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Hepatic transcriptomic profiles from barramundi, *Lates calcarifer,* as a means of assessing organism health and identifying stressors in rivers in northern Queensland

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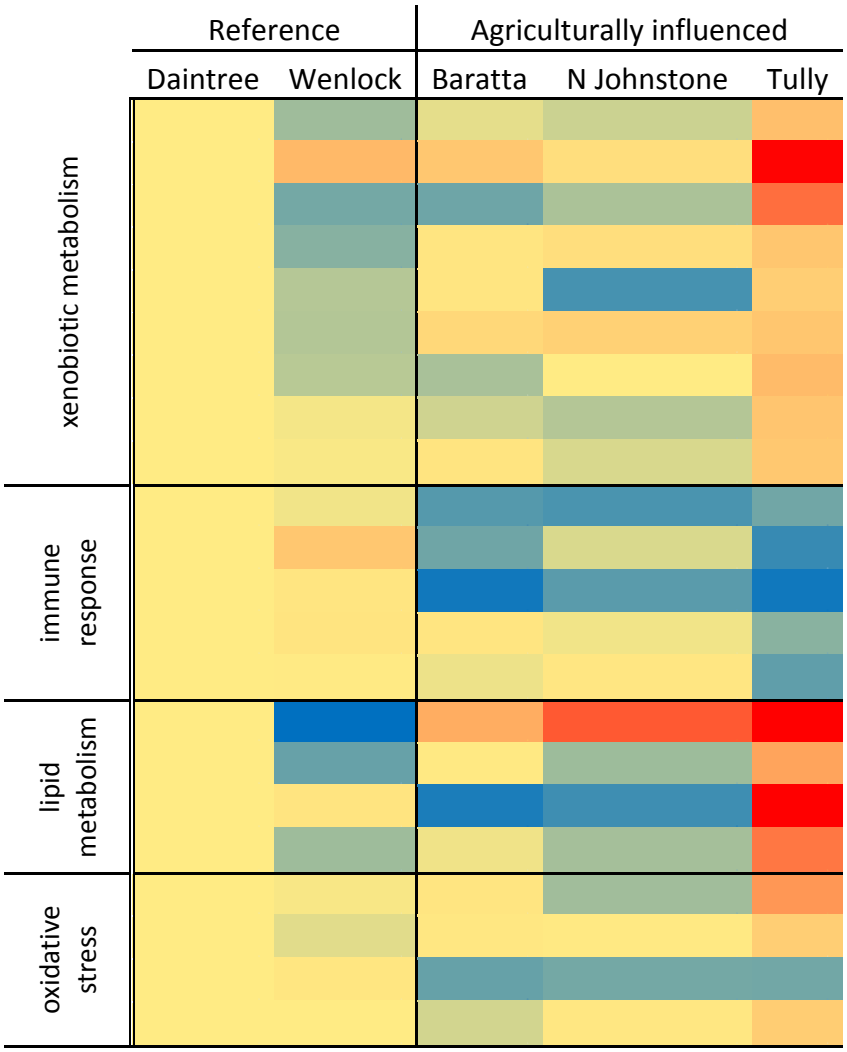
Abstract

Resource managers need to differentiate between sites with and without contaminants and those where contaminants cause impacts. Potentially, transcriptomes could be used to evaluate sites where contaminant-induced effects may occur, to identify causative stressors of effects and potential adverse outcomes. To test this hypothesis, the hepatic transcriptomes in Barramundi, a perciforme teleost fish, (*Lates calcarifer*) from two reference sites, two agriculturally impacted sites sampled during the dry season, and an impacted site sampled during the wet season were compared. The hepatic transcriptome was profiled using RNA-Seq. Multivariate analysis showed that transcriptomes were clustered based on site and by inference water quality, but not sampling time. The largest differences in transcriptomic profile were between reference sites and a site sampled during high run-off, showing that impacted sites can be identified via RNA-Seq. Transcripts with altered abundance were linked to xenobiotic metabolism, peroxisome proliferation and stress responses, indicating putative stressors with the potential for adverse outcomes in barramundi.

Graphical Abstract:



Highlights:

- Fish were collected from catchments with different agricultural land use patterns
- The hepatic transcriptome differed with different land use and water quality
- Transcripts for exposure biomarkers were used to tentatively identify stressors
- Functional pathways could contribute to predictions of adverse health outcomes

Keywords:

Transcriptomics; RNA Seq; Barramundi; Great Barrier Reef; pesticides; herbicides; agricultural contamination

1. Introduction:

Few aquatic ecosystems are truly pristine. Many face increased pressure from modifications of waterways, changes in land-use patterns as a consequence of coastal development, industrial and agricultural discharges, introduced species and global climate change (Dafforn et al., 2012). Determining what impact, if any, these stressors are having on the health of key species or on ecosystem function as a whole can be challenging for resource managers, who often lack the logisitic support to characterise every aspect of a system. Since many of the stressors in the system may interact, their behaviour in the field may not be easily predicted from laboratory-based studies. For example, the impact of stressors such as endocrine disrupting chemicals (EDCs) may not be apparent from traditional toxicity tests (Hook et al., 2014a). Resource managers need metrics to use, both to classify systems as "unimpacted" or "impacted" so that they can be prioritised for protection or remediation, as well as to identify the causative agents of any declines in either organism or ecosystem health.

Several metrics for classifying the health of ecosystems already exist. One of these is the index of biotic integrity (IBI), which measures the capacity of an area to sustain populations of organisms with comparable composition, diversity, and function to natural areas within the same system. These comparisons are done by comparing the composition and biomass of selected taxa from impacted and unimpacted sites (Hughes et al., 1998; Bilkovic et al., 2006). This approach has been effectively used in a variety of systems (e.g. Hughes et al., 1998; Mebane et al., 2003; Bilkovic et al., 2006; An et al., 2002). However, this approach has disadvantages in that it cannot convey causal relationships between anthropogenic or natural stressors and changes in biotic integrity (Hughes et al., 1998). Also, this metric must be developed independently for each system in which it is intended to be used, and intensive field sampling, as well as in-depth prior knowledge of ecosystem composition and function, are required (Hughes et al., 1998).

Individual biomarker-based responses in fish have been used to differentiate between impacted and unimpacted sites (reviewed in Hook et al., 2014a), for example vitellogenin (Jobling and Tyler, 2003; Kroon et al., 2015a; Sumpter and Jobling, 1995) or the condition of gonads (Blazer et al., 2014), both of which were used to infer exposure to EDCs and the potential for impacts on reproduction. Vitellogenin, cytochrome p450 1A, biliary Fluorescent Aromatic Compounds, DNA damage, and histology have also been used to indicate exposure to sewage borne contaminants near waste water outfalls in Orange County, CA, USA (Roy et al., 2003). However, choosing the appropriate biomarker-based assay requires knowledge of the causative agent of toxicity and in some cases a change at the molecular level may be difficult to link to population level outcomes (reviewed in Hook et al., 2014a).

DNA metabarcoding has also been used recently to classify systems as being more or less degraded (e.g. Chariton et al., 2010; Baird and Hajibabaei, 2012; Dafforn et al., 2014; Gibson et al., 2015). While this approach has the advantage of being high throughput and taking more of a "whole ecosystem" approach (Dafforn et al., 2014; Gibson et al., 2015; Chariton et al., 2015), it only reports on the presence or absence of organisms, nothing about their condition, so these approaches cannot act as "early warning" of impending species loss. Further, while there are approaches to correlate changes in species abundance with environmental parameters (Chariton et al., 2015), it can be difficult to determine causality as many these parameters co-vary, such as increased nutrient levels,

decreased oxygen levels and increased micropollutants (as per Luo et al., 2014) that would all be expected to be found near sewage treatment plants (Dafforn et al., 2014).

Here, we hypothesise that the transcriptome of a selected species could be used to not only classify ecological sites as comparatively pristine or impacted, but also to identify potential causative agents of changes in organism physiology. Since all transcripts are profiled, this approach does not require a priori knowledge of the stressor or its modes of action. Since there is a vast amount of literature on gene expression and ecotoxicology (Hook, 2010; Bahamonde et al., 2016;), transcripts that are indicative of exposure to specific contaminants or classes of contaminants, such as those that correspond to established exposure biomarkers (Hook et al., 2014a), could be identified. Also, changes in the transcriptome could be used to identify potential population level impacts, via the adverse outcome pathway concept (Ankley et al., 2010). A transcriptomic approach has been used successfully in prior studies. For example, caged fathead minnow have been used to profile differences in microarray based gene expression profiles due to agricultural land use patterns in Nebraska, USA, watersheds (Jeffries et al., 2012). Multivariate models of microarray--based gene expression fingerprints have also been used to predict the causative agent of toxicity in flounder collected from contaminated sites in the United Kingdom (Falciani et al., 2008). In addition, qPCR based transcriptional profiles have been used to predict the health outcomes of steelhead Oncorhynchus mykiss from the Columbia River basin (Connon et al., 2012). However, both of these approaches require prior knowledge of the genome sequence of the organism being studied, which is frequently not available for non model organisms, especially in Australia. Instead, we used RNA Seq to characterise the transcriptome (reviewed in Mehinto et al., 2012). RNA Seq, where transcripts are reverse transcribed into cDNA libraries and quantified directly, has the advantage of being able to be performed on organisms with an uncharacterised genome (Mehinto et al., 2012; Hook et al., 2014b) and has a greater dynamic range than either qPCR or microarray hybridisation (Tarazona et al., 2011).

To test the hypothesis that the transcriptome would indicate the presence of stressors in an organism's habitat, the hepatic transcriptome of fish collected from different rivers in tropical North Queensland was examined. Most of the study sites discharge into the Great Barrier Reef (GBR). Some rivers in the system, including those included in this study, have poor water quality principally due to increased loads of nutrients, sediment, and pesticides arising from agricultural land use (reviewed in Brodie et al., 2012; Kroon et al., 2012; Smith et al., 2012). Rainfall in this area is typically very seasonal, with frequent, heavy rain in November through March, and much lower amounts the rest of the year. Concentrations of pesticides and other water quality related stressors, such as sediments and high nutrient load, are typically highest during the rainy season (Smith et al., 2012; Davis et al., 2012 Furthermore, aqueous concentrations of some stressors, such as diuron, atrazine, terbuthiuron and metolachlor, are frequently measured above water quality guidelines (Lewis et al., 2012; Smith et al., 2012). Concentrations of water soluble pesticides, such as atrazine and imidacloprid, are typically below detection during the dry months. However, it is uncertain whether elevated concentrations of stressors are sufficiently high to cause changes in the physiology of fish species (Kroon et al., 2015a). In addition to changes in water quality, these watersheds have been modified by changes in land use patterns, (e.g. deforestation, altered biogeochemical cycles), which could have impacts on aquatic food webs, and as a consequence, fish health (Jeffrey et al., 2015). We postulated that changes in the transcriptome that were a result of declining water quality would be most apparent in fish collected from agriculturally impacted watersheds during the wet

season, whereas those that resulted from persistent contaminants or habitat changes would be apparent in all seasons.

In this paper, three hypotheses were tested: 1) that the hepatic transcriptome would differ between impacted and reference sites; 2) that the changes in the transcriptome would indicate seasonal stressor exposure; and 3) that changes in the transcriptome would contain transcripts for known contaminant exposure biomarkers (e.g. Hook et al., 2014a) that could be used to help identify putative causative stressors and other transcripts to could be related to physiological changes that might link to potential adverse outcomes. The study extended previous work examining changes in vitellogenin and aromatase levels in field-collected fish (Kroon et al., 2015a) and an initial survey of differences in global transcriptomic patterns at comparatively impacted (the Tully River) and a reference site (the Daintree River) (Hook et al., 2017). The previous study found large differences in the transcriptomic patterns, but we could not be certain that these were not just variability between different sites. We also could not be certain that the changes we measured in the previous study were related to water quality and not habitat modification. To address some of these uncertainties, this study examined an additional reference site but in this study two additional sites were examined during the dry season when the contaminant concentrations were expected to be low based on previous monitoring studies (Lewis et al., 2009) and the same two sites previously used by Hook et al. (2017). We used known exposure biomarkers in the transcriptomes and functional pathways with altered transcriptome abundance to identify sites and potential causative agents.

2. Materials and methods:

2.1 Study organism

Barramundi *(Lates calcarifer)* (Bloch) (Family Latidae), a perciforme teleost fish, is of cultural importance to Australia's aboriginal peoples, and is fished extensively recreationally and commercially (Kailola et al., 1993). In northern Australia, barramundi lives in river and estuarine habitats. In northeast Queensland, they spawn near the mouths of estuaries prior to and during the start of the wet season, between November and January. Larval barramundi are found in coastal wetlands after December, with some migrating further upstream into freshwater habitats between March and June. The species is protandrous hermaphrotide, with juveniles maturing as males >250 mm total length (TL) in the first 2–4 years, and changing into females in the 680–900 mm TL size range.

2.2 Study site description

The five catchments from which barramundi were collected are located in North Queensland, Australia (Figure 1). These catchments differ in surface area, land uses, mean annual flow and river loads of sediment, nutrients and pesticides (Table 1). In the catchments of the two reference rivers agricultural land use is either completely absent (Wenlock) or covers a relatively small area (7%, Daintree). In contrast, agricultural land uses cover relatively larger areas in the Tully, North Johnstone and Barratta catchments, with horticulture crops (primarily bananas) and sugarcane being cultivated on the coastal floodplains. Conservation land use occupies large areas of the upper

catchments in the Daintree, North Johnstone and Tully Rivers. All rivers are perennial, with most of the discharge occurring during the summer wet season (December – April). River pollutant loads of sediment and nutrients to the GBR lagoon have increased substantially since European settlement in the 1850s (Kroon et al., 2012; Waters et al., 2016), with increases particularly evident in the North Johnstone, Tully and Barratta. In addition, over 50 pesticides have been detected in these three rivers, with substantial loads of pesticides being transported to the GBR lagoon every year (Waters et al., 2016).

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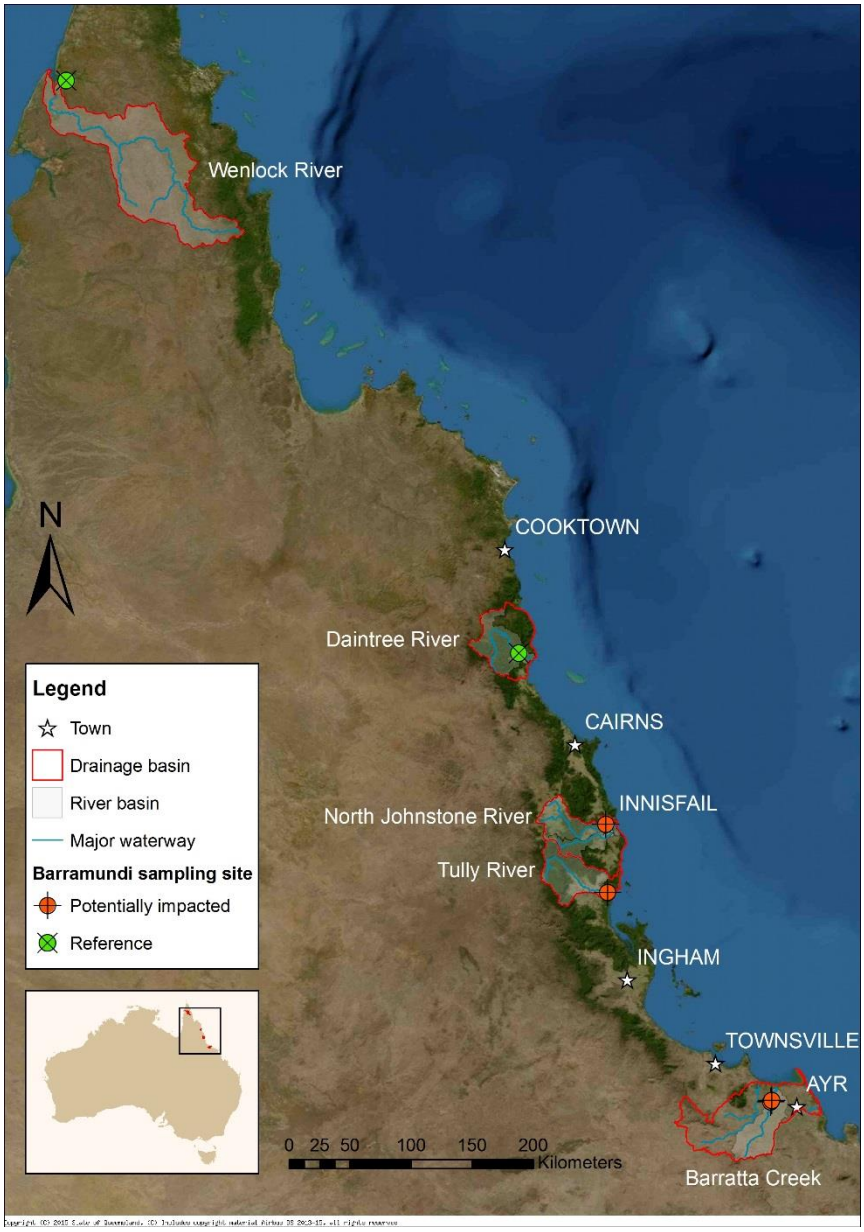

178

179 Figure 1. Barramundi collection sites. Drainage basins are outlined in red.

180 Green spots indicate reference sites, whereas red spots indicate potentially impacted sites.

Table 1. Catchment information for collection sites of barramundi (*Lates calcarifer*). Catchment surface area, mean annual discharge, main land uses, and river pollutant loads are given. TSS is total suspended sediment, TN is total nitrogen, TP is total phosphorus, and PSII herbicides is photosystem II herbicides. PSII herbicide load is expressed as the annual toxicity-based herbicide loads (diuron equivalent loads, Escher et al., 2008). Land use data was taken from Kroon et al., (2015); river pollutant loads from Waters et al. (2014).

River	Catchment surface area (km^2)	Main land uses (% of total catchment area)					Long-term mean annual discharge (GL)	River pollutant loads			
		Conser-vation	Forestry	Grazing	Horti-culture	Sugar-cane		TSS ($t\ km^{-2}$)	TN ($kg\ km^{-2}$)	TP ($kg\ km^{-2}$)	PSII herbicides ($kg\ y^{--2}$)
Wenlock~	7,525	64	<1	33	<1	<1	3,200[d]	Not measured	Not measured	Not measured	Not measured
Daintree	2,107	67	23	10	<1	2	925~	29	642	45	Not measured
North Johnstone	2,325	53	<1	40	<1	1	1,800	150	1,100	290	79
Tully	1,683	72	2	5	<1	13	3,100	67	1,100	100	270
Barratta (Haughton)	4,051	0	<1	73	<1	25	160	10	240	25	38

~ based on long term monitoring from 108002A Daintree River at Bairds, note this will be an underestimate of total discharge from the basin

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2.3 Fish collections

Barramundi were collected using monofilament gill nets (50 mm stretched mesh) in 2011 or 2012 via purse seining, where fish were captured and immediately transferred to a water bath containing anaesthetic (Kroon et al., 2015a) with the approval of the CSIRO Sustainable Ecosystems animal ethics committee (permit #13-12) (Table 2). All fish collected were immature or mature males, and smaller than the smallest length at which female gonadal features are first recognizable (680 mm TL; Kailola et al., 1993), Fish were anaesthetised with clove oil (Kroon, 2015), then sacrificed by gill slitting and cervical dislocation. Fish were immediately measured (mm, total length, TL), weighed (g), then liver tissues were collected and small samples preserved in *RNA later*© (Ambion). Samples were kept on ice until delivery to the laboratory then stored at -20°C until further processing.

Table 2. Collection information for barramundi (*Lates calcarifer*).

Collections		Classification				Fish	
Rivers	Dates	Status	Season	Number of fish	Size range (mm, TL)	K[1] (x 10^{-3})	Sample ID
Wenlock	23/04/2012	Reference	Changing towards dry	5	357–492	1.1–1.3	WC2–4; 55, 56
Daintree	22/03/2011	Reference	Wet	6	295–460	1.0–1.2	2–7
North Johnstone	27/06/2011	Impacted	Dry	5	192–350	1.0–1.2	402–406, 408
Tully	23/03/2012	Impacted	Wet	8	264–445	1.1–1.2	97–104
Barratta	25/05/2011	Impacted	Dry	6	240–421	1.0–1.2	353–355, 358–360

[1.] Fulton's condition index (K) was calculated as per Froese et al. (2006).

2.4 RNA extraction

The workflow used during the RNA-Seq experiment is described in Hook et al (2017) (Fig. S1). RNA was extracted as described previously (Kroon et al., 2014; Kroon et al., 2015a;). Briefly, approximately 20 mg of liver tissue was immersed in TRIzol© (Invitrogen) reagent, homogenized using MP Biomedical© bead beater and lysing matrix E tubes, then extracted following the TRIzol© protocol through the removal of the aqueous phase. The RNA was subsequently purified using the Ambion Purelink kit After extraction, the TURBO DNA free© (Ambion) kit was used to eliminate genomic DNA contamination. RNA purity was determined using a nanodrop spectrophotometer (260/280 ratio greater than 2.0) and integrity determined using an Agilent bioanalyzer© (RIN greater than 7.0).

2.5 Sequencing

Libraries for sequencing were created from hepatic RNA from each fish in Table 2 as an individual (samples were not pooled). Sequencing for this project was performed at the Australian Genome Research Facility. Briefly, cDNA synthesis and library preparation was carried out using a starting template concentration of 1 µg RNA. Illumina's TruSeq stranded mRNA sample preparation was used and the manufacturer's protocol was followed. The samples were run on an Illumina HiSeq 2000 with 100 base pair reads. The sample reads were deposited in the CSIRO data access portal and at NCBI at PRJNA352735 (SRP093513). Data are also available via the CSIRO data access portal at http://doi.org/10.4225/08/583766535a240.

2.6 Quality Assessment and Read Mapping

The Trimmomatic algorithm (Bolger et al., 2014) on the CSIRO instance of Galaxy (Goecks et al., 2010) was used to filter reads for quality (Figure S1). Sequences were trimmed on a sliding window, with a window size of 20 bases, a minimum quality score of 20 (Blankenberg et al., 2010) and the number of bases to exclude set to 1. Read mapping against the whole barramundi transcriptome derived in Hook et al. (submitted) (PRJNA290400) was performed using Bowtie (Langmead et al., 2009) and quantified using the RSEM abundance estimation algorithm (Li and Dewey, 2011) with the aid of scripts provided with Trinity (Grabherr et al., 2011; Haas et al., 2013); (r2014_04_13). The numbers of total reads and mapped reads for each individual fish are in Table S1.

2.7 Differential Expression Analysis

Data (from both this and the previous study, Hook et al., 2017) were loaded into CLC Genomics workbench version 8.0.3 for differential expression analysis. The previously published data were included to expand the number of sites surveyed, and to ensure that more than one reference site was examined. The previous data had a greater sequencing depth (22–31 M reads per sample) than the current study did (12–14 M reads per sample). To account for differences in overall number of reads per sample, FPKM (fragments per kilobase of transcript per million mapped reads) normalised values were used in all subsequent analysis. Transcripts that did not have an average FPKM value of one across all treatments were discarded to eliminate problems with false positives associated with low read abundances (Tarazona et al., 2011). A total of 18,031 transcripts were included in the differential abundance analysis. Approximately 95% of mapped reads (or 137.4 million reads overall) mapped to these 18,031 contigs. Differential expression was calculated using Gaussian statistics, with an FDR rate of 0.05.

2.8 Annotation

In addition to annotating with BLAST2GO, as described previously (Hook et al., 2017), the differentially abundant transcript lists were annotated by BLAST against the *Oreochromis niloticus,* another perciforme fish, Ref Seq transcriptome, downloaded from NCBI on March 2, 2016 with an e value cut off of $1e^{-5}$. Both annotations are included in lists of differentially expressed genes for completeness. Differentially abundant contigs (putative transcripts assembled from sequencing reads) were also BLASTx searched with an evalue cut off of $1e^{-5}$ against the UniProtKB/Swiss-prot database using the CSIRO instance of Galaxy, their closest orthologue (or best match) identified, and submitted to DAVID against a background of all contigs with the minimum read count subjected to the same search criteria (Huang et al., 2009) for further functional annotation. Functional pathways were also annotated by analysis against KEGG pathways in BLAST2GO (Conesa et al., 2005).

3. Results

3.1 Sequencing results

The Illumina 2500 HiSeq run generated 241 million reads. Once the reads were trimmed to remove the first base pairs that are non-random due to primer bias, virtually all reads were of sufficient quality to be used in mapping (Table S1). The number of reads per sample was lower in this study (12–14 million) than in our previous study (25–31 million) because the number of samples multiplexed per sequencing lane was higher in this study (16) than in the previous (14). The percentage that mapped in this study (53–64%), with the exception of one sample, was higher than the previous study (40–48%), despite the fact that the same Perl script and mapping algorithm were used. To account for these differences in read number and percent mapping, FPKM-corrected values were used in all subsequent analyses. Since large fold changes in low abundance transcripts are often artificial, only contigs with an average FPKM of one in at least one condition were considered. A total of 18,031 transcripts met this condition.

3.2 Transcriptomic profiling

The reads were mapped onto a previously developed transcriptome (Hook et al., submitted) which is available via the CSIRO DAP available via the CSIRO data access portal (http://doi.org/10.4225/08/583b5570b27e5). Briefly, that transcriptome contains 102,061 contigs. 27891 ORFs could be extracted from these contigs, representing coding sequences. Of these transcripts, 27,034 (96.9%) have an orthologue in the BLAST database. The read mapping revealed differences in the transcriptomic profiles between different fishes (Figure 2). There are large differences in the hepatic transcriptomes of fish collected from the Tully River relative to fish collected from other sites. These trends are also apparent if the data are visualised using hierarchical clustering (Figure 3) instead of PCA. If data are input based on the size of the fish collected or the date the fish was collected, no clear clusters are apparent (Figures S2 and S3).

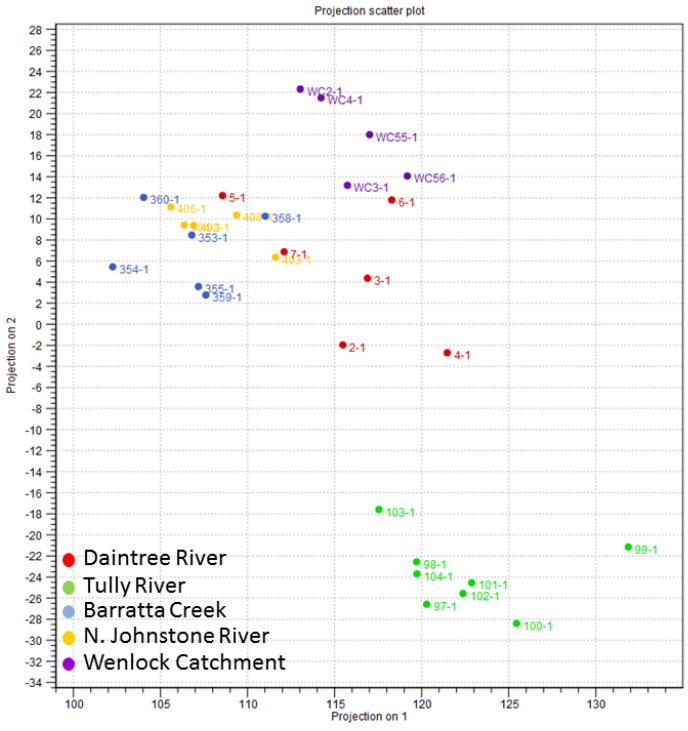


Figure 2. Principal components based visualisation of the plus one, log 10-transformed hepatic transcriptome based on site where the fish was collected. Each dot represents an individual fish.

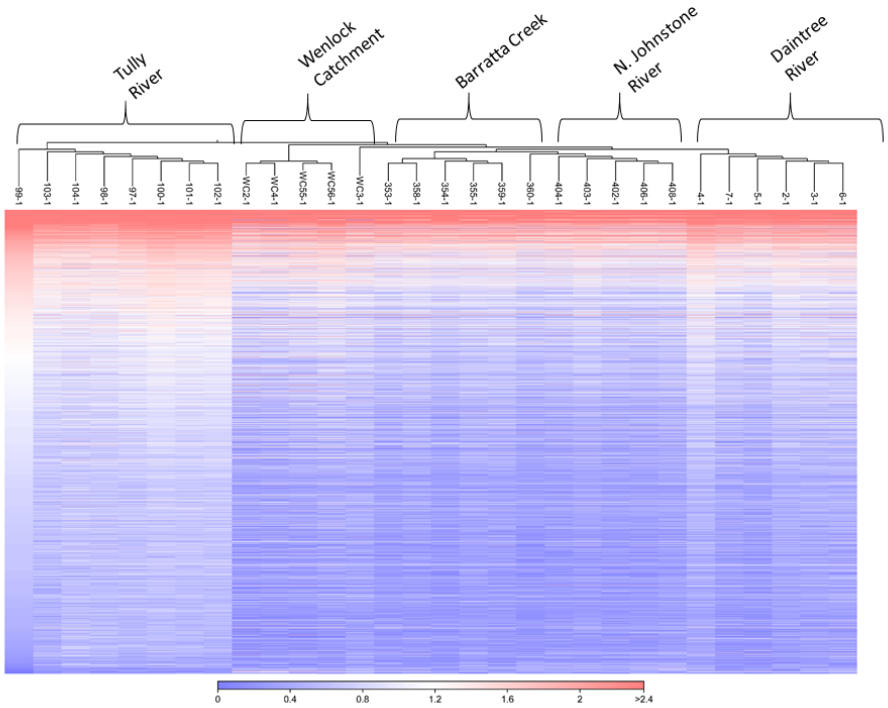


Figure 3. Hierarchical clustering algorithm showing relationships between the hepatic transcriptome of barramundi collected from different sites. Only transcripts with significantly different abundances relative to both reference sites are plotted. The colour bar indicates +1 log transformed FPKM read count.

3.3 Differentially Abundant Transcripts

There were more differentially abundant transcripts in the transcriptome of barramundi collected from the Tully River compared to either Barratta Creek or the North Johnstone River (Table 4). These transcripts, their closest orthologue as determined via BLAST against the *Oreochromis niloticus* Ref Seq transcriptome, their mean abundances (as FPKM) in fish from each river, are presented in Table S2. The Venn diagram shows little overlap amongst the transcripts differentially abundant in each river (Figure S4).

Table 4. Matrixes showing the number of transcripts with significantly different abundances for each comparison, relative to a total number of 18,031 transcripts.

By site:

Reference sites	Impacted sites			Reference Sites	
	Tully River	North Johnstone River	Barratta Creek	Daintree River	Wenlock River
Daintree River	4974	1846	817	-	84
Wenlock River	6964	4037	3591	88	-
Both	2610	303	861	-	-

Differences in the transcriptome in fish of different size were difficult to discern, as the main differences were between fish captured at different sites (Figure S2). When the transcriptome was compared by date collected, the patterns were compounded by site (Figure S3).

3.4 Functional Annotation of Differentially Abundant Transcripts

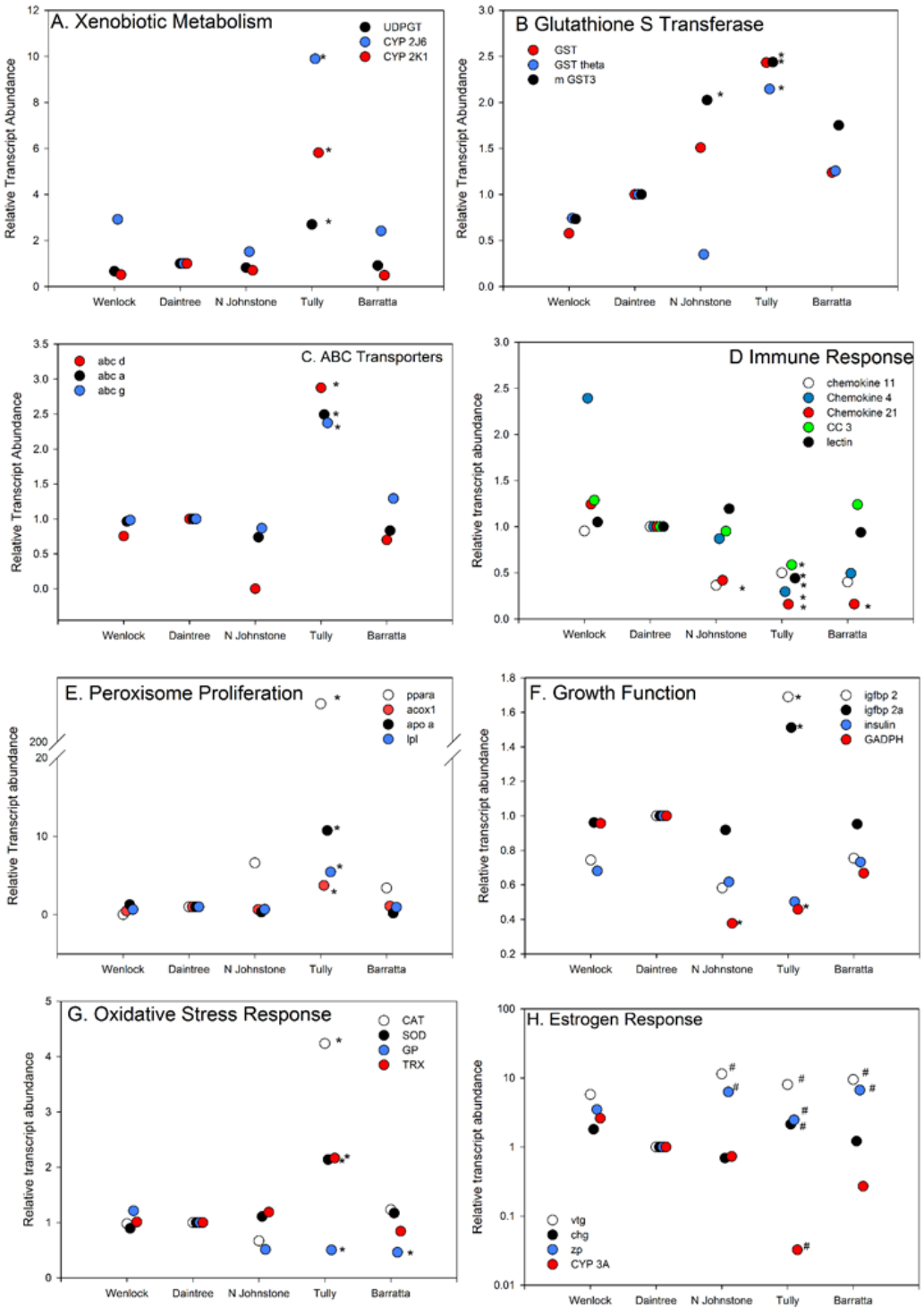
Transcripts for the pathway of excretion of xenobiotic compounds, which are established biomarkers for exposure to organic contaminants, were elevated in fish from the agriculturally impacted North Johnstone, Tully, and Barratta relative to the Daintree and Wenlock rivers (Figures 4A –C). Transcripts encoding pesticide and nicotine-metabolising enzymes (cytochrome p450 2J,(CYP2J) a Phase I oxidoreductase, and multiple isoforms of glutathione S transferase (GST) and UDPGT (uridine diphosphate glucuronosyltransferase), conjugating enzymes involved in phase II metabolism), are all significantly more abundant in fish from the Tully River relative to all other rivers (Figures 4 A and B respectively). Transcripts encoding ATP binding cassettes (ABC transporters), which excrete xenobiotic compounds from cells, were also more abundant in the hepatic transcriptomes of fish from the Tully River (Figure 4C).

As reported previously (Hook et al., 2017), barramundi transcripts encoding gene products with an immune related function were less abundant in fish from the agriculturally intensive rivers, the North Johnstone, Tully and Barratta relative to the Daintree and Wenlock rivers (Figure 4D). Chemokines, chemokine receptors, granzyme and t cell receptors encoding transcripts are all less abundant in fish collected from the North Johnstone, Tully and Barratta compared to the Daintree and Wenlock (Figure 4D and Table S2). A large number of transcripts encoding enzymes involved in the metabolism of energy stores (lipid, protein or carbohydrate) were elevated in the livers of fish collected from the Tully River, as reported previously (Hook et al., 2017), but not from the North Johnstone and Barratta (Figure 4E and Table S2). These transcripts encoded peroxisome proliferator

activated receptor (PPAR) alpha, tyrosine aminotransferase, long-chain fatty acid ligase (lpl), and apolipoproteins (apo - lipid binding proteins). Growth function genes, such as insulin-like growth factor binding factor protein (igfbp), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), had altered abundance in the Tully River when compared to the other rivers (Figure 4F). Transcripts encoding leptin, the saiety hormone, was less abundant in the Tully River compared to other sites (Table S2). Oxidative stress related transcripts, catalase (CAT), heme oxygenase (HO) and superoxide dismutase (SOD), were also measured in increased abundances in the transcriptomes of fish collected from the Tully River only (Figure 4G).

Transcripts encoding estrogen responsive gene products such as *vtg* and the zona pellucida (zp) proteins were elevated in all rivers relative to the Daintree River (Figure 4H) , although not to the Wenlock Catchment – the other reference site. Transcripts involved oogenesis including vitellogenin were elevated in the North Johnstone, Tully and Barratta, while transcripts encoding the egg membrane proteins were elevated in the Tully and Barratta. Cytochrome p450 3A (CYP3A) transcripts were less abundant in fish from the Tully and Barratta compared to the Daintree and Wenlock.

The majority of transcripts with differential abundance between the two reference sites could not be annotated, making their functional significance difficult to discern. However, several ATPases were approximately 5 fold more abundant in the transcriptomes of barramundi collected from the Wenlock catchment relative to the Daintree River. Not enough of the transcripts with differential abundance between reference sites could be sufficiently annotated to allow for mapping to functional pathways.



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343 Figure 4. Mean transcript abundance for fish collected from each river, relative to the mean
344 abundance in the hepatic transcriptome of fish from the Daintree River (chosen because of the
345 larger samples size) of selected transcripts in different functional categories. Panel A: xenobiotic
346 metabolising enzymes; B: s glutathione S transferases C: ABC transporters; D: immune function
347 transcripts; E the peroxisome proliferator activated receptor alpha pathway; F growth function
348 transcripts; G: transcripts for oxidative stress response genes and panel H shows estrogen
349 responsive transcripts* indicate statistical significance FDR corrected $p<0.05$ from both the Daintree

and Wenlock values, # indicates statistical significance from the Daintree fish only. Standard deviations are not shown for clarity. Data are slightly offset so that all symbols are visible.

3.5 Alignment of Differentially Expressed Transcripts to Functional Pathways

Because comparatively few contigs had an orthologue in the UniProt_Swiss Prot database, as identified via a BLASTx search, the DAVID functional annotation platform (Huang et al., 2009) identified very few functional categories that were significantly enriched amongst the differentially abundant transcripts (Table 5, Table S3). Transcripts involved in protein tagging (phosphoproteins) and alternative splicing, as well as hydrolases, were most significantly enriched (lowest p value , Benjamani Hochberg multiple test correction) in fish from the Tully River (Table S3). Transcripts involved in disease, lipid metabolism and oxido-reductase activity were also significantly different (p value <0.05 , Benjamani Hochberg multiple test correction) (Table 5). Immune-related transcripts, including chemokines and cytokines, had decreased abundance in the transcriptomes of fish collected from the Tully River (Table 5 and S3). No functional groups were significantly enriched amongst those transcripts with increased abundance in the North Johnstone River or Barratta Creek, and only cell adhesion was significantly enriched among the transcripts that were less abundant in fish from the North Johnstone River (Table 5 and S3). In Barratta Creek, Pleckstrin homology (cell signalling) and GTPase activity were the only functional categories significantly less abundant in the transcriptome at that site (Table 5 and S3).

Table 5. A summary of the results of DAVID and KEGG pathways functional mapping. Full results (including counts and p values) are given in Tables S3 and S4.

River	Relative abundance compared to the reference sites	Pathway	Evidence from
Tully	More abundant	Hydrolase, transferase, oxidoreductase, lipid metabolism	DAVID
		Lipid metabolism, glycolysis, glutathione metabolism, amino acid metabolism, Metabolism of xenobiotics by cytochrome P450, Steroid hormone biosynthesis	KEGG

	Less abundant	Chemokines (immune related transcripts)	DAVID
		glycolysis, amino acid metabolism	KEGG
N Johnstone	More abundant	N.A.	
	Less abundant	Cell adhesion	DAVID
Barratta Creek	More abundant	N.A.	
	Less abundant	GTPases, actin binding	DAVID
		Glycolysis / gluconeogenesis, cell signalling pathways	KEGG

N.A., not applicable, no significantly enriched functional categories were identified.

As with the DAVID analysis, comparatively few of the differentially abundant contigs could be assigned an enzyme code, which is required for mapping to KEGG pathways. Nevertheless, a few trends were apparent from this analysis, as summarised in Table 5. More transcripts that were differentially abundant in the hepatic transcriptomes of Tully River fish could be mapped than the other impacted or reference collection sites. As discussed previously (Hook et al., 2017), transcripts that were more abundant in the hepatic transcriptomes of fish collected from the Tully River could be mapped to a variety of pathways, including fatty acid degradation, glycolysis, metabolism of xenobiotics via cytochrome p450, and steroid hormone biosynthesis (Table 5 and S4). Some of the transcripts that were less abundant in the hepatic transcriptome of fish from the Tully could be mapped to oxidative phosphorylation, the metabolism of different amino acids, and glycolysis (Table 5 and S4). By comparison, far fewer KEGG pathways were identified in the transcripts from the other impacted rivers. Few transcripts with increased abundance that could be mapped to KEGG pathways were observed in the hepatic transcriptomes from fish from North Johnstone River and Barratta Creek. KEGG pathways for the metabolism of simple sugars such as galactose, starch and pentose were less abundant in fish from the North Johnstone, whereas fish from Barratta Creek had fewer transcripts involved in oxidative phosphorylation, T cell receptor signalling and the oxidation of simple sugars (Table 5 and S4).

4. **Discussion**

In this study, we used the hepatic transcriptomic profiles of barramundi from rivers with different land use patterns collected during the dry (low run off) and wet (high run off) seasons both to (i) differentiate between impacted and reference sites; (ii) determine the extent to which the transcriptome reflected seasonal changes in water quality; and (iii) identify potential stressors in these systems using transcripts encoding known exposure biomarkers. Overall, the largest

differences in the global hepatic transcriptome were in barramundi from the Tully River collected during the wet season, demonstrating that the transcriptome can be used to identify impacted sites, supporting the first hypothesis. Compared to the Daintree catchment (a reference site), where barramundi were also collected during the wet season, the Tully (an impacted site) contains more sugarcane and horticulture upstream of the collection site than the Daintree collection site (Waters et al., 2016). Barramundi collected from the Tully River during the wet season had more differences in their transcriptomes relative to the two reference catchments and to those collected from the two other agriculturally impacted catchments (i.e., North Johnstone and Barratta) during periods of low run off, when pollutant levels are lower, supporting the second hypothesis. Few differences in the transcriptome could be attributed to wet versus dry season overall (e.g. pooling both reference and impacted areas) or attributed to different sequencing runs.

4.1 Transcripts for xenobiotic metabolising enzymes

Xenobiotic metabolising enzymes are commonly used, well established exposure biomarkers, and increases in their transcription levels can be used to surmise exposure to pharmacologically relevant doses of their substrate (Schlenk et al., 2008, Hook et al., 2014). The presence of these exposure biomarkers in the transcriptome can assist with putative stressor identification and thus supports the third hypothesis. Xenobiotics are often metabolised in a three phase pathway (Livingstone, 1991; Schlenk et al., 2008). The first phase occurs via oxidation, typically performed by a member of the cytochrome p450 family (Schlenk et al., 2008). Transcripts encoding CYP 2j and k transcripts of enzymes that metabolise pesticides in fish (Haasch et al., 1998; Lemaire et al., 2004) and nicotine in mammals (Hukkanen et al., 2005) were elevated in the hepatic transcriptomes of fish from all the agriculturally impacted sites compared to the reference sites – but only significantly ($p \leq 0.05$) in the fish from the Tully River. The second phase of metabolism is glucorondiation, which is often performed by a member of the GST family (Schlenk et al., 2008). Different transcript isoforms of GST were significantly ($p \leq 0.05$) more abundant in fish collected from the Tully River than the reference sites, although some contigs were elevated in fish from all agriculturally impacted rivers, which suggests exposure to lower levels of pesticides year round, consistent with the known patterns of contamination (e.g. Lewis et al., 2009) or another oxidative stressor. Exposure to atrazine is known to induce GST in fish (Egaas et al., 1993; Wiegand et al., 2000). Transcripts of UDPGT are also more abundant in fish from the Tully River, and like CYP2 proteins they are also involved in the metabolism of nicotine (Hukkanen et al., 2005) and potentially neonictinoid insecticides such as imidacloprid. The third phase of metabolism is excretion from the cell, which is typically carried out by an ABC transmembrane protein (Schlenk et al., 2008). Several different members of the ABC transporters, which excrete organic contaminants from cells (Leslie et al., 2005) had increased abundance in fish from the Tully River (Figure 4C). Collectively, transcripts of fish from the impacted sites indicate exposure to organic contaminants, most likely to commercial pesticide formulations that include both active ingredients and additives such as solvents, surfactants, or preservatives, as there are no other known sources of organic contaminants in the area (Cox and Surgan, 2006; Kroon et al., 2015a, b). Other xenobiotic metabolising enzymes were also induced in fish from the impacted sites. The elevations in these transcripts in the Tully River barramundi were discussed in our previous study (Hook et al., 2017).

4.2 Estrogen-responsive transcripts

Estrogen-responsive transcripts, such as zona pellucida and vitellogenin, were amongst the most differentially abundant transcripts (Table S2). Normally, in female fish approaching spawning, circulating levels of estrogen increase, setting off a cascade of events, including stimulating the liver to start synthesising proteins required for oogenesis (reviewed in Swanson et al., 2003; Villeneuve et al., 2007). These transcripts can also become more abundant following exposure to xenoestrogens, including in juveniles and males (Jobling and Tyler, 2003). Transcripts encoding vitellogenin and the egg shell proteins were significantly elevated in the male fish from the agricultural catchments relative to male fish from the Daintree (Figure 4H). A common commercial herbicide formulation used in the area contains surfactants such as alkylphenol ethoxylates (Kroon et al., 2015b). Akylphenol ethoxylates are weakly estrogenic, and have been shown to cause elevations in the abundances of transcripts encoding *vtg* and egg yolk proteins in both lab and field studies (Meucci and Arukwe, 2005; Li et al., 2012; Schlenk et al., 2012). This includes controlled laboratory studies on juvenile barramundi, during which similar, modest changes in transcript abundance were observed following exposure to both the "surfactant booster", and the combination of herbicides and the "surfactant booster" (Kroon et al., 2015b). In addition to an aqueous pathway, alkylphenol ethoxylates have been shown to be persistent in sediments (Ferguson et al., 2003; Ferguson and Brownawell, 2003; Ferguson et al., 2001) so fish could be exposed during foraging or feeding during the dry season. The increased abundance of these transcripts in livers of male fish suggests exposure to a xenoestrogen such as alkylphenol ethoxylates and thus supports the third hypothesis.

4.3 Immune responsive transcripts

Decreased abundance of some immune related transcripts were measured in all of the impacted rivers. Decreased abundances of immune related transcripts may indicate toxicant induced immunomodulation (reviewed in Segner et al., 2012). Previous studies have also noted changes in the abundance of immune related transcripts following contaminant exposure. For instance, following exposure to crude oil, decreased abundances of immune related transcripts was noted in Japanese flounder (Nakayama et al., 2008), and similar changes in the transcriptome were also measured in the head kidney of olive flounder exposed to benzo[α]pyrene (Hur et al., 2008) and in rainbow trout exposed to xenoestrogens (Hook et al., 2008). Decreased levels of immune related transcripts may also be a generalised stress response (Aluru and Vijayan, 2009). Without histological examination, it can not be determined whether these changes indicate increased incidence of disease.

4.4 Transcripts with a metabolic function

Some of the other changes in the fish transcriptome at agriculturally impacted sites suggested the potential for adverse outcomes on both individuals and populations, also supporting the third hypothesis. Changes in metabolism can be indicative of increased energy expenditure via stress. Stressed animals would be expected to have increased utilisation of energy stores (lipid and protein), as well as increased levels of gluconeogenesis (Philip et al., 2012). Increases in transcript abundance of genes involved in lipid and amino acid metabolism, which is an expected stress response in fish (Aluru and Vijayan, 2009), were measured only in the Tully River (Table 5, tables S3 and S4). A previous study has noted an increase in protein metabolism in fish exposed to atrazine (McCarthy and Fuiman, 2008), which is frequently detected in the Tully River (Smith et al., 2012). Other studies

have noted changes in osmoregulatory capacity and as a consequence decreased feeding in fish exposed to atrazine (Nieves-Puigdoller et al., 2007). Whether the metabolic changes noted are directly attributable to exposure to stressors or a consequence of changed feeding behaviour is not known.

Changes in lipid metabolism and energy utilisation can also be indicative of a metabolic disorder. PPARs regulate lipid and carbohydrate metabolism (Urbatzka et al., 2015), so changes in PPARs are considered a form of metabolic endocrine disruption (Casals-Casas et al., 2008). Activation of PPAR alpha has been shown to alter insulin resistance and alter patterns of lipid storage (Pavlikova et al., 2010). Notably, these included an increase in the abundance of PPAR and acyl-coenzyme oxidase (Pavlikova et al., 2010). Changes in PPARs have been noted following exposure to a variety of anthropogenic contaminants, including organotins (Pavlikova et al., 2010), oil (Bilbao et al., 2010), and nonylphenol (Cocci et al., 2013). A large increase (greater than 200 fold, table S2, figure 4E) in the abundance of the transcript for PPARα was measured in fish from the Tully River, as well as an increased abundance of transcripts for acyl-coenzyme a oxidase, fatty acid synthase, and for several lipases. All of these transcripts have been induced in a previous study following exposure to environmental contaminants and implicated in metabolic disorders such as steatosis disease (Maradonna et al., 2015). Changes in transcript abundance for insulin-like growth factor-binding protein and GADPH were also measured in fish from the Tully River. Previous studies have hypothesised these proteins have a negative effect on gametogenesis, heart rate, and energy supply for swimming (Urbatzka et al., 2015).

Both generalised stress and induction of the peroxisome proliferator pathway are thought to have deleterious consequences for the immune system (reviewed in Aluru and Vijayan, 2009, DeWitt et al., 2009). For example, exposure to Perfluorooctanoic acid and Perfluorooctane sulfonate, two well-known peroxisome proliferating compounds, altered transcription of immune related genes in cultured hepatocytes of the minnow (*Gobiocypris rarus*) (Wei et al., 2009). Cortisol was shown to modulate the expression of cytokines and alter the inflammatory response in trout hepatocytes (Philip et al., 2012). Previous studies have measured a decreased abundance of immune function related transcripts such as various complement components and macroglobulin in rainbow trout exposed to ethinyl estradiol (Hook et al., 2007), in flounder exposed to fuel oil (Nakayama et al., 2008), in juvenile rainbow trout exposed to both atrazine and nonylphenol (Shelley et al., 2012) and in delta smelt exposed to permethrin, a pyrethroid pesticide (Jeffries et al., 2015). We recorded a decrease in the abundance of immune related transcripts in fish from the three agriculturally impacted rivers relative to the fish from the reference rivers, with these decreases typically greatest in fish collected from the Tully River, as was noted in our previous study (Hook et al., 2017).

Elevations in the levels of oxidative stress related transcripts (Table 5) could be a direct contaminant response, or they could be indicative of higher levels of stress experienced in fish from the agricultural catchments. For instance, in fish from the Tully River, an increased abundance of transcripts of SOD and CAT were measured. These enzymes act in concert to detoxify superoxides with superoxide dismutase reducing radical oxygen species to hydrogen peroxide, then catalase reducing hydrogen peroxide to water and oxygen (Dalton et al., 2002). Oxidative stress responses are a well known indicator of exposure to and effects of environmental contaminants (e.g. Maria et al., 2009; Marigomez et al., 2013; Regoli et al., 2002). Previous studies have found increased abundance of these transcripts in the hepatic transcriptome of brown trout exposed to the herbicide

glyphosate (Uren Webster and Santos, 2015). These transcripts were only measured in fish from the Tully River that were collected during the wet season. Changes in oxidative stress related transcripts may also be indicative of stress. For instance, increased abundance of transcripts for catalase were measured in migrating salmon in the Fraser River in British Columbia (Veldhoen et al., 2010), though the authors were not certain whether these were associated with contaminant body burdens or the metabolic costs of migration. Typically, in North Queensland, pesticide, nutrient, and sediment loads in the catchments are highest during the wet season (Turner et al., 2012, 2013; Wallace et al., 2014, 2015, 2016 and Garzon-Garcia et al., 2015). Hence, we suggest that Tully fish were exposed to the highest levels of stressors in the study.

Changes in transcript abundance could be a direct response of exposure to commercial pesticide formulations used in North Queensland (Kroon et al., 2015b), as previous studies have measured these changes in fish exposed to active ingredients such as atrazine and additives such as nonylphenol (Cocci et al., 2013). Alternatively, changes in transcript abundance could be a response to turbidity or other stressors in the environment known to correlate with elevated pesticide levels (Garzon-Garcia et al., 2015). Elevations in stress-related transcripts (though not the ones measured in this study) have been measured in delta smelt larvae exposed to high levels of turbidity (Hasenbien, 2016). Other changes that may result in stress, such as changes in habitat or loss of a prey species could also underpin the changes observed (Aluru and Vijayan, 2009). Nevertheless, changes in the abundance of stress related transcripts were only apparent in fish collected in the Tully during the wet season, and not in the two other agricultural catchments during the dry season. This suggests that any potential effect of habitat or prey alterations is overridden by effects of water quality. Regardless of the cause, prolonged exposure to stress can lead to the allocation of energy away from growth, reproduction and development, and could have negative consequences for the population (Hasenbien et al., 2016).

4.5 *Using transcriptomic signatures in environmental management*

At the onset of this research, it was not certain if there would be any differences between sites or if the site related differences would be masked by inter-individual variability. The results of the previous efforts (Hook et al., 2017) and this research clearly show that despite individual differences and presumably, different population structures (e.g. Keenan, 1994), the transcriptomic profiles had greater differences between sites than between individuals. The number of individual replicates (5-8) used in this study likely contributed to being able to differentiate between sites, individual variability would have had more influence if fewer individuals were being compared.

The results of this study also demonstrate the potential utility in using transcriptomic profiles in site management. As we stated in the introduction, at the onset of this study water quality in the GBR catchment area was known to be degraded (reviewed in Brodie et al., 2012; Kroon et al., 2012; Smith et al., 2012), but it was not clear whether these changes were having any impact on the fish in the catchment. Because of natural variability in fish populations, declines in abundance and their causative agents can be difficult to identify (Hamilton et al., 2016). Measuring changes in fish physiology has advantages over traditional endpoints such as length at age or abundance because these endpoints are slow to respond to stressors and do not provide any information about causality (Jeffreys et al., 2015). However, since most of the pesticides in the catchment have not been well studied in fish, such that mode of action has been identified (Kroon et al., 2015; Pathiratne and

Kroon, 2016) there were a large number of physiological endpoints that could be monitored. The RNA Seq study helped to resolve the uncertainity by showing that fish were exposed to a pharmacologically relevant dose of pesticides (e.g. transcripts for xenobiotic metabolising enzymes were more abundant). However, demonstrating exposure is not sufficient to prove chronic impact, a link need to be made to another ecologically relevant endpoint, such as altered reproduction, disease incidence or growth (Hamilton et al., 2016). The transcriptomic data suggested physiological pathways that may be altered, including lipid, amino acid and primary metabolism, and these may indicate a reduced energy budget (Groh et al., 2015a). This study also helped to direct future monitoring efforts by identifying molecular endpoints (changes in lipid content and inflammation) that will be surveyed in future studies. Also, since our previous work (Hook et al., 2017) was a single comparison, this study provides further evidence that the changes observed can be attributed to differences in water quality, as opposed to site specific changes.

This study also demonstrates how RNA Seq data can be integrated into adverse outcome pathway (AOP) development to better understand the implications of contaminant exposure on fish populations. As stated previously, a goal in ecotoxiciology is to translate from exposure (a molecular initiating event in AOP terminology) to population level impacts (or adverse outcomes) (reviewed in Garcia Reyero, 2014). The elevated transcripts for xenobiotic enzymes suggest exposure to pesticides. This exposure may serve as a molecular initiating event in the adverse outcome pathway (e.g. Villeneuve et al., 2014). The oxidative stress, altered metabolic pathways, and altered abundance of immune transcripts are all suggestive of "key events" (e.g. physiological changes that could alter organism health) (Villeneuve et al., 2014). If these effects are measurable at the whole organism level and sufficient in duration and severity, may indicate a reduced energy budget in these fish and reduced ecological resilience (Groh et al., 2015 et al., 2015 a,b, Hamilton et al., 2016). In future work, we will integrate other physiological indices along our RNA Seq studies to assess the potential for adverse outcomes.

Changes in water quality are likely only one of the factors driving differences in transcriptomic profile. Other factors include natural variability as well as differences in hydrology, diet and other natural variability (Hamilton et al., 2016). These factors may have contributed to the differences in transcriptomic profile between the two reference sites, though the significance of these changes are difficult to predict as many transcripts could not be identified.

4.6 Conclusions

The hepatic transcriptome profiles were able to indicate: 1) in which sites there is the greatest potential for changes to fish health as indicated by alterations in transcript abundance (i.e. the Tully River) supporting the first hypothesis; 2) the transcriptomes were most different in fish collected from agriculturally impacted sites during the wet season, supporting the second hypothesis; 3) which stressors are influencing the transcriptome (i.e., exposure biomarkers such as the xenobiotic metabolising enzymes indicate exposure to pesticides, the xenoestrogens suggest exposure to alkylphenol ethoxylates) supporting the third hypothesis; and 4) what are the potential adverse outcomes associated with these stressors (i.e., decreased energetic reserves and immune capacity) if substantiated in future studies using additional endpoints, also supporting the third hypothesis. Collectively, these findings suggest that transcriptomic patterns may be a useful tool for resource managers. The transcripts with altered abundance in the different rivers correspond to known

stressors in the catchments (e.g. pesticides and their surfactant co-factors), and confirms the findings of our previous study (Hook et al., 2017). Furthermore, the transcripts suggest that although some exposure to these compounds is occurring year round, barramundi may be experiencing the most deleterious consequences during the wet season, as suggested by changes in lipid metabolism and immune response. We hypothesise that since the altered transcript abundance were most prevalent in fish collected from the Tully River during the wet season, pesticide runoff from agriculture, as opposed to alterations in barramundi habitat, is the primary stressor. Our study demonstrated that the hepatic transcriptome, as determined via RNA-Seq, could be an effective means of classifying the relative degree of degradation of a site, and should be considered as a management tool. Overall, this study not only provides information on the health of barramundi in selected Great Barrier Reef catchments, it also demonstrates the utility of using the transcriptome to monitor organism health and act as an early warning of potential deleterious biological impacts.

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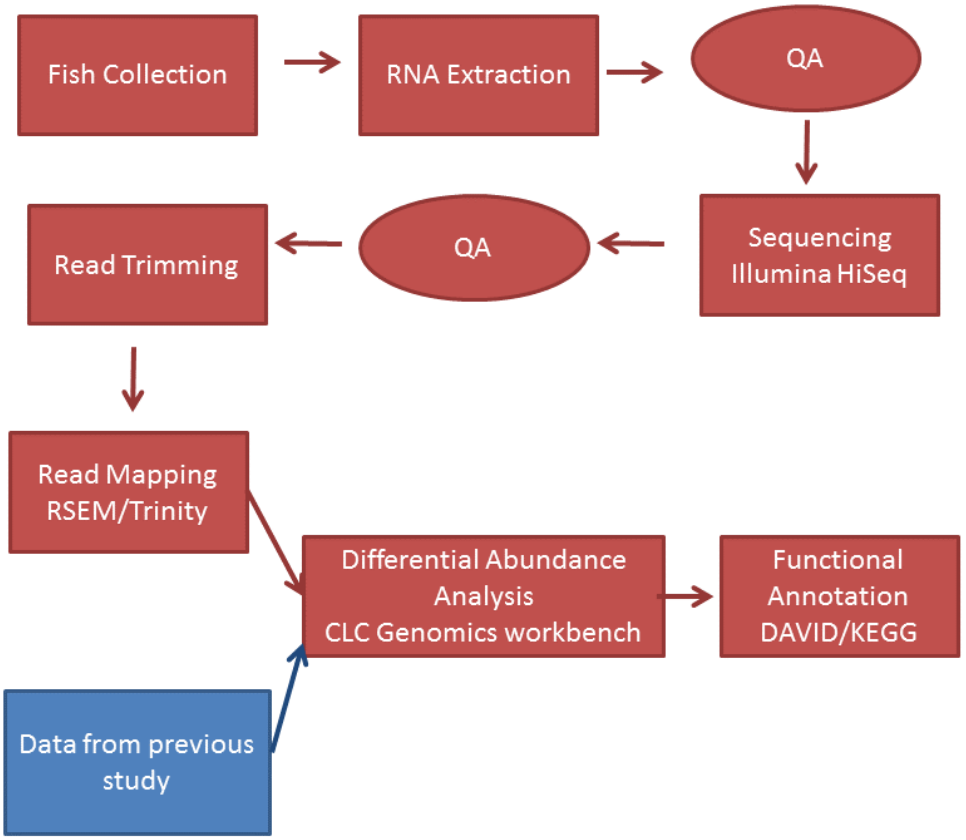

Figure S1. Workflow for this study, showing how data from the previous study were incorporated. These data were previously generated using the same analysis pipeline. Adapted from Hook et al., 2017.

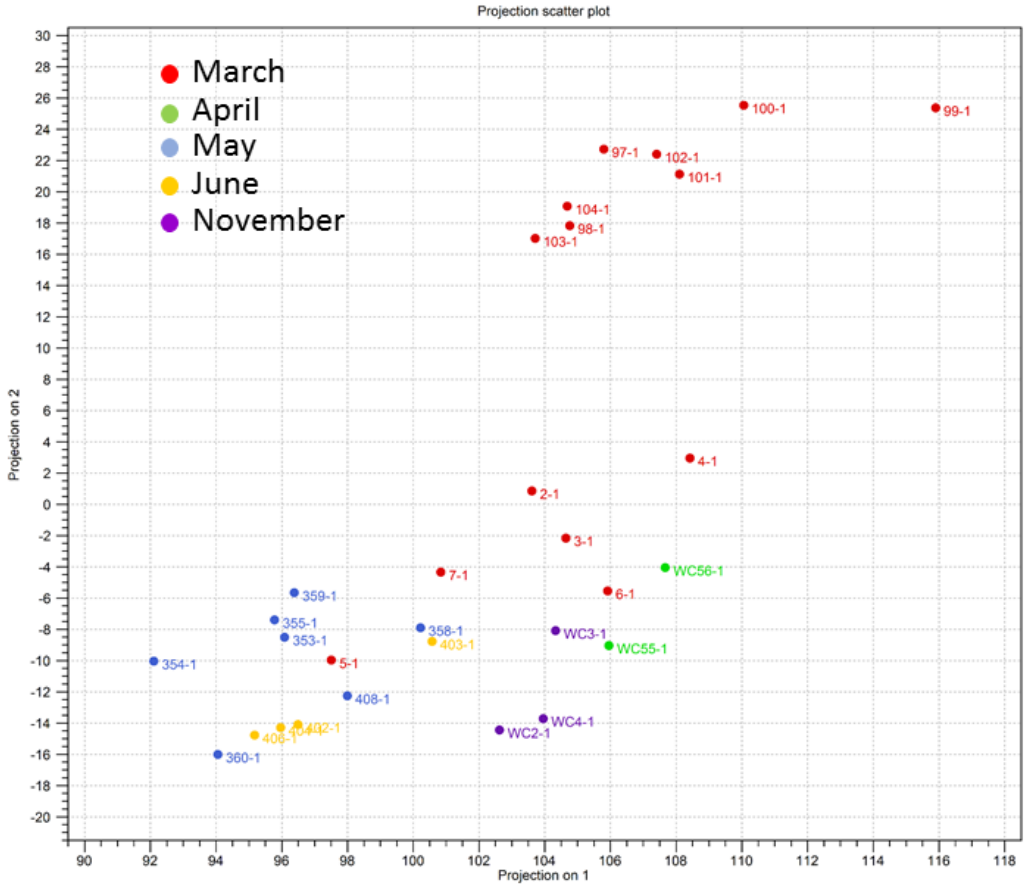

Figure S2. Principle components based visualisation of the plus one, log transformed hepatic transcriptome based on the time of year when the fish was collected. Each dot represents an individual fish.

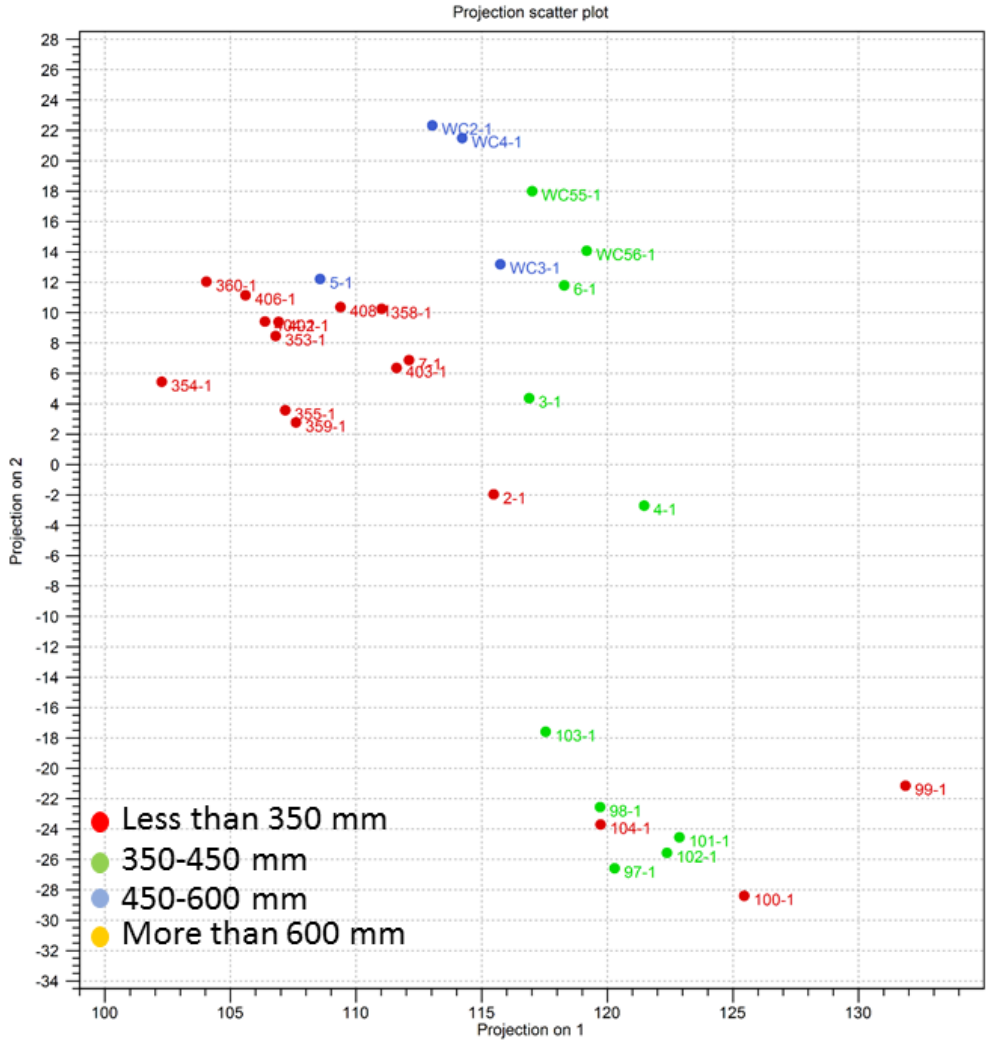

Figure S3. Principle components based visualisation of the plus one, log transformed hepatic transcriptome based on the size (in standard length) of the fish collected. Each dot represents an individual fish.

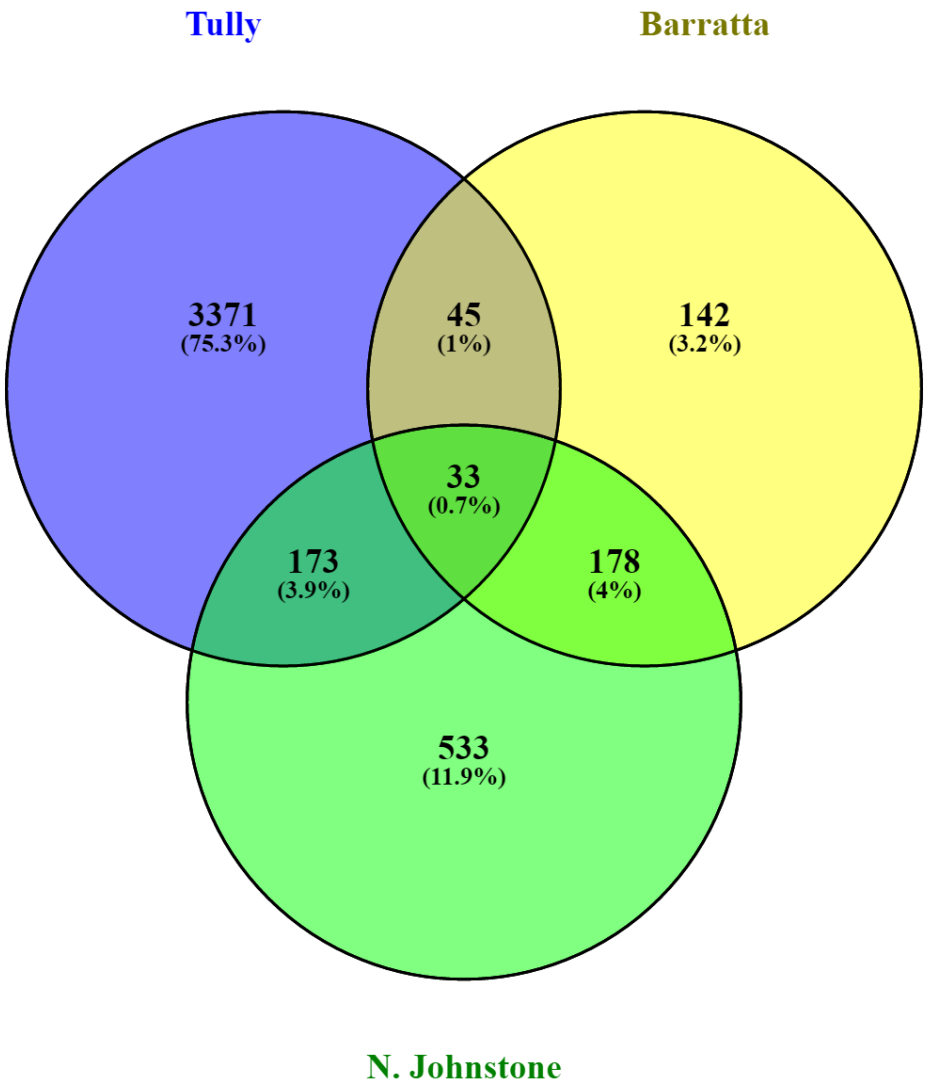

Figure S4. Similarities in the profiles of differentially abundant transcripts in livers for fish from each impacted river relative to both reference sites. Figure was created using Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Table S1. The number of reads for each sample and the percentage that aligned

M is million abbreviated. Fish numbers 2-7 were collected from the Daintree River, fish numbers 97-104 were collected from the Tully River.

Previous study (Hook et al., 2017)

Sample	2	3	4	5	6	7	97	98	99	100	101	102	103	104
Number of reads	28M	27M	26M	25M	25M	25M	28M	28M	31M	26M	28M	25M	26M	26M
% aligned	41.73%	47.50%	46.35%	47.28%	45.35%	42.35%	47.22%	47.81%	46.99%	46.17%	47.23%	47.92%	39.95%	47.88%

Current study

Sample	353	354	355	358	359	360	402	403	404	406	408	WC1	WC2	WC3	WC4	WC5	WC51	WC55	WC56
Number of reads	14M	14M	13M	13M	13M	13M	12M	13M	12M	12M	13M	12M	12M	12M	12M	12M	13M	13M	13M
% aligned	58.55%	60.22%	64.34%	59.7%	60.71%	57.89%	57.40%	56.01%	61.30%	62.12%	62.12%	62.97%	57.30%	58.17%	48.71%	58.59%	57.01%	53.14%	58.08%

Supplementary table S2. Gene lists go here.

Table S3. Functional pathways to which differentially abundant transcripts in the list of differentially abundant transcripts relative to the transcriptome as a whole could be significantly mapped

More abundant in the Tully River

Term	Number of transcripts	% of transcripts	Fold Enrichment	Benjamini-Hochberg corrected p value
phosphoprotein	362	19.30	1.60	1.45E-27
acetylation	151	8.05	1.84	7.16E-12
protein transport	40	2.13	2.65	9.64E-06
cytoplasm	154	8.21	1.48	1.75E-05
lysosome	19	1.01	4.09	8.06E-05
atp-binding	74	3.94	1.79	9.42E-05
nucleotide-binding	88	4.69	1.68	1.05E-04
alternative splicing	289	15.41	1.24	1.10E-04
hydrolase	82	4.37	1.69	1.40E-04
transferase	71	3.78	1.64	2.06E-03
compositionally biased region:Pro-rich	59	3.14	1.96	2.46E-03
splice variant	289	15.41	1.24	2.48E-03
actin-binding	21	1.12	2.73	3.90E-03
disease mutation	77	4.10	1.55	4.04E-03
endoplasmic reticulum	42	2.24	1.89	4.10E-03
oxidoreductase	35	1.87	2.00	5.62E-03
ligase	23	1.23	2.42	6.98E-03
lipid metabolism	15	0.80	3.19	7.39E-03
sequence variant	424	22.60	1.13	0.01
compositionally biased region:Glu-rich	24	1.28	2.71	0.02
kinase	38	2.03	1.77	0.02
GO:0005773~vacuole	22	1.17	2.57	0.03
GO:0005829~cytosol	72	3.84	1.59	0.03
nadp	14	0.75	2.88	0.03
GO:0000267~cell fraction	59	3.14	1.60	0.03
mutagenesis site	95	5.06	1.48	0.03
GO:0000166~nucleotide binding	113	6.02	1.43	0.03
polymorphism	395	21.06	1.10	0.04
GO:0001882~nucleoside binding	84	4.48	1.48	0.04
GO:0001883~purine nucleoside binding	83	4.42	1.47	0.04
GO:0005764~lysosome	19	1.01	2.65	0.04
GO:0000323~lytic vacuole	19	1.01	2.65	0.04
GO:0017076~purine nucleotide binding	97	5.17	1.43	0.04

Less abundant in the Tully

Term	Number of transcripts	% of transcripts	Fold Enrichment	Benjamini-Hochberg corrected p value
109.Chemokine_families	4	1.35	11.46	3.47E-03
PIRSF001950:small inducible chemokine, C/CC types	4	1.35	32.16	0.01
chemotaxis	5	1.69	19.08	0.02
IPR000827:Small chemokine, C-C group, conserved site	4	1.35	39.78	0.02
SM00199:SCY	4	1.35	22.71	0.03
cytokine	6	2.03	9.11	0.04
IPR001811:Small chemokine, interleukin-8-like	4	1.35	24.26	0.05

Less abundant in Barratta Creek

Term	Number of transcripts	% of transcripts	Fold Enrichment	Benjamini-Hochberg corrected p value
protein transport	36	2.18	2.78	1.37E-05
atp-binding	61	3.69	1.72	3.82E-03
IPR011993:Pleckstrin homology-type	25	1.51	2.92	4.59E-03
GO:0030695~GTPase regulator activity	28	1.69	2.39	0.01
GO:0060589~nucleoside-triphosphatase regulator activity	29	1.75	2.42	0.02
actin-binding	18	1.09	2.73	0.02
GO:0017048~Rho GTPase binding	8	0.48	7.25	0.02
SH2 domain	11	0.67	3.72	0.03
GO:0051020~GTPase binding	12	0.73	3.79	0.03
GO:0005096~GTPase activator activity	18	1.09	2.82	0.03
GO:0017016~Ras GTPase binding	11	0.67	4.16	0.04
IPR000198:RhoGAP	10	0.60	5.28	0.04
GO:0003779~actin binding	22	1.33	2.32	0.05
GO:0031267~small GTPase binding	11	0.67	3.75	0.05

Less abundant in N Johnstone

Term	Number of transcripts	% of transcripts	Fold Enrichment	Benjamini-Hochberg corrected p value
cell adhesion	12	3.07	3.99	0.02

Table S4. KEGG functional categories to which the enriched transcripts in fish from each transcriptome can be mapped.

Pathway	Number of sequences in gene list	Number of unique enzymes in gene list	Number of sequences in transcriptome	Expected number of sequences
More abundant in fish from the Tully River				
Purine metabolism	30	19	237	24
Glycerophospholipid metabolism	24	15	88	9
Glycerolipid metabolism	19	9	94	10
Glycolysis / gluconeogenesis	18	12	76	8
Fatty acid degradation	18	9	41	6
Lysine degradation	16	10	50	7
Glycine, serine and threonine metabolism	16	11	45	6
Pyruvate metabolism	16	11	47	5
Sphingolipid metabolism	15	6	59	8
Tryptophan metabolism	15	7	45	6
Pentose phosphate pathway	14	10	47	5
Citrate cycle (TCA cycle)	14	11	48	7
Glutathione metabolism	14	7	43	6
Arginine and proline metabolism	13	11	46	6
Ether lipid metabolism	13	6	27	4
Valine, leucine and isoleucine degradation	13	11	27	4
Phosphatidylinositol signalling system	13	8	109	11
Oxidative phosphorylation	13	8	63	9

Aminoacyl-tRNA biosynthesis	12	13	45	6
Inositol phosphate metabolism	12	7	94	10
Propanoate metabolism	12	9	36	5
Glyoxylate and dicarboxylate metabolism	12	9	26	4
Arachidonic acid metabolism	12	6	37	5
mTOR signalling	12	2	31	4
beta-Alanine metabolism	6	6	25	4
Amino sugar and nucleotide sugar metabolism	11	11	53	7
Tyrosine metabolism	11	12	26	4
Alanine, aspartate and glutamate metabolism	10	13	40	6
Biosynthesis of unsaturated fatty acids	10	4	25	4
Drug metabolism - cytochrome P450	10	5	32	5
Metabolism of xenobiotics by cytochrome P450	10	5	36	5
Aminobenzoate degradation	10	5	28	4
Starch and sucrose metabolism	10	11	37	5
alpha-linolenic acid metabolism	9	4	20	3
Chloroalkane and chloroalkene degradation	9	3	11	1
Pentose and glucuronate interconversions	6	3	24	3
Steroid hormone biosynthesis	8	5	30	4

Butanoate metabolism	8	10	20	3
Drug metabolism - other enzymes	8	8	53	5
Galactose metabolism	7	6	62	9
Retinol metabolism	7	4	32	4
Porphryrin metabolism	7	7	24	3
Steroid biosynthesis	7	5	10	1
Selenocompound metabolism	6	5	14	2
Nicotinate and nicotinamide metabolism	6	3	31	3
N-glycan biosynthesis	6	4	25	3
Other glycan degradation	6	5	34	5
Histidine metabolism	6	4	12	1
Ascorbate and aldarate metabolism	5	2	12	1
Folate biosynthesis	5	5	10	1
Cysteine and methionine metabolism	5	7	40	4
Phenylaline metabolism	5	7	32	4
Linoleic Acid	5	2	14	2
Various types of N-glycan biosynthesis	5	3	21	3
Limonene and pinene degradation	5	2	6	1
Arginine biosynthesis	5	5	46	6
Cyanoamino acid metabolism			20	3
Less abundant in fish from the Tully River				
Glycolysis / gluconeogenesis	18	12	76	8

Cysteine and methionine metabolism	5	4	40	4
Less abundant in fish from the N Johnstone River				
Glycolysis / gluconeogenesis	6	6	76	8
Less abundant in fish from Barratta Creek				
Purine metabolism	35	22	237	24
Glycolysis / gluconeogenesis	19	12	76	8
Phosphatidylinositol signalling system	16	7	109	11
Fructose and mannose metabolism	14	8	55	8
Inositol phosphate metabolism	13	6	94	13
Oxidative phosphorylation	13	4	63	9
Galactose metabolism	12	6	62	9
T cell receptor signalling pathway	12	2	82	8
Amino sugar and nucleotide sugar metabolism	9	12	53	7
Pyrimidine metabolism	11	8	105	10
Starch and sucrose metabolism	11	8	37	5
Pentose phosphate pathway	10	5	47	5
Pyruvate metabolism	7	6	47	5
Glycine, serine and threonine metabolism	8	8	45	5
Pentose phosphate pathway	8	3	47	5
Drug metabolism - other enzymes	7	5	53	5

Arginine and proline metabolism	6	4	46	6
Pentose and glucuronate interconversions	6	4	24	3
Ester lipid metabolism	6	5	27	4
Lysine degradation	6	4	50	7
Aminoacyl-tRNA biosynthesis	5	5	45	6
Nicotinate and nicotinamide metabolism	5	4	31	3
Glutathione metabolism	5	4	43	6
Trytophan metabolism	5	3	45	6

Only those pathways with five or more sequences that are relevant to eukaryotes are presented. The number of sequences expected to be included in the gene list due to chance alone is presented for comparison.