid composition of muscle tissues was determined using gas chromatography (GC), with component confirmation by GC-mass spectrometry (GC-MS). All samples were analysed following the direct transmethylation technique outlined in Parrish et al. (2015). Tissue samples were homogenised and approximately 40–50 mg placed into pre-weighed and tared glass tubes. All tubes were reweighed and samples directly transmethylated in methanol:dichloromethane: concentrated hydrochloric acid (10:1:1 v/v/v). All samples were kept for 2 hrs at 80°C. Tubes were cooled and 1 mL of Milli-Q® water added, along with 1.8 mL hexane:dichloromethane (4:1 v/v). Tubes were vortexed and centrifuged at 2000 rpm for 5 minutes to break phase, with the upper, organic layer removed. Additional hexane was added, and the procedure repeated twice. The combined organic layers from all three extractions were reduced under a stream of nitrogen gas. Chloroform, with a known concentration of internal injection standard (19:0 FAME) was then added.

FAME samples were analysed using an Agilent Technologies (Palo Alto, CA, USA) 7890B GC equipped with a non-polar Equity™-1 fused silica capillary column (15 m x 0.1 mm internal diameter and 0.1 µm film thickness). Samples (0.2 µl) were injected at an oven temperature of 120°C with helium as the carrier gas. The oven temperature was raised to 270°C at a rate of 10°C per minute, then to 310°C at 5°C per minute. The oven was maintained at 310°C for 10 minutes. A selection of extracts was initially screened for concentration and then an appropriate concentration of internal injection standard (19:0 FAME) was added to each sample prior to all samples being analysed. Peaks were quantified using Agilent Technologies ChemStation software, with initial identification based on comparison of retention times with known laboratory standards. Confirmation of component identification was performed by GC-MS of selected samples and was carried out on a ThermoScientific 1310 GC coupled with a TSQ triple quadrupole. Samples were injected using a Tripleplus RSH auto sampler with a non-polar HP-5 Ultra 2 bonded-phase column (50 m x 0.32 mm i.d. x 0.17 µm film thickness) used. The HP-5 column was of similar polarity to the column used for GC analyses. The initial oven temperature of 45°C was held for 1 minute, followed by temperature programming at 30°C per min to 140°C, then at 3°C per minute to 310°C where it was held for 12 minutes. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy 70 eV; emission current 250 µA, transfer line 310°C; source temperature 240°C; scan rate 0.8 scan/sec and mass range 40–650 Da. Mass spectra were acquired and processed with Thermo Scientific Xcalibur™ software (Waltham, MA, USA). Differences in the amounts of fatty acids were analysed via one way analysis of variance, followed by Holm-Sidak's method for pairwise comparison in SigmaPlot version 12.3.

2.11 Histology

Fixed tissue samples were prepared through a desiccation series of increasing ethanol from 70% to 100%, cleared in Histolene®, embedded in paraffin wax, manually sectioned to 4 µm, stained using standard haematoxylin and eosin (H&E), and mounted with DPX® (Mondon et. al., 2001). Microscopy examination of stained sections was conducted using a Ziess AxioPlan microscope fitted with AxioCamERc5s image capture software. 'Liver and gill samples were examined for evidence of circulatory disturbance, including haemorhrage and thrombus, regressive changes (including decrease in cell number and necrosis), progressive changes (such as hyperplasia and proliferation), prevalence of tumour and metaplasia, and parasites.

3. Results

3.1 Water Quality and Pesticide analysis

The pesticide concentrations measured in time-matched manual grab samples are presented in Table 1. None of the pesticides measured in the 25 October 2015 Repulse Creek samples were above the levels of reporting (LOR) and there were no exceedances of the Australian and New Zealand and Great Barrier Reef water quality guideline trigger values (ANZECC/ARMCANZ, 2000; GBRMPA, 2010). In contrast, Sandy Creek had many pesticides that exceeded the LORs and had elevated concentrations relative to water quality guideline trigger values (ANZECC/ARMCANZ, 2000; GBRMPA, 2010), including atrazine, diuron, and metolachlor.

Based on the GBRCLMP upstream sampling, pesticide concentrations in Sandy Creek were up to 100 fold higher in December 2015 than in March 2016 (Figure 2). The first fish samples were collected from Sandy Creek in December 2015 - after the first flush of pesticides (denoted by the left arrow in Figure 2). The second fish samples were taken in March 2016 (denoted by the second arrow in Figure 2) after pesticide concentrations had dissipated with the progression of the wet season, but were still notably above the dry season background concentrations (Figure 2). Based on these data, it is likely that organisms downstream of the GBRCLMP monitoring site were exposed to elevated concentrations of pesticides for up to three or more months prior to the March fish sampling event.

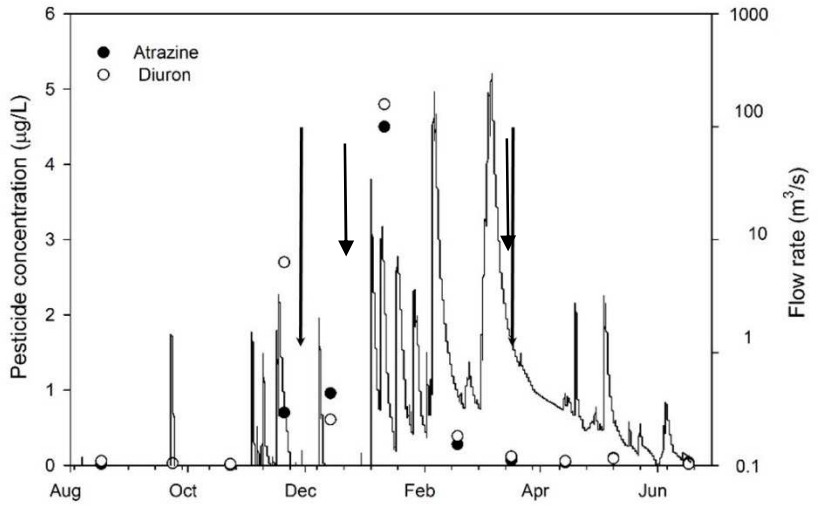


Figure 2 – Temporal trends in pesticide concentrations (open and filled circles) and flow rate (black line) in the upstream region of Sandy Creek (above the tidal zone) measured in 2015/2016 as part of routine catchment monitoring. The barramundi sampling events are shown with arrows. For clarity, only the concentrations of the two most abundant pesticides (atrazine and diuron) are plotted.

Table 1. Pesticide concentrations (µg/L) recorded at fish sampling sites and their dates of collection. Values of <0.01 µg/L indicate concentrations below the levels of reporting (LOR). Only pesticides with values above the LOR for at least one sampling period are reported.

Pesticide	Location	Repulse Creek (fish collection site)	Sandy Creek (fish collection site)	Sandy Creek (upstream monitoring site)[+]	Water Quality Trigger Values[a]
	GPS coordinates	-20.413, 148.769	-21.280, 149.083	-21.283, 149.022	
	Date	26 October 2015	9 December, 2015	17 March, 2016	
Ametryn		< 0.01	0.07	< 0.01	0.5
Atrazine		< 0.01	1.9	0.08	0.6
Desethyl Atrazine		< 0.01	0.2	n.d.[£]	n.a.[$]
Desisopropyl Atrazine		< 0.01	0.1	n.d	n.a.
Diuron		< 0.01	1.8	0.18	0.9
Hexazinone		< 0.01	0.5	0.09	1.2
Imazapic		< 0.01	0.25	< 0.01	n.a.
Imidacloprid		< 0.01	0.31	0.12	0.1[b]
Metolachlor		< 0.01	0.08	0.03	0.02[c]
2,4-D*		< 0.01	0.44	0.02	0.8
Fluroxypyr		< 0.01	0.15	0.04	n.a.
MCPA#		< 0.01	0.04	0.02	1.4[c]
Isoxaflutole		< 0.01	0.02	< 0.01	n.a.

* 2,4-D is 2,4-Dichlorophenoxyacetic acid, # MCPA is 2-methyl-4-chlorophenoxyacetic acid, + The Sandy Creek (as depicted in Figure 1) data collected as part of the routine catchment monitoring approximately 5 km upstream of the fish collection site, [£] the pesticide was not determined (n.d.), [$] a water quality guideline trigger value was not available (n.a.).

[a] Water quality guidelines for the protection of 99% of species, as defined by GBRMPA (2010), unless otherwise noted.

[b] European Water Quality guidelines, obtained from Smit et al. (2015).

[c] Australian and New Zealand water quality guideline trigger values s (ANZECC/ARMCANZ, 2000) calculated using the assessment factor approach and so not designed to protect any particular percentage of species.

Selected water quality physico-chemical parameters were also measured at each site at the same time fish samples were collected (Table 2). There are marked differences between the two sampling periods in Repulse Creek with temperature, conductivity, and percent oxygen saturation all lower in the second sampling period (May 2016) relative to the first (October 2015). In Sandy Creek, only percent oxygen saturation decreased markedly in the second sampling period (March 2016) relative to the first (December 2015).

Table 2. Water quality parameters at the fish sampling sites at the time of collection

Location	Repulse Creek		Sandy Creek	
Date	26 Oct 2015	4 May 2016	9 Dec 2015	22 March 2016
Temperature (^{o}C)	30.8–32.7	25.2–25.4	27.5–28.1	27.4–27.7
pH	7.89–8.72	7.85–8.09	7.37–7.7	7.16–7.25
EC (µs/cm)	297–391	188–193	382–384	416–422
% O_2 saturation	116–124	65–93	90.3–96.5	59–64

3.2 Morphometric characteristics of barramundi

The number of barramundi caught including their morphometric and condition (K) parameter values from each sampling site and time are provided (Table 3). Sandy Creek fish were significantly longer (t-Test: Two-Sample Assuming Equal Variances; p=0.0001), and heavier (t-Test: Two-Sample Assuming Equal Variances; p=0.0002) than those from Repulse Creek, at both sampling times. However, given that an earlier extensive field survey (over 5000 fish) only found one female at a size less than 730 mm (Moore, 1979), it is exceptionally unlikely that Sandy Creek fish were female.

The mean K index values were not statistically significant between the two sites (p=0.183), however the minimum – maximum range of K index values was higher for Repulse Creek (1.01 – 1.47 K index), relative to Sandy Creek (1.0 – 1.19 K index).

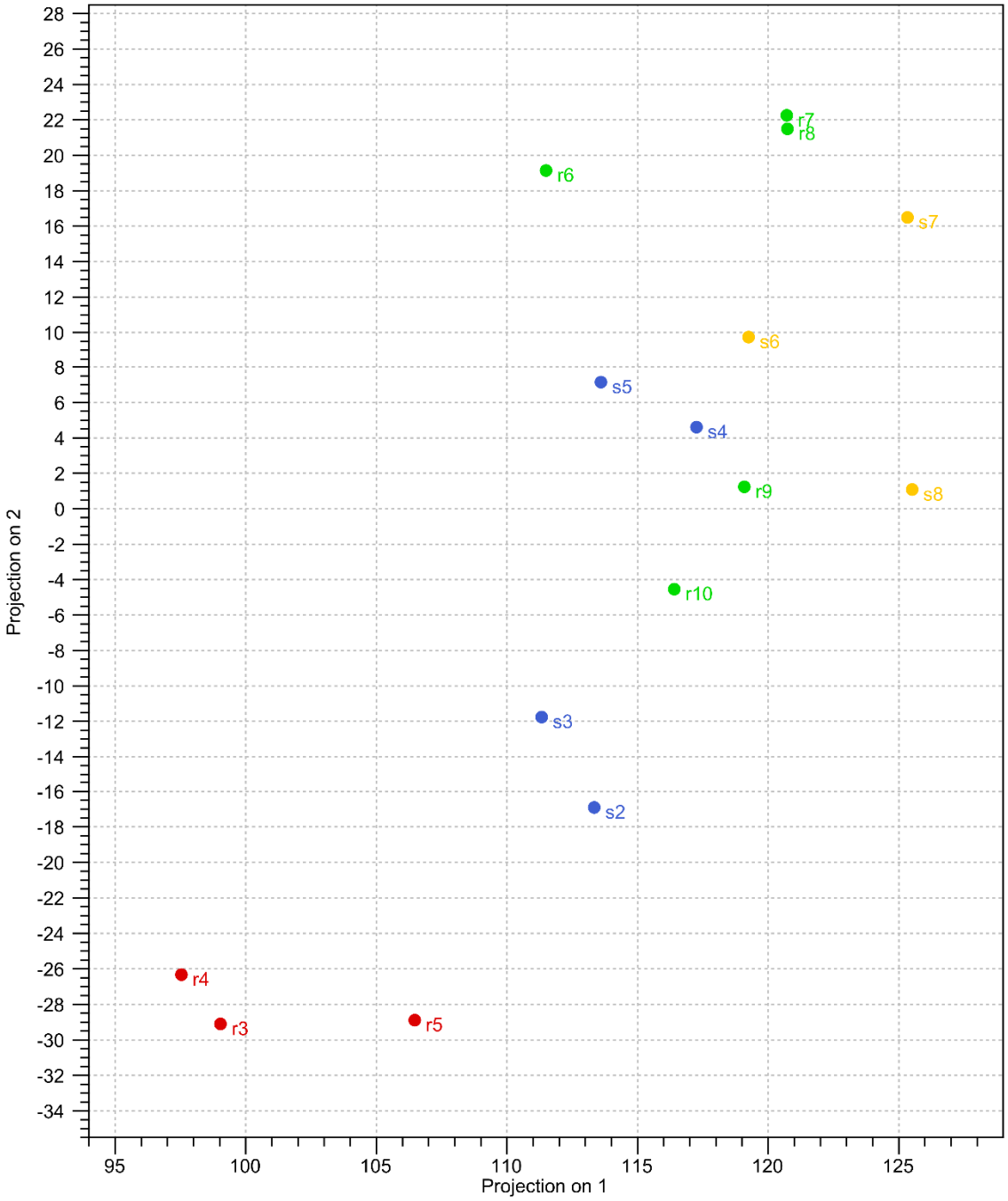
Table 3. Collection date and location, length, weight and condition factor^{+} (K) of each barramundi used in this study

Sample collection date	Site	Fish ID	Length (mm)	Weight (g)	K
26-Oct-15	Repulse Creek	R1	485	1275	1.12
		R2	425	1125	1.47
		R3	390	700	1.18
		R4	330	400	1.11
		R5	281	317	1.43
9-Dec-15	Sandy Creek	S1	570	1850	1.00
		S2	620	2720	1.14
		S3	521	1505	1.06
		S4	500	1450	1.16
		S5	480	1300	1.18
22-Mar-16	Sandy Creek	S6	650	2800	1.02
		S7	640	2850	1.09
		S8	420	880	1.19
4-May-16	Repulse Creek	R6	260	225	1.28
		R7	380	575	1.05
		R8	420	750	1.01
		R9	290	305	1.25
		R10	365	550	1.13

+ Condition Factor was calculated as per Frose (2006)

3.3 Transcriptome Analysis

The number of reads per library and the percentage of reads mapped is shown in Table S2. On average, each sample generated approximately 15 million reads, of which 60% could be mapped to the transcriptome. A higher mapping percentage may have been obtained if we used more stringent read filtering, however, the current approach was sufficient to provide some differentially abundant transcripts. To account for differences in the overall number of reads as well as those mapped, FPKM (fragments per kilobyte mapped) were used in all analyses. There was a small difference in the transcriptome due to size or age as only 16 of the transcripts were differentially abundant (Supplemental Table S3) when fish of different size were compared. By contrast, 654 transcripts were differentially abundant in the livers of December Sandy Creek fish when compared to those collected from Repulse Creek in October; 296 transcripts were differentially abundant in the livers of March Sandy Creek fish when compared to those collected from Repulse Creek in October, and 1468 transcripts are differentially abundant in the livers of May Repulse Creek fish compared to those collected in October (Figure S2). The overall patterns in transcript abundance are shown in the principle components analysis (PCA), which was used to visualise differences in the patterns of transcript abundance in the hepatic samples (Figure 3). There is clear separation between the transcriptomes from barramundi collected at Repulse Creek in the first sampling period (October 2015, samples r3 to r5) and both samplings at Sandy Creek (samples s2 to s10) and the second, May 2016, sampling at Repulse Creek (samples r6 to r8). However, there is overlap between the fish collected from the second Repulse Creek sampling (samples r6 to r8) and the Sandy Creek fish from the other two sampling times (samples s2 to s10). There is also less spatial separation between the first and second collections of Sandy Creek fish than between the first and second Repulse Creek samples. Similar trends are seen if the samples are analysed via hierarchical clustering (Figure S3). The three fish from the initial Repulse Creek sampling period form a distinct clade. Fish from the two sampling periods at Sandy Creek form another clade, and the largest clade contains fish from the two sampling periods in Sandy Creek as well as fish from the later sampling period in Repulse Creek. Two of the fish from the later sampling period in Repulse Creek form an outgroup (Figure S3). As shown in the Venn diagram in Figure S2, the fish collected from Repulse Creek in May had the most unique transcriptome, with 1106 transcripts (75%) being uniquely significantly differentially abundant in these fish. By contrast, fish from Sandy Creek showed more overlap in their transcriptome. Forty-seven % (312 transcripts) of transcripts are unique in the December Sandy Creek transcriptome, compared to only 11% (34 transcripts) of the March Sandy Creek transcriptome (Figure S2).

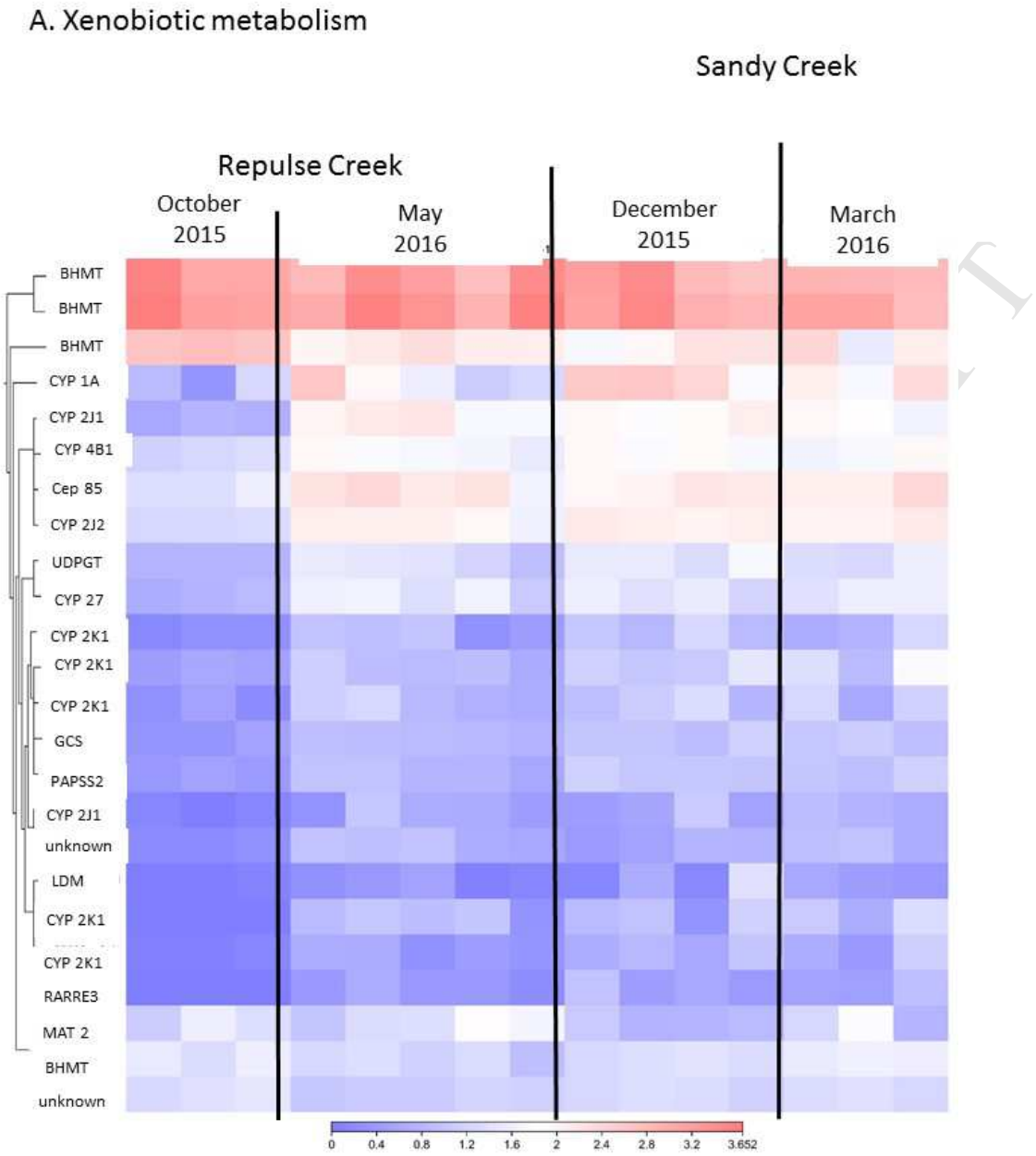


406

407 Figure 3. Principle component analysis of the hepatic transcriptomes of barramundi. For sample
408 codes refer to Table 3. The analysis was performed using 19,599 contigs and 15 samples. Principal
409 component 1 contains 90% of the relative variance, principal component 2 contains 2.5%. Samples
410 r3–r5 were collected in October 2015 and r6–r10 in May 2016 from Repulse Creek. Samples s2–s5
411 were collected in December 2015 and samples s6–s8 were collected in March 2016 from Sandy
412 Creek. Samples r1, r2, and s1 were excluded from the analysis because we were unable to extract
413 sufficient quantities of intact RNA.

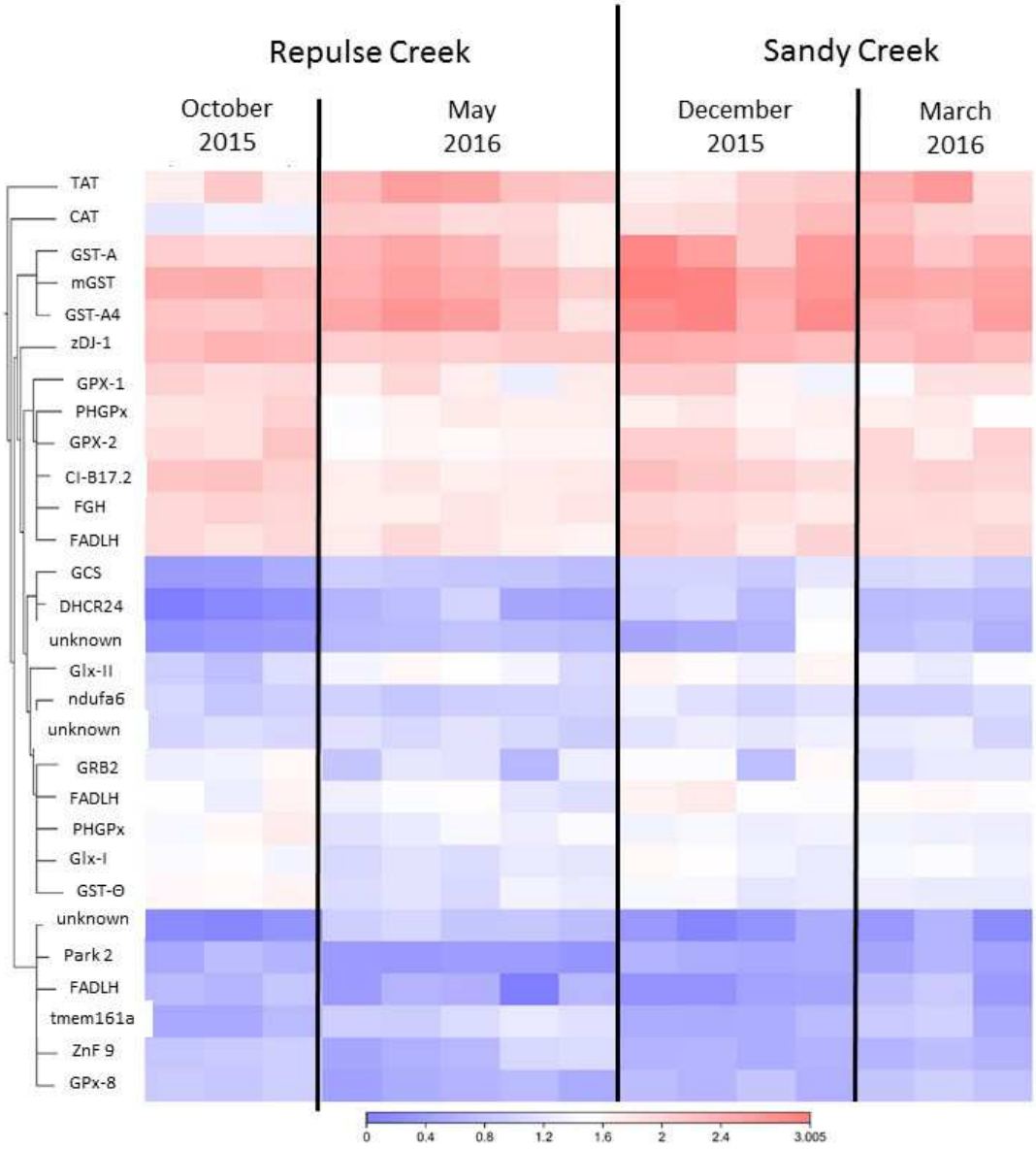
414

As described previously (Hook et al., 2017b), over 97% or the putative contigs in this assembly have an orthologue in the BLAST database, and 8236 could be annotated with Gene Ontology. The differentially abundant contigs were grouped into five functional groups to provide biological context (e.g., Ubrihien et al., 2017): xenobiotic metabolism; oxidative stress, lipid metabolism, glucose metabolism, and immune response (Figure 4). These categories were chosen because of their biological significance and because they were among the most frequently represented in the GO and DAVID analysis (see Section 3.4). For clarity, full gene names are provided in the figure legend for Figure 4 and abbreviations are used here. Several contigs with roles in xenobiotic metabolism, such as CYP 1A, CYP 2J and CYP 2K, and UDPGT, have increased abundances in livers of fish from Sandy Creek at both sampling time periods and in fish from the second sampling time at Repulse Creek compared to fish from the first Repulse Creek sampling (Figure 4a). BHMT transcripts are less abundant in the hepatic transcriptomes of fish from Sandy Creek at the second sampling period (in March) than in the hepatic transcriptomes of other fish (Figure 4a). Trends with contigs with a role in oxidative stress response are less consistent. Some contigs, such as CAT, GST-A, and Glx-II, are more abundant in the livers of fish from the second Repulse Creek sampling in May relative to fish from the first Repulse Creek sampling in October (Figure 4b). Others, such as GPX isoforms, GST Θ and FADLH, are less abundant in the hepatic transcriptomes of fish from the second collection of Repulse Creek fish (in May) relative to the other three collection periods (Figure 4b). Immune responsive transcript levels in the liver varied depending on where and when the fish were collected. For instance, transcripts encoding CCL 20 and MyD88 were more abundant in fish collected in the first Repulse Creek collections, whereas CFH, THBS-1 and Apo-2 were more abundant in the hepatic transcriptomes of fish collected second from Repulse Creek (in May) and second from Sandy Creek in March (Figure 4c). Many transcripts with a role in lipid metabolism or in the peroxisome were consistently less abundant in fish from Repulse Creek collected during the first sampling period (e.g., SCD, AOX, EL, FABP), or consistently more abundant at the same period (e.g. DECR1, GT, LBP) (Figure 4d). Abundances of contigs involved in carbohydrate metabolism showed more variability in the hepatic transcriptomes with sample site (Figure 4e). Some transcripts, such as PP1-3C-B and 3B, PKM, AKR1A1, were always more abundant in the livers of fish from Sandy Creek than those from Repulse Creek. NFκp65, PCB, and GNMT were more abundant in fish from Repulse Creek from the second sample collections (in May) relative to the other sampling periods, whereas PGM1, PGK1, and TIMB were less abundant in fish from Repulse Creek collected at the same time (Figure 4e). Furthermore, transcripts such as those for GADPH, GYS-2 and MDH1 were frequently more abundant in livers of fish from the first collection of fish from Repulse Creek (in October) relative to fish from other time periods, whereas PGAM1 is less abundant in these fish initial Repulse Creek fish (Figure 4e).

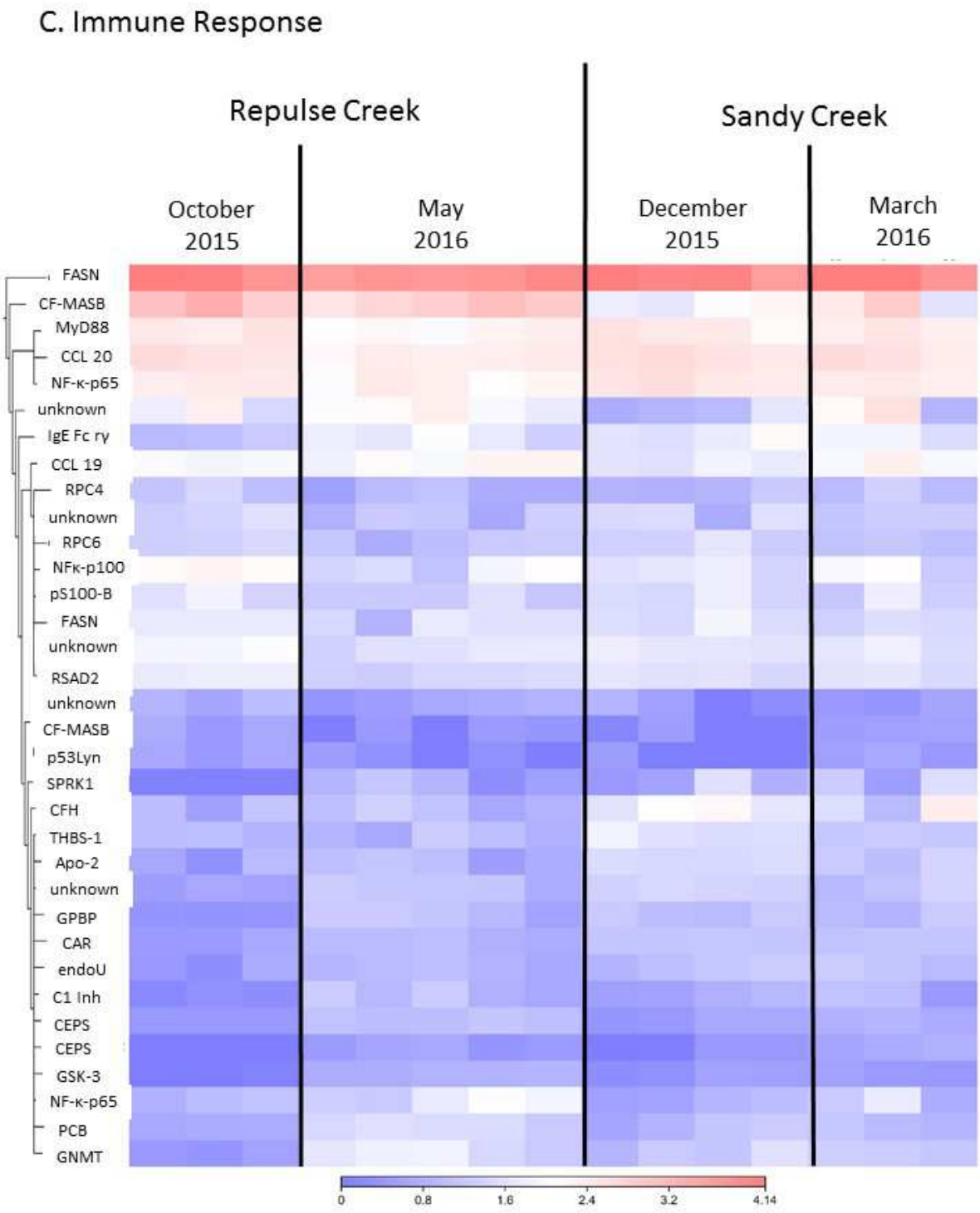

A. Xenobiotic metabolism
Sandy Creek
Repulse Creek
October 2015
May 2016
December 2015
March 2016
BHMT
BHMT
BHMT
CYP 1A
CYP 2J1
CYP 4B1
Cep 85
CYP 2J2
UDPGT
CYP 27
CYP 2K1
CYP 2K1
CYP 2K1
GCS
PAPSS2
CYP 2J1
unknown
LDM
CYP 2K1
CYP 2K1
RARRE3
MAT 2
BHMT
unknown
0
0.4
0.8
1.2
1.6
2
2.4
2.8
3.2
3.652

450

B. Oxidative stress

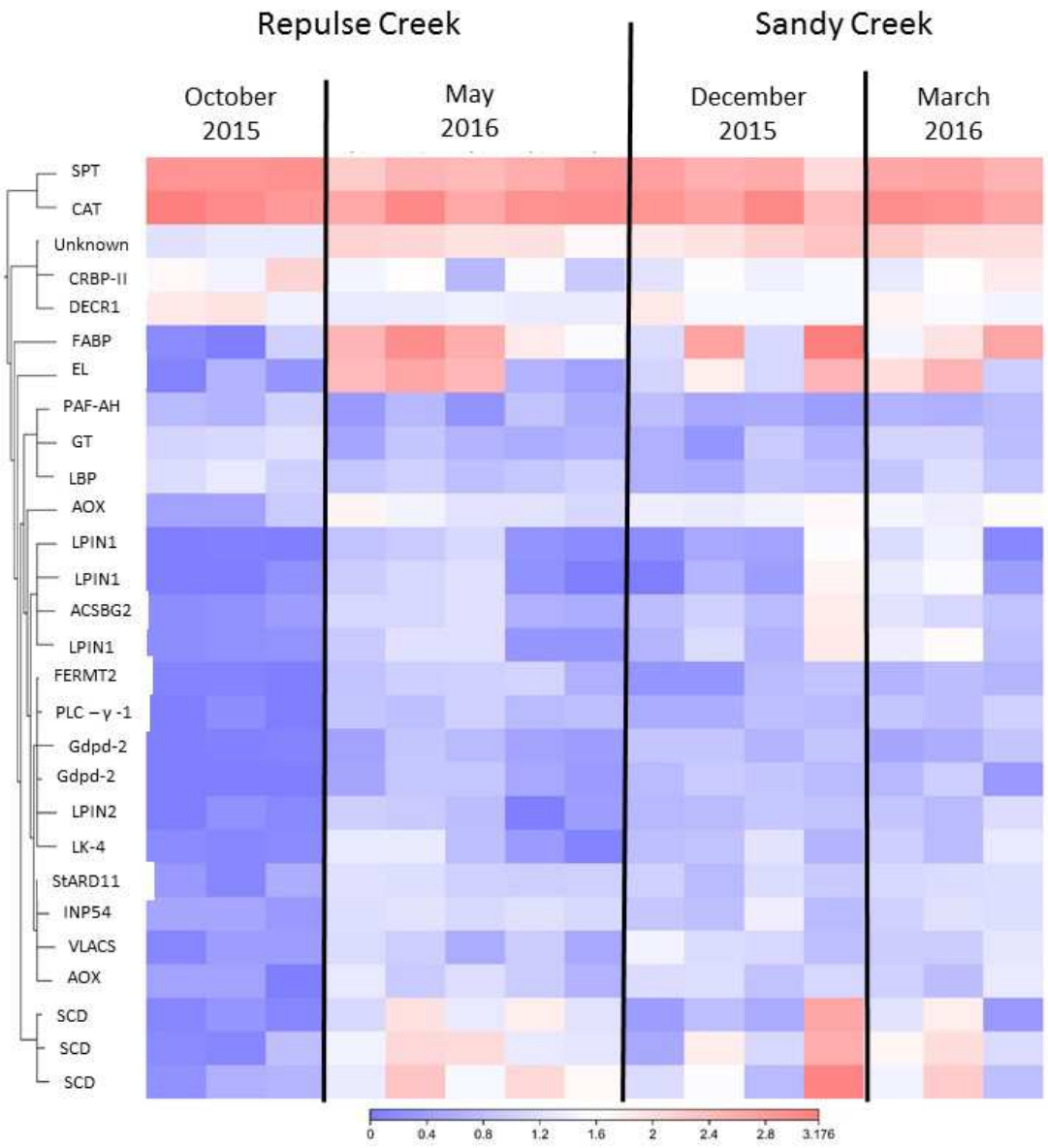


451


C. Immune Response
Repulse Creek
Sandy Creek
October 2015
May 2016
December 2015
March 2016
FASN
CF-MASB
MyD88
CCL 20
NF-κ-p65
unknown
IgE Fc rγ
CCL 19
RPC4
unknown
RPC6
NFκ-p100
pS100-B
FASN
unknown
RSAD2
unknown
CF-MASB
p53Lyn
SPRK1
CFH
THBS-1
Apo-2
unknown
GPBP
CAR
endoU
C1 Inh
CEPS
CEPS
GSK-3
NF-κ-p65
PCB
GNMT
0
0.8
1.6
2.4
3.2
4.14

452

D. Lipid metabolism



453

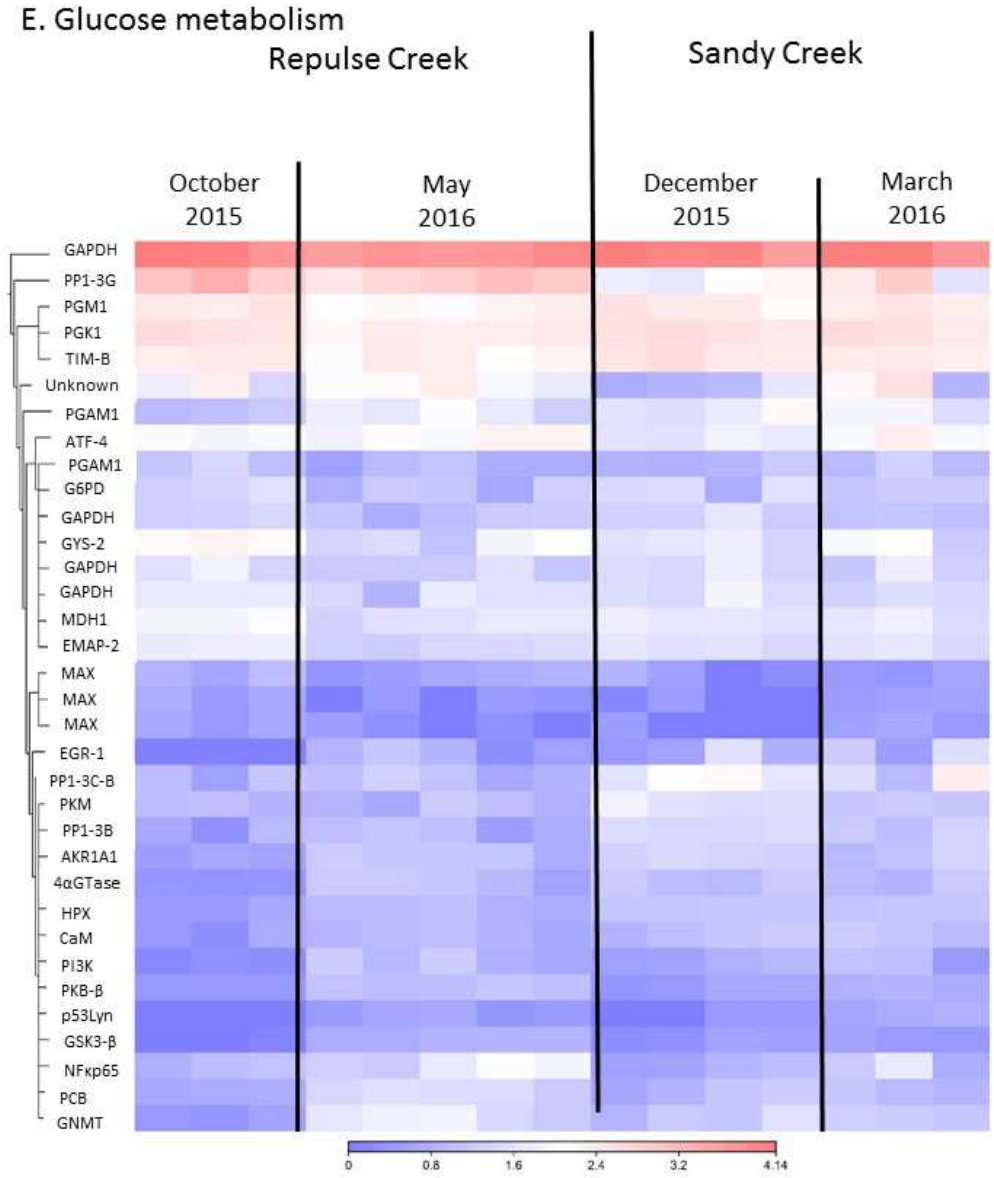


Figure 4. Hierarchical cluster diagrams showing selected differentially abundant contigs, grouped into functional categories. Colour and intensity show the log abundance of each contig, in fragments per kilobase mapped, with dark blue indicating very low abundance and dark red indicating very high. These heat maps are based on 3, 5, 4 and 3 individual fish for October 2015 (Repulse Creek), May 2016 (Repulse Creek), Dec 2015 (Sandy Creek) and March 2016 (Sandy Creek), respectively. The gene abbreviations are abbreviated as follows, and the notation following the gene abbreviation specifies the isoform that was measured. Multiple gene names in the figures denote that multiple isoforms of the same gene were significantly enriched. Unknown genes were not fully annotated, but were still assigned to these functional categories by GO analysis. ***Xenobiotic metabolism***: BHMT = betaine--homocysteine S-methyltransferase 1; CYP = Cytochrome p450; Cep85 = Centrosomal protein of 85 kDa; UDPGT = uridine diphosphate glucuronosyltransferase; GCS = Glutamate--cysteine ligase catalytic subunit; PAPSS 2= functional 3'-phosphoadenosine 5'-phosphosulfate synthase 2; LDM = Lanosterol 14-alpha demethylase; RARRE = Retinoic acid receptor responder protein; MAT 2= Methionine adenosyltransferase 2 ***Oxidative Stress*****:** TAT= Tyrosine aminotransferase; CAT = catalase; GST = Glutathione S Transferase; m GST = microsomal

Glutathione S Transferase; zDJ-1 = Protein deglycase DJ-1zDJ-1; GPx-1= Glutathione peroxidase 1; PHGPx = Phospholipid hydroperoxide glutathione peroxidase, mitochondrial; C1-B17.2= NADH-ubiquinone oxidoreductase subunit B17.2; FGH = formylglutathione hydrolase; FALDH = Glutathione-dependent formaldehyde dehydrogenase; GCS = Glutamate--cysteine ligase catalytic subunit; DHCR24 = Delta(24)-sterol reductase; Glx II =Glyoxalase II; ndufa6 = NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 6; GRB2 = Growth factor receptor-bound protein 2; park2= E3 ubiquitin-protein ligase; tmem161a =transmembrane protein 161A; ZnF = Zinc finger like protein; ***Immune Response*** FASN = Fatty acid synthase; CF = Complement factor; MyD88 = Myeloid differentiation primary response protein; CCL= CC- chemokine; NF-κ = Nuclear factor NF-kappa; IgE Fc rγ = High affinity immunoglobulin epsilon receptor subunit gamma; RP = RNA polymerase; pS100B = S100 calcium-binding protein B; RSAD = Radical S-adenosyl methionine domain-containing protein; p53Lyn = Tyrosine-protein kinase Lyn; SPRK1 = Serine/arginine-rich protein-specific kinase 1; THBS-1 = Thrombospondin-1; Apo = apoptosis-inducing ligand; GPBP = Goodpasture antigen-binding protein; CAR = Coxsackievirus and adenovirus receptor homolog; endoU = Poly(U)-specific endoribonuclease; C1 Inh = Plasma protease C1 inhibitor; CEPS= Ubiquitin-60S ribosomal protein L40; ***Lipid Metabolism*** SPT = Serine--pyruvate aminotransferase; CAT = catalase; CRBP-II = Cellular retinol-binding protein II; DECR1 = 2,4-dienoyl-CoA reductase; FABP = Fatty Acid Binding Protein; EL = Endothelial lipase; PAF-AH = Platelet-activating factor acetylhydrolase; GT = Gastrotropin; LBP = Lipopolysaccharide-binding protein; AOX = Peroxisomal acyl-coenzyme A oxidase; LPIN1 = Lipin-1; ACSBG2= Long-chain-fatty-acid--CoA ligase; FERMT2 = Fermitin family homolog 2; PLC = Phospholipase C; gdpd 2 = Glycerophosphodiester phosphodiesterase 2; LK4 = Lipid kinase 4; StARD11 =StAR-related lipid transfer protein 11; INP54 = Phosphatidylinositol 4,5-bisphosphate 5-phosphatase; VLACS = Very long-chain acyl-CoA synthetase; SCD = Acyl-CoA desaturase ***Glucose Metabolism*** GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; PP1 = Protein phosphatase 1; PGM 1 = Phosphoglucomutase-1; PGK1 = Phosphoglycerate kinase 1; TIM-B = Triosephosphate isomerase B; PGAM1 = Phosphoglycerate mutase 1; ATF = cAMP-dependent transcription factor; G6PD = Glucose-6-phosphate 1-dehydrogenase; GYS = Glycogen synthase; MDH = Malate dehydrogenase; EMAP-2 = Endothelial monocyte-activating polypeptide 2; MAX = Myc-associated factor X; EGR-1 = Early growth response protein 1; PKM = Pyruvate kinase; AKR1A1 = Aldo-keto reductase; GTase = glucanotransferase; HPX = Hemopexin; CaM = Calmodulin; PI3K = Phosphatidylinositol 3-kinase; PKB = Protein kinase B beta; p53Lyn = Tyrosine-protein kinase Lyn; GSK = Glycogen synthase kinase; PCB = Pyruvic carboxylase; GNMT = Glycine N-methyltransferase

3.4 Pathway Analysis

The functions of the differentially abundant contigs were further analysed by mapping to Gene Ontology (GO) terms (Table 4, Table S4) and using DAVID based functional annotation (Table 5, Table S5). The two annotation platforms frequently delivered similar results. For instance, in fish collected from Sandy Creek in December 2015, transcripts with altered abundances relative to the Repulse Creek October 2015 fish mapped, which were in the best health, to GO terms for metabolic processes (glycogen and lipid processing), oxidative stress response, translation and calcium ion dependant processes, and to the DAVID functional categories of oxidoreductase pathways, the peroxisome, and fatty acid metabolism pathways (Table 4, Table 5, full results in Tables S3–S4). In fish collected from Sandy Creek in March 2016, differentially abundant transcripts could be annotated with the GO categories for growth, lipid metabolism, ATP synthesis and cell surface processes, as well as the DAVID functional categories for the mitochondrion signal peptides (Table 4, Table 5, Tables S3–S4). There was not as much agreement between the two annotation pipelines for the transcriptomes of barramundi collected from Repulse Creek in May 2016. This transcriptome had increased abundances of transcripts with GO annotations related to metabolic alterations,

translation, ATP synthesis, oxidative stress response and lipid metabolism, whereas the DAVID functional analysis identified enrichment of transcripts involved in cell shape and blood coagulation (Table 4, Table 5, S4–S5). Also, the DAVID functional analysis identified changes in the complement components (Table 5, Table S5), which was not identified by the GO pipeline.

Table 4. Selected enriched GO terms, in comparison to the hepatic transcriptome of reference fish collected from Repulse Creek in October 2015, the site and time that is best as a reference and most disparate from the other samples. Number indicates the number of transcripts that were in excess of the number from the transcriptome as a whole that would be expected due to chance alone, and a hyphen indicates that the term was not significantly enriched in the transcriptome of fish from that sampling period. The P value refers to the probability that the number of enriched transcripts would occur due to chance alone. BP = biological process, MF = molecular function. Full results are provided in Table S4.

Oxidative stress response						
GO term	Sandy Creek December 2015		Sandy Creek March 2016		Repulse Creek May 2016	
	Number	P value	Number	P value	Number	P value
oxidation-reduction process (BP - GO:0055114)	14	1.26E-04	-	-	13	0.0069
response to oxidative stress (BP - GO:0006979)	-	-	-	-	5	0.019
oxidoreductase activity (MF - GO:0016491)	14	3.90E-05	-	-	12	0.012
peroxidase activity (MF - GO:0004601)	-	-	-	-	5	9.96E-05
glutathione peroxidase activity (MF - GO:0004602)	-	-	-	-	4	3.59E-04
ATP Synthesis						
GO term	Sandy Creek December 2015		Sandy Creek March 2016		Repulse Creek May 2016	
	Number	P value	Number	P value	Number	P value
electron transport chain (BP - GO:0022900)	-	-	-	-	8	0.00012
respiratory electron transport chain (BP - GO:0022904)	-	-	-	-	5	0.0012
ATP synthesis coupled proton transport (BP - GO:0015986)	-	-	-	-	6	5.96E-06
mitochondrial electron transport, ubiquinol to cytochrome c (BP – GO:0006122)	-	-	2	2.33E-03	-	-
ADP binding (MF-GO:0043531)	-	-	3	8.59E-04	4	7.65E-03
NADH dehydrogenase (ubiquinone) activity (MF-GO:0008137)	-	-	-	-	9	5.94E-07
cytochrome-c oxidase activity (MF- GO:0004129)	-	-	-	-	7	1.99E-05
Lipid metabolism						
GO term	Sandy Creek December 2015		Sandy Creek March 2016		Repulse Creek May 2016	
	Number	P value	Number	P value	Number	P value
fatty acid biosynthetic process (BP-GO:0006633)	5	1.77E-04	-	-	-	-
plasma membrane repair (BP - GO:0001778)	2	9.74E-03	3	1.96E-05	3	0.0049
lipid homeostasis (BP- GO:0055088)	2	0.020	-	-	2	0.015
fatty acid binding	2	0.016	-	-	2	0.015

(MF-GO:0005504)						
phospholipase A2 activity (MF -GO:0004623)	-	-	2	2.12E-03	-	-
fatty acid metabolic process (BP - GO:0006631)	-	-			-	-
Translation						
GO term	Sandy Creek December 2015		Sandy Creek March 2016		Repulse Creek May 2016	
	Number	P value	Number	P value	Number	P value
ribosome biogenesis (BP- GO:0042254)	5	1.62E-03	-	-	6	0.0043
rRNA processing (BP-GO:0006364)	4	8.92E-03	-	-		
translation initiation factor activity (MF- GO:0003743)	3	0.030373	-	-	7	1.44E-03
Primary Metabolism						
GO term	Sandy Creek December 2015		Sandy Creek March 2016		Repulse Creek May 2016	
	Number	P value	Number	P value	Number	P value
glycogen biosynthetic process (BP- GO:0005978)	3	5.89E-03	-	-	3	0.013
gluconeogenesis (BP- GO:0006094)	-	-	-	-	3	0.036
tricarboxylic acid cycle (BP - GO:0006099)	-	-	2	0.037	-	-
Oxygen Transport						
GO term	Sandy Creek December 2015		Sandy Creek March 2016		Repulse Creek May 2016	
	Number	P value	Number	P value	Number	P value
oxygen transporter activity (MF-GO:0005344)	-	-	-	-	3	9.28E-03

530

531 Table 5. Summary of the DAVID functional annotation clusters. Transcriptomes are compared to the
532 reference fish from Repulse Creek, October 2015, the site and time that is best as a reference. The
533 numbers provided are enrichment factors that were greater than two when compared to the results
534 from the reference using DAVID functional annotation clusters. The arrows indicate whether the
535 transcripts were increased or decreased in abundance. Only those clusters with an enrichment
536 scores greater than 2 are presented. Full results are provided in Table S5.

Cluster	Sandy Creek, December 2015	Sandy Creek, March 2016	Repulse Creek, May 2016
Oxidoreductase	↑ 6.76		
Aldo/keto reductase	↑ 3.72		
Peroxisome	↑ 3.22		
Innate immunity	↑ 3.19		
long-chain fatty acid-CoA ligase	↑ 2.32		
Complement pathway	↑ 2.19	↑3.71	↑2.63
Cell shape (Actin and Myosin)			↑2.81
Heparin Binding			↑2.63

Blood Coagulation			↑2.31
Nucleoside transport			↑2.10
signal peptide		↑3.06	
Lipid biosynthesis	↑ 2.09		
Mitochondrion	↓ 2.10	↓6.12	↓22.69
Mitochondrion inner membrane			↓18.73
Mitochondrion electron transport			↓11.16
Oxidative phosphorylation (disease associated)			↓7.99
rRNA processing	↓5.71		↓56.95
Translation			↓ 3.41

537

538

539 *3.5 Lipid Class Analysis*

540 Results of the lipid class analyses are shown in Figure 5. Although there is noticeable individual
541 variability between fish caught at the same sampling location and time period, as indicated by the
542 spread in the data, there are no consistent trends in total lipid content between sampling collection
543 times and sites (ANOVA, P=0.52, Figure 5A). Polar lipid concentrations also did not vary between
544 fish sampled at different sites and times (P=0.71, Figure 5B). However, when triacylglycerides (TAG)
545 were examined, a trend towards increased concentrations, as increased median values, in the two
546 Sandy Creek sampling periods was observed, although this was not significant (ANOVA, P=0.12,
547 Figure 5C). Increased TAG:ST ratios were also apparent in Sandy Creek samples, relative to Repulse
548 Creek samples, which were significantly different for the second Sandy Creek (March 2016) samples
549 (ANOVA, P=0.024; Figure 5D).

550 There were also non-significant (ANOVA, p= 0.06) decreases in the saturated fatty acid content in
551 the muscle of barramundi from Sandy Creek and the second Repulse Creek sampling relative to the
552 reference (the first Repulse Creek sampling) (Figure 6A). However, these differences were marginally
553 significant if only the Sandy Creek March 2016 samples and the first Repulse Creek October 2015
554 samples are compared (t test, p=0.05), but not if all four sampling times are compared. There were
555 no significant differences in either poly or mono unsaturated fatty acid content between sites and
556 times (Figure 6 B & C).

557

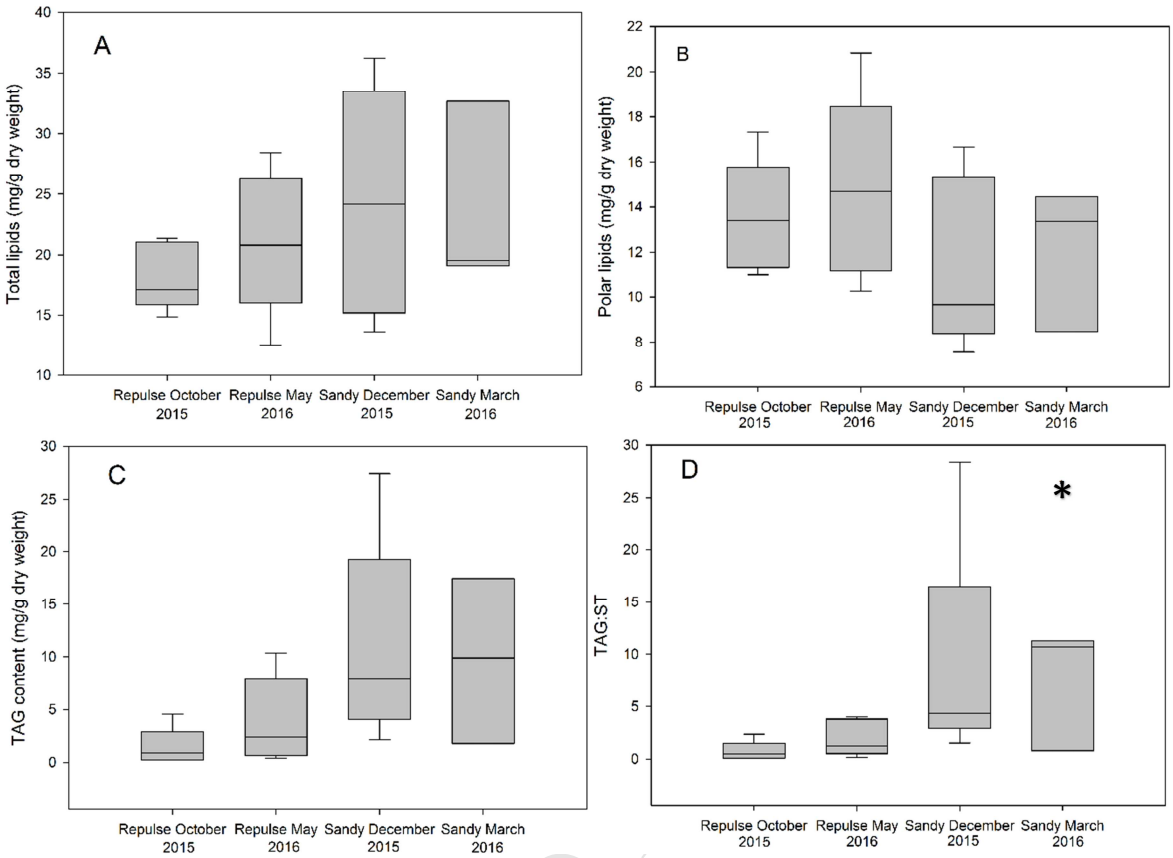


Figure 5. Total lipid content (A) and lipid class profiles (B–C) from barramundi from different collection sites and times. Panel B shows polar lipids content, panel C shows triacylglycerol (TAG) content, and panel D shows the TAG:ST (ST = sterol) ratio. The centre line of each box plot indicates the median value, the edges of the boxes are the 25th and 75th percentiles, and the whiskers show the 10th and 90th percentiles. All data are presented as mg/g dry weight. * indicates statistically significant differences at p≤0.05, Kruskal Wallis ANOVA on ranks.

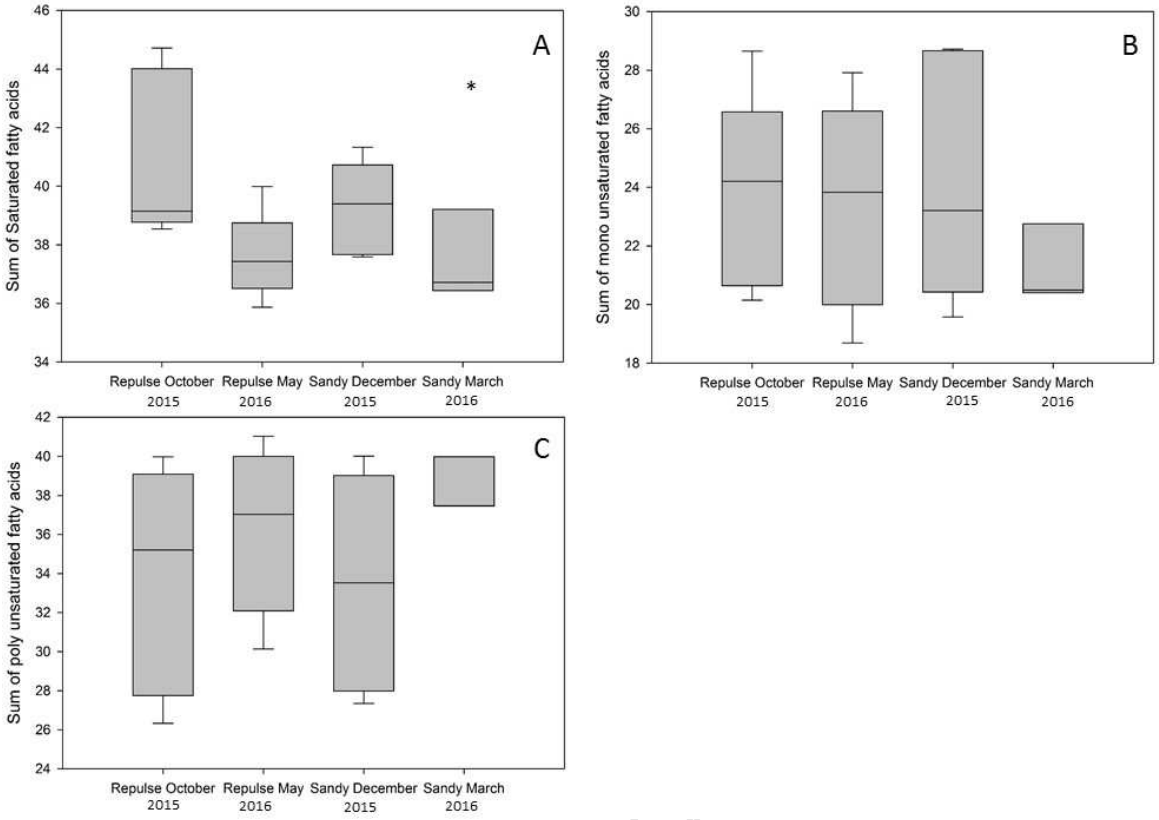


Figure 6. Saturated (A) and mono unsaturated (B) and polyunsaturated fatty acids (C) from barramundi muscle from different sites and times. The centre line of each box plot indicates the median value, the edges of the boxes are the 25th and 75th percentiles, and the whiskers show the 10th and 90th percentiles. All data are presented as mg/g dry weight. The sum of saturated fatty acids showed a strong trend towards decreased concentrations in the March and May fish relative to the others (ANOVA, p =0.064), and a one tailed t-test showed a significant decrease in summed saturated fatty acids in Sandy Creek fish collected in March relative to Repulse Creek fish collected in October (p=0.0498), as dented by the asterix. No other samples were significantly different (P > 0.05) by ANOVA.

3.6 Histology

Fish collected from the first Repulse Creek sampling period in October 2015 displayed relatively normal tissue histology. Gills exhibited either normal morphology, with very slight partial epithelial lifting on the secondary lamellae (Figure 7A), or were infected with microcystis-like cysts (cyanobacteria) on and between the secondary lamellae (Figure 7B & C). Their livers exhibited relatively normal morphology consisting of hepatocytes (liver cells) and hepatopancreas centres (darker pancreatic cells), with limited focal necrosis (small patches of liquefaction) and melanomacrophage centres (clusters of brown pigmented macrophages) (Figure 7D & E). Two individual fish exhibited granuloma of unknown etiology (Figure 7F). Fish collected from Repulse Creek during the second sampling period in May 2016 however, displayed clear pathologies. Gills from each fish exhibited abnormal morphology; increased number of epithelial cells (epithelial hyperplasia) resulting in fusion of the secondary gill lamellae (creating a 'clubbed' appearance of the primary and secondary lamellar structure) (Figure 8A, B & C). Some haemorrhaging was present (Figure 9A). Two small encysted parasites were present in the epithelia cell mass between the

secondary lamellae of one fish (Figure 8C). Liver's exhibited a mixture of normal morphology consisting of hepatocytes (liver cells) and hepatopancreas centres (darker pancreatic cells) and degenerative tissue (which has a liquid appearance). A granulomatous foci (mass of granulation tissue) was evident in one individual showing different stages of inflammation and breakdown (Figure 8D). Two small parasites are seen in cross section (Figure 8D) in one individual. Replacement of hepatocytes and hepatopancreatic tissue with adipose tissue (fatty tissue appearing as white 'enlarged vacuolated cells'), and limited liquefaction was evident (Figure 8E). Limited focal caseous necrosis (small patches of 'pale cheese-like' necrosis beneath liquefactive (purple)) was also evident in some tissue (Figure 8F). There was no evidence to suggest that parasites were causing the significant differences in pathologies between sites and time of sampling. Both endoparasites and ectoparasites were present. Endoparasites were encapsulated, with limited associated inflammatory response in the liver. However, this was not associated with replacement of hepatocytes with adipose cell tissue. The epithelial hyperplasia in gills was definitively linked to the presence of ectoparasites were present feeding on gill lamellae.

Fish tissues collected from Sandy Creek showed abnormalities at both sampling times. In the first sampling period, December 2015, gills uniformly exhibited extensive epithelial hyperplasia resulting in lamellar fusion and an almost club-like appearance for the majority of primary lamellae (Figure 9 A, B & C). External parasites (flukes) have squeezed between the lamellae and are feeding on secondary lamellae (Figure 9 B lower left-hand corner; Figure 9C upper left-hand corner). Liver from these fish exhibited a mixture of normal hepatocyte tissue, and replacement of hepatocyte and hepatopancreas cells with adipose tissue (Figure 9D & F), several small melanomacrophyte centres (Figure 9 D, E & F) and focal areas of necrosis (liquefaction) replacing hepatopancreatic tissue and hepatocyte cells (Figure 9 E & F). The fish collected at Sandy Creek in the second sampling period, March 2016, displayed similar histological alteration to those collected in the first sampling. Gills exhibited a mixture of partial to extensive epithelial hyperplasia resulting in lamellar fusion (Figure 10A, B & C) and a pinched club-like appearance at the apex of the secondary lamella (Figure 10B). External parasites (fluke) are also present between the lamellae (Figure 10, centre in C); internal parasites are evidenced by the presence of encapsulated granuloma (Figure 10, centre A). Liver exhibited a mixture of normal hepatocyte tissue and focal areas of necrosis (liquefaction) replacing hepatopancreatic tissue and hepatocyte cells (Figure 10 D, E & F). Several small melanomacrophyte centres are present (Figure 10 D, E & F). Very low level fatty necrosis (lipodosis) is evident by the presence of small round clear vesicles distributed across the hepatocyte tissue (Figure 10F). Again, there was no evidence that these pathologies were caused by disease or parasites.

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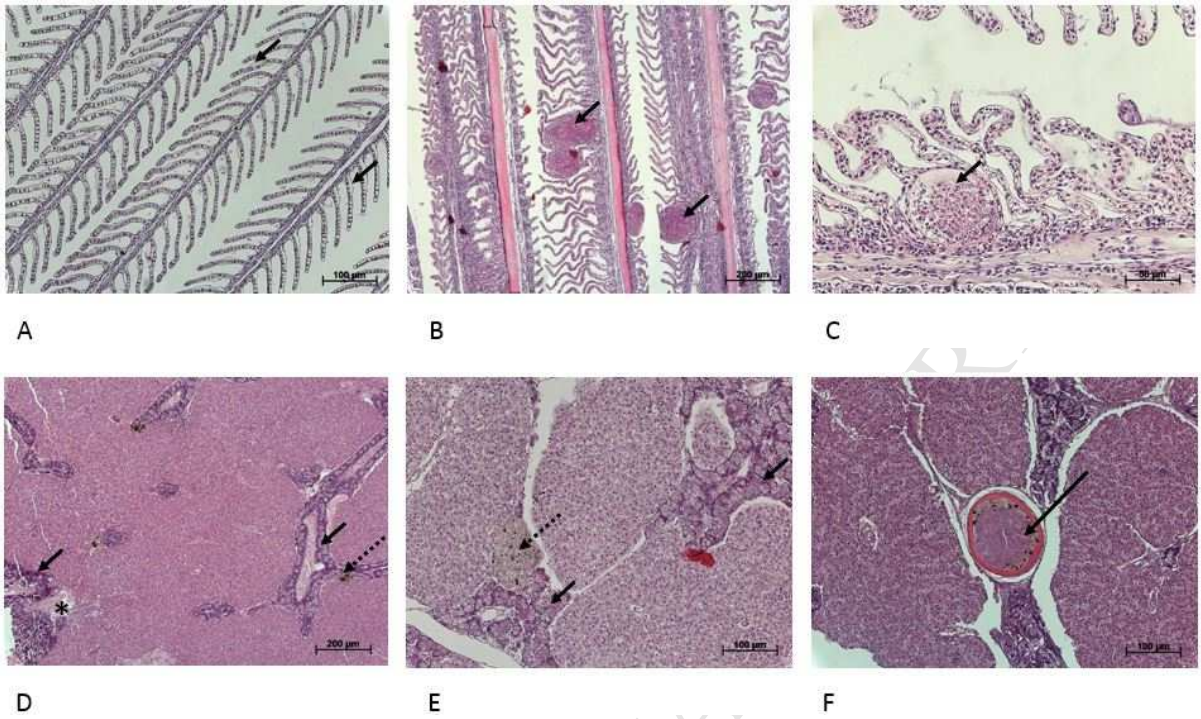

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632 Figure 7. Representative histology from fish collected in Repulse Creek in October 2016. Gill tissue
633 is shown in panels A–C and liver tissue is shown in panels D–F. Partial epithelial lifting on secondary
634 gill lamellae is indicated by arrows in panel A; microcystis-like infection indicated by arrows in panels
635 B and C. Liquefactive necrosis of hepatocytes is indicated by an asterix in panel D; hepatopancreas
636 tissue is indicated by short arrows, and melanomacrophage centres indicated by dashed arrows in
637 panels D and E; granuloma is indicated by an elongated arrow in panel F.

638

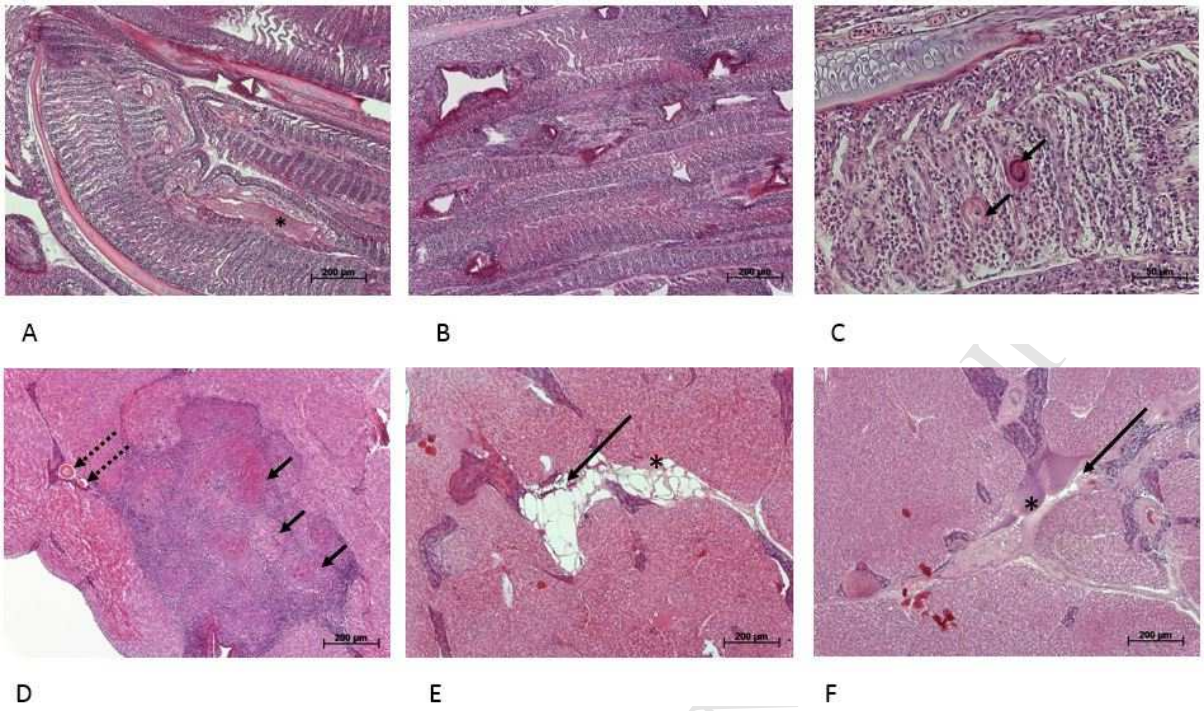

Figure 8. Representative histology from fish collected in Repulse Creek in May 2016. Gill tissue is shown in panels A–C, and liver tissue is shown in panels D–F. Epithelial hyperplasia and lamella fusion is evident in all gill sections (panels A–C); haemorrhage indicated as an asterix in panel A; encysted parasites indicated by arrows in panel C. Multifocal granuloma at varying stages of inflammation and necrosis indicated by arrows, and encapsulated parasites indicated by dashed arrows in panel D; replacement of hepatocytes and hepatopancreas with adipose tissue indicated by elongated arrow in panels E and F; liquefaction and limited caseous necrosis indicated by an asterix in panels E and F.

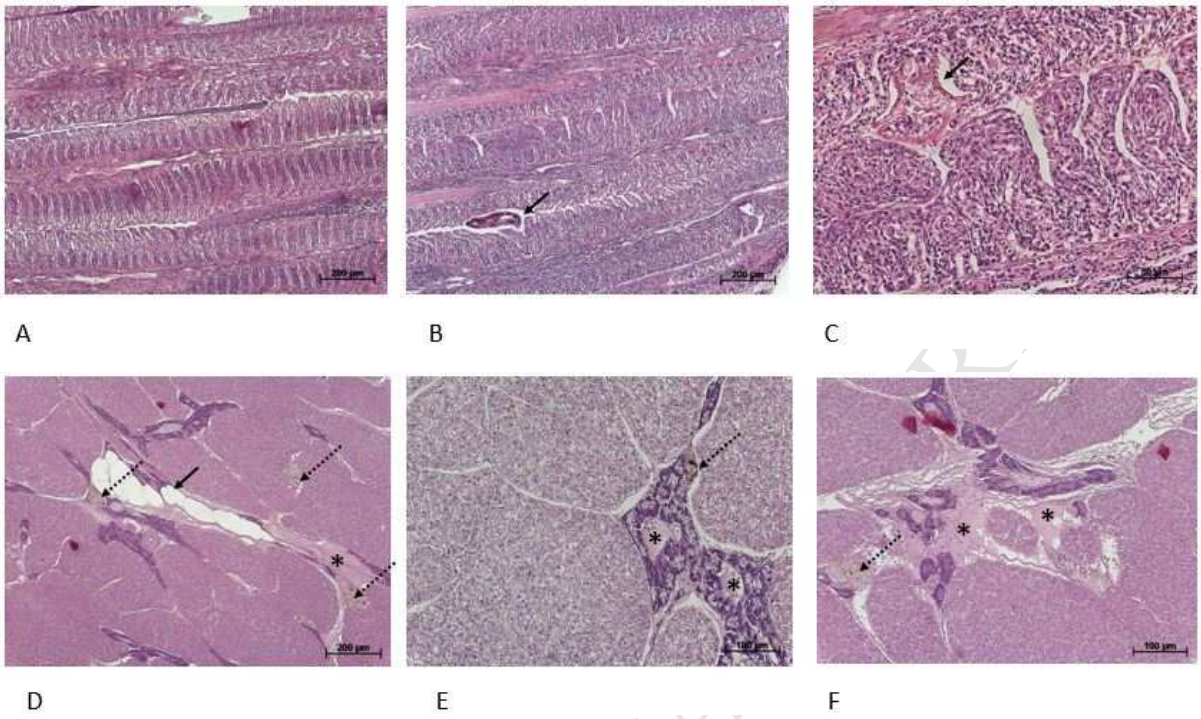

Figure 9. Representative histology from fish collected in Sandy Creek, December 2015. Gill tissue is shown in panels A–C, and liver tissue is shown in panels D–F. Extensive epithelial hyperplasia resulting in lamellar fusion in all gill sections (panels A–C); gill flukes evident on the secondary lamellae indicated by arrows in panels B and C. Replacement of hepatocytes and hepatopancreas with adipose tissue indicated by solid arrow in panel D; melanomacrophyte centres indicated by dashed arrows in panels D–F); focal areas of liquefactive necrosis (liquefaction) indicated by asterix in panels D–F.

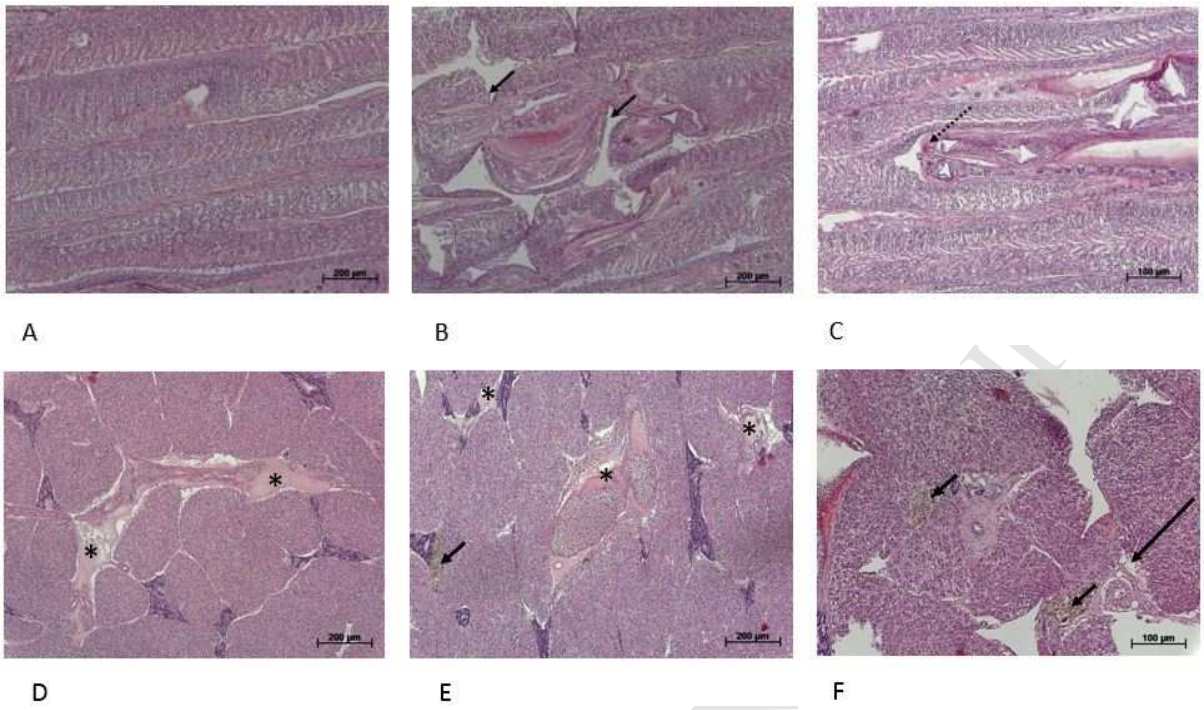

Figure 10. Representative histology from fish collected in Sandy Creek, March 2016. Gill tissue is shown in panels A–C, whereas liver tissue is shown in panels D–F. Epithelial hyperplasia associated with lamellar fusion in all gill sections (A–C); pinched 'club-like' secondary lamella indicated by arrow in panel B. Gill fluke indicated by dashed arrow in panel C. Focal areas of liquefactive necrosis replacing hepatopancreatic tissue and hepatocyte cells indicated by asterix in panel D and E; melanomacrophyte centres indicated by arrows in panels E and F; low level fatty necrosis (lipodosis) in panel F, indicated by arrows.

4. Discussion

We compared the hepatic transcriptomes, tissue structure, and lipid class levels in fish from two sites (Sandy Creek, in the Plane basin, a pesticide hot spot, and Repulse Creek in the Proserpine basin, a relatively pristine site) at two sampling times. The first fish collected from Repulse Creek in October 2015 had a transcriptomic profile that was clearly different to all the other fish collected. These fish also had normal gill and liver tissue for a wild–caught fish (e.g., some indication of parasites, but apparently healthy). Fish were collected from Sandy Creek at the beginning of the wet season (December 2015, when pesticide concentrations first became elevated) and at the end of the wet season (March 2016), when fish had been exposed to pesticides for months but at declining concentrations since the beginning of the wet season. Oxygen saturation was also lower in Sandy Creek at the second sampling time than in the first. The transcriptomic profiles from fish collected from Sandy Creek at both sampling times were distinctly different to the transcriptome of fish collected from Repulse Creek in October 2015. Fish collected in Sandy Creek had elevated levels of CYP transcripts and other transcripts related to xenobiotic metabolism, as well as lipid metabolism, and inflammation. The fish collected from the first Sandy Creek sampling in December 2015 had some differences in the transcriptome relative to the fish collected from Sandy Creek during the second sampling effort in March 2016. In the first sampling of Sandy Creek, when pesticide concentrations were high, transcripts involved with the metabolism of simple sugars, lipid metabolism, and oxidative stress response were elevated, whereas fish collected from the same site

later in the wet season (which had been exposed to pesticide concentrations over a longer period), showed changes in transcripts with functions in ATP synthesis and cell growth, in addition to those measured in the first sampling period. Fish collected from both Sandy Creek samplings had histological alterations in both the liver and gill, and those collected in March 2016 had altered TAG:ST ratios, and a strong trend (ANOVA, p=0.06) towards decreased levels of saturated fatty acids, which was significant if October Repulse Creek and March Sandy Creek samples are compared (t test, p=0.05). Surprisingly, given the land-use of the Repulse Creek catchment (i.e. a National Park) with no major agriculture, fish from the second sample of Repulse Creek also showed altered transcriptomic profiles and histological damage, comparable to those measured in the fish from Sandy Creek. The transcripts with altered abundance (relative to the Repulse Creek October 2015 samples) included CYP 2J and K enzymes, as well as those involved in oxidative stress response, lipid and sugar metabolism, and ATP synthesis. Transcripts for the CYP 2 enzymes have had increased abundance following diuron and atrazine exposure in human cell line studies (Abass et al., 2012). Although a substantial number of transcripts with significant differences in abundances were identified, the small sample size and differences in size of the fish mean that not all transcriptomic differences were identified, due to reduced statistical power of the tests and increased inter-individual variability. More transcripts, and potentially additional altered physiological pathways, could have been identified with a greater number of fish included in the survey. However, the fish we did survey portray a population under stress, presumably resulting from the pesticide exposure.

While it is apparent that the fish from the second sampling of Repulse Creek in May 2016 cannot act as a reference for healthy fish given the preceding results, which was the original intention, there is no clear explanation as to what caused the observed degradation. In addition to the histological and transcriptomic changes noted in this study, there were decreased oxygen saturation and lower fish species diversity and abundance in the second sampling time relative to the first found in a separate study conducted using the same sampling trips (Marsh, 2016). As discussed in more detail in subsequent sections, neither the transcriptomic profile nor the histology suggest that the low dissolved oxygen was causing the observed differences. Discussions with the Conway National Park rangers indicate that glyphosphate is used in the area (although it had not been applied immediately before the May 2016 sampling) and that bridges are treated with “Timber oil”, which contains turpentine and the pesticide octhilinone (R. Perry, Head Ranger, Conway National Park, pers. comm.). Octhilinone is a thiazole fungicide. Since there had been rain in the area in the weeks prior to the May 2016 fish collection, fish may have been exposed to compounds from the timber oil. Alternately, barramundi, may have been exposed to water containing pesticides that had been discharged from the nearby Proserpine River (Table S6), e.g. through migration to or from Repulse Bay and/or Proserpine River itself (see Figure S1). Data presented are for the 2016/17 wet season, but are indicative of contaminant levels in the Prosperine Catchment. Although barramundi have been thought to be resident within a single river system (reviewed in Keenan, 1994), recent studies have highlighted that barramundi, which are catadromous, swim upstream during rain events outside of their spawning season (C. Dench, Reef Catchments River Restoration coordinator, pers. comm). The Proserpine River catchment has a high proportion of sugar cane land use (DSITI, 2016). Pesticide monitoring did not commence in the Proserpine River until July 2016 (after the last barramundi sampling date), from which it was discovered that pesticide contamination was high, the variety of pesticides detected were indicative of sugar cane land use (similar to Sandy Creek) and the estimated loads of pesticides discharged to Repulse Bay are comparatively high for the Mackay Whitsunday region (e.g. Wallace et al., 2016). It is possible if barramundi in Repulse Creek commonly move out into Repulse Bay that they would be exposed to pesticides originating from the Proserpine River. Another possible explanation is that pesticides in Repulse Bay that originated from Proserpine

River may have entered Repulse Creek due long-shore drift and tidal movements. If either of these explanations are true, this finding is significant as it indicates that fish from waterways that would be expected to have little exposure to pesticides can be exposed to pharmacologically active concentrations. Therefore, risks to fish and other aquatic flora and fauna in waterways with limited pesticide contamination but are located in close proximity to other waterways with significant pesticide contamination may be underestimated.

The transcriptomic patterns recorded in barramundi from Sandy Creek at both collection times and at the latter time period in Repulse Creek are similar to what we observed in our previous work in barramundi from the Tully River, which has similar land use and pesticide loads (Hook et al., 2017a,b). In those studies, the hepatic transcriptome of barramundi collected from the Tully River at the end of the wet season was compared to the hepatic transcriptome of barramundi from the Daintree River, which drains a national park. Fish from the agriculturally impacted Tully River had an increased abundance of xenobiotic metabolism transcripts, transcripts involved in the metabolism of lipids and amino acids, and transcripts involved in ATP synthesis, but a decreased abundance of transcripts involved in immune response, relative to those collected from the Daintree River (Hook et al., 2017a). Fish collected from agriculturally impacted rivers during the dry season, when water quality is better, did not show the same transcriptomic patterns, suggesting that the patterns are related to water quality, not habitat alteration (Hook et al., 2017b).

The elevated levels of transcripts for xenobiotic metabolising compounds indicate that the barramundi from Sandy Creek and from the later Repulse Creek collection were exposed to pharmacologically relevant concentrations of pesticides (reviewed in Hook et al., 2014; Schlenk et al., 2008). Previous studies have measured increased abundances of CYP 2 or GST transcripts in fish exposed to atrazine and other pesticides (Egaas et al., 1993; Haasch et al., 1998; Lemaire et al., 2004; Wiegand et al., 2000). While the other changes in the transcriptome, lipid levels and tissue structure cannot be definitively attributed to pesticide exposure, the increased abundance of these transcripts acts as a biomarker of exposure to a pharmacologically relevant concentration of pesticides (e.g. Hook et al., 2014).

Although diuron and atrazine have been in use for over 50 years, there are few mechanistic studies on their impacts on fish in the literature, aside from their potential roles as reproductive endocrine disruptors (e.g. Rohr and McCoy, 2010, Tillitt et al., 2010, Kroon et al., 2014, Ali et al., 2018). However, some studies in the mammalian toxicology literature suggest atrazine has additional modes of action linked to some of the transcriptiomic as well as some of the histological changes observed in the fish we studied. One possible mode of action is that atrazine is acting as a metabolic endocrine disruptor. Environmental factors, including exposure to endocrine disrupting compounds, are increasingly thought to be contributing to metabolic disorders, including non-alcoholic fatty liver disease (Heindel et al., 2017). Exposure to metabolism disrupting compounds is thought to alter transcriptional patterns, and enzyme and biosynthesis that results in metabolic disease, potentially by modulating the activities of the peroxisome proliferator activated receptors (Nadal et al., 2017). Atrazine has been associated with fatty liver and mitochondrial disfunction in rodent studies (Heindel et al., 2017). In a study where mice were exposed to atrazine via their drinking water for twenty weeks, there was an increase in hepatic lipid content and triacylglycerol (Jin et al., 2014), similar to the replacement of hepatocytes with adipose tissue observed in the livers of fish from Sandy Creek and the second sampling event at Repulse Creek, as well as with the increases (though not significant) in triacylglycerides that were measured in the livers of these fish. The study in mice also measured increases in hepatic transcripts involved in carbohydrate to lipid conversions and fatty acid metabolism, including glucose transporters, carbohydrate-responsive element-binding

protein, acetyl-CoA carboxylase, and a stearoyl-CoA desaturase (Jin et al., 2014), many of which are regulated by PPARα. These transcripts were all more abundant, although with differing degrees of significance, in the hepatic transcriptome of fish from Sandy Creek and in fish from the second sampling period at Repulse Creek relative to fish from the first sampling period at Repulse Creek. However, the mice also had increased transcript abundance of PPAR δ (Jin et al., 2014), whereas in the fish in our study only had increased transcript abundance of PPAR α.

Furthermore, a link between contaminant exposure and the impacts on lipid metabolism, inflammation and other whole organism effects may be occurring via the peroxisome proliferation activated receptors (PPARs) (Casals-Casas et al., 2008). PPARs mediate lipid and glucose metabolism and fat storage in the cell, and change the sensitivity of cells to insulin (Adeogun et al., 2016; Pavlikova et al., 2010). PPARγ regulates fat cell differentiation, whereas PPARα regulates the synthesis of very low density lipoproteins, triglycerides, fatty acids and cholesterol (Heindel et al., 2017). Altered regulation of these receptors would lead to steatosis or a fatty-liver disease-like condition (Nadal et al., 2017). Prolonged activation of these receptors has been shown to lead to changes in the energy available in fish for swimming and reproduction (Pavlikova et al., 2010). Some of the compounds detected in the catchment are known to cause peroxisome proliferation. For example, atrazine, which was measured at Sandy Creek (Table 1) has been shown to cause the growth of renal peroxisomes in rainbow trout (Oulmi et al., 1995; Olivares-Rubio and Vega-Lopez, 2016). Alkylphenol ethoxylates have been shown to cause peroxisome proliferation (Cocci et al., 2013; Maradonna et al., 2015), and these are break down products of a commonly used adjuvant of pesticides sprayed in the region (Kroon et al., 2015). Surprisingly, although significantly increased abundance of PPARα transcripts were not noted in this study, it was measured in fish collected from the Tully River with similar pesticides present (Hook et al., 2017a). Increased abundances of ACOX-1, a transcription factor modulated by PPARα (Olivares-Rubio and Vega-Lopez, 2016), and other transcripts related to lipid metabolism were consistently observed in fish from Sandy Creek and the later Repulse Creek collection (e.g. Tables 4 and 5). Fish exposed to peroxisome proliferating compounds have been shown to accumulate lipids in the liver (e.g. Maradonna et al., 2015), as noted in this study, and undergo similar transcriptomic changes to those observed in this work, such as increases in ACOX-1 and lipase genes (Urbatzka et al., 2015). More studies are needed to determine the potential for the pesticides detected in this region to act as metabolic endocrine disruptors.

Another possible mode of action is that the photosystem II inhibiting herbicides are causing mitochondrial toxicity. Photosystem II herbicides target the thykaloid membrane, which is structurally similar to the mitochondrial membrane (Lim et al., 2009). In rats exposed to atrazine via the drinking water for 5 months, there was a decrease in basal metabolic rate, an increase in body weight, abdominal fat and insulin resistance. Atrazine treatment also blocked oxidative phosphorylation complexes I and III, as well as insulin mediated phosphorylation of Akt (Lim et al., 2009). Atrazine exposure also caused structural problems in the mitochondria revealed by electron microscopy (Lim et al., 2009). Early studies with diuron showed that it is capable of uncoupling oxidative phosphorylation (reviewed in Simoes et al., 2017). In a study performed on perfused rat liver, diuron inhibited gluconeogenesis and ammonia detoxification, and decreased ATP synthesis, all of which are mitochondrial functions (Simoes et al., 2017). The transcriptomic changes we measured, including alterations in ATP synthesis, lipid metabolism and primary metabolism (Table 4) suggest mitochondrial disfunction. Hypothetically, the metabolic endocrine disruption or mitochondrial toxicity could be acting independently or in concert to cause the transcriptomic and histological changes observed.

Some of the other changes in the transcriptomic profile observed in the fish from Sandy Creek at both sampling periods and the second Repulse Creek collection are similar to those observed in fish collected from other pesticide contaminated sites. Alterations in transcripts involved in lipid metabolism and transcription were also measured in the hepatic transcriptomes of fathead minnows caged in agricultural areas with a mixture of contaminants, including atrazine (Jeffries et al., 2012). Alterations in the abundance of transcripts in lipid metabolising pathways have also been measured in fish exposed to polychlorinated biphenyls and polybrominated diphenyl ethers, and organochlorine pesticides in the Columbia River (WA, USA) (Christiansen et al., 2014), and in fish exposed to sewage effluent (Moens et al., 2007; Garcia-Reyero et al., 2008). The consistency of these transcriptomic changes in fish collected from pesticide contaminated areas suggests that exposure to pesticides may alter lipid metabolism, and as a consequence, decrease metabolic energy reserves and consequently, ecological resilience (e.g. Jeffrey et al., 2015).

The transcriptomic profiles from fish collected in May 2016 from Repulse Creek and from Sandy Creek at both time periods also resemble profiles of fish exposed to herbicides in controlled laboratory experiments, adding to the weight of evidence that the transcriptomic changes observed in this study may result from pesticide exposure. For instance, brown trout exposed to glyphosate showed changes in the abundance of transcripts involved in oxidative stress, cell signalling, cell proliferation and turnover, and a subsequent metabolic response (Uren Webster and Santos, 2015). More changes in the oxidative stress responsive transcripts were apparent in fish collected from Repulse Creek in the second sampling effort relative to the three other sampling periods (Figure 5B), consistent with the hypothesised mode of action of glyphosate (Uren Webster and Santos, 2015). As we observed in this study, changes in the abundance of transcripts with a role in immune system processes and decreases in ribosomal biogenesis were also measured in rainbow trout exposed to high concentrations of atrazine (Shelley et al., 2012).

Even though at two sampling periods, fish were collected from water with low oxygen saturation, they did not show a characteristic transcriptomic response to hypoxia. Neither of the hypoxia inducible factors (Kodama et al., 2012) nor the hormone leptin (Gracey et al., 2011) were induced (data not shown). Previous studies have found that Atlantic Salmon exposed to hypoxia have increased abundance of transcripts related to protein ubiquination, which were not enriched in the transcriptome of the fish in this study. Also Olsvik et al. (2013) found that oxidative stress transcripts decreased under hypoxic conditions. However, in our study, fish that experienced low oxygen waters had significantly increased abundance of these transcripts.

Additionally, the observed histological alteration in gill morphology is indicative of exposure to a toxicant rather than hypoxia. Hypoxia-induced change is likely to result in a reduction in cell proliferation in the filament epithelium; reduction in the number of pavement cells comprising the epithelial layer, elongation of the lamellae, and an increase in gill surface area facilitating oxygen transport (Sollid et al., 2003). With the exception of the first fish collections from Repulse Creek in October 2016, all gill sections exhibited an epithelial proliferation resulting in partial to full lamellar fusion and filament fusion. This thickening of the respiratory epithelium is an effective protection against damage to the lamellae from toxicant or microbial insult, but is singularly ineffective for gas and ion exchange under low dissolved oxygen conditions (Evans, 1987; Matey et al., 2008). Although we cannot rule out hypoxia as a contributing factor to the changes in the transcriptome and the tissue structure, comparison to literature studies, although conducted with different species of fish, does not suggest that hypoxia alone is causing the observed differences.

Some of the changes observed in the transcriptome reflect the changes observed by histological examination of the tissue. For example, the increased abundance of transcripts related to the

complement system in all sampling periods relative to the reference fish collected in the first round of sampling in Repulse Creek may be related to the inflammatory response (reviewed in Magnadottir, 2006) identified in the liver tissue of fish collected from Sandy Creek at both sampling periods and from Repulse Creek during the second sampling effort in May. Complement activation stimulates phagocytic activity to remove foreign material via opsonisation of pathogens (Janeway et al., 2001), potentially evidenced, in this case, by the presence of phagocytic cells (focal pigmented macrophage centres), and focal necrosis as the phagocytic activity endpoint. The changes in abundance of transcripts associated with lipid metabolism and peroxisome proliferation may be related to the lipidosis in the liver in fish from Sandy Creek and the increased abundance of lipid granules in barramundi collected from Repulse Creek in May. Previous studies have measured alterations in the abundance of transcripts involved in fatty acid metabolism in association with increased fatty deposits in the liver of fish exposed to endocrine disrupting compounds in the diet (Maradonna et al., 2015). There were also increases in the abundances of transcripts associated with oxygen transport. These may be related to a response to the epithelial cell proliferation present in the gill and as a consequence, a decrease in oxygen availability (Evans et al., 2005).

Fish collected in Sandy Creek had a higher TAG:ST ratio than fish collected from Repulse Creek at both time points, and lower levels of saturated fatty acids than fish from the reference collection period in Repulse Creek (March) . Previous studies have linked decreases in the ratio to lower habitat quality (Amara et al., 2007). However, exposure to peroxisome proliferating activating compounds may alter the normal lipid storage patterns (Maradonna et al., 2015). Exposure to PPAR agonists has also been shown to alter insulin sensitivity (Pavlikova et al., 2010). While PPAR was not consistently detected in these fish, other transcripts in this pathway, including ACOX-1, had increased abundance. Also, the livers in these fish had increased lipidosis, providing further evidence that normal lipid metabolism was altered.

Recent reviews have advocated using transcriptomic analysis and other physiological metrics to evaluate the health of wild fish populations for conservation efforts because of the transcriptome's rapid response to stressors and ability to provide causal information (Connon et al., 2018; Jeffrey et al., 2015). This study also suggests the utility of using the transcriptome to evaluate fish populations. As discussed above, if an adverse outcome pathway framework (e.g. Ankley et al., 2010; Villeneuve et al., 2014) is used to assess the potential for impact on populations, the transcriptome effectively acts as a "cellular response", linking the toxicant exposure and organ level responses (in this case altered tissue structure). These sorts of linkages facilitate the predictions of population level impacts (e.g. decreased resilience or increased frequency of disease). The consistency of the patterns observed in this study and our previous work (where fish were collected from rivers with different land use patterns and as a consequence, were exposed to a similar mixture of pesticides (Hook et al., 2017a,b)) provides further support for the use of transcriptomics to identify fish populations under pressure in conservation and management. One of the short comings of this study is that it used relatively few fish, and that they were of different sizes, likely reflecting different ages (Moore, 1979) and thus represent a heterogenous population. None the less, the transcriptomic patterns were consistent with our previous studies of fish exposed to similar stressors (Hook et al., 2017 a,b), and to fish exposed to these pesticides in the laboratory (Shelley et al., 2012), and consequently could be linked to changes in histology and lipids in an adverse outcome pathway type framework (Ankley et al., 2010; Villeneuve et al., 2014). This suggests that evaluation of the transcriptome could be a valuable tool to evaluate organism physiology and stress response, even in fish with heterogenous backgrounds.

5. Conclusions

When compared to fish from a low pesticide exposure reference site, fish from waterways with frequent pesticide exposure had an altered hepatic transcriptome, including increased abundance of transcripts with a role in xenobiotic metabolism and altered lipid metabolism. There were also differences in some of the lipid class metrics measured, and in the histological condition of the tissue in fish collected at the different sampling locations and time periods. These physiological changes, if persistent, would be expected to alter the performance of individual fish in the system and to decrease energy reserves, and as a consequence, their capacity to respond to other stressors. Some of the changes in the transcriptomic profile (e.g. the altered lipid metabolism and inflammation related transcripts) are reflected in the changes in histology and lipid metabolism, suggesting that the transcriptome can be used in a "weight of evidence" type approach to assess organism physiology.

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Highlights: 1. Fish from a pesticide hot spot were compared to those in fish from a national park.

2. Fish from the hotspot had alterations in histology, transcriptome and some lipid classes.

3. Fish from the second collection from the national park showed similar alterations.

4. The physiological alterations may coincide with pesticide exposure or health declines.

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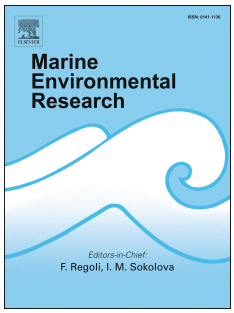

Transcriptomic, lipid, and histological profiles suggest changes in health in fish from a pesticide hot spot

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