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# ASSESSMENT OF MOLECULAR BIOMARKER RESPONSES IN COMMON BULLY (*GOBIOMORPHUS COTIDIANUS*) FROM THE MATAURA RIVER

**Louis A. Tremblay[1,2*], James M. Ataria[1], Gen Kume[3,5], Olivier Champeau[1], Katherine Trought[3], Grant L. Northcott[4] and Andrew Barrick[1]**

[1.] Cawthron Institute, Nelson, New Zealand

[2] School of Biological Sciences, University of Auckland, Auckland, New Zealand

[3] Landcare Research, Lincoln, New Zealand

[4] Northcott Research Consultants Limited, Hamilton, New Zealand

[5] Current address: Faculty of Fisheries, Kagoshima University, 4-50-20 Shimoarata, Kagoshima 8900056, Japan

* Author for correspondence: email louis.tremblay@cawthron.org.nz

## ABSTRACT

New Zealand has a reputation for having a comparatively clean environment but there is growing recognition that the freshwater ecosystems are under threat and continue to decline. Many monitoring frameworks use ecosystem-based assessments to demonstrate adverse impacts, but this approach has limitations to identify causalities. The aim of this study was to measure metallothionein, cytochrome P450 and vitellogenin molecular biomarkers in the New Zealand native common bully *Gobiomorphus cotidianus* sampled across a pollution gradient on the Mataura River. *G. cotidianus* demonstrated site-specific induction of metallothionein and cytochrome P450 with significant effects occurring at sites more downstream from point sources including a sewage outfall and an industrial estate. This outcome suggests potential biological responses are occurring in the Mataura River that could potentially be linked to anthropogenic activity. The results of the study provide evidence that there may be a response gradient as the Mataura River flows towards the ocean, supporting the need to assess catchments from mountains-to-sea.

**Keywords**: New Zealand; bioindicator; qPCR; metals; organic pollutants; diffuse pollution; native species

## INTRODUCTION

There is evidence of the impacts of land-use activities on the environment (Foley et al. 2005). While the extent of contamination in New Zealand is not as severe as in other more industrialised nations, there is still a need to assess the risks of anthropogenic activities on local ecosystems (Hickey et al. 1995). Ecosystems are under threat and water quality continues to decline across most New Zealand regions as a result of the intensification of diffuse discharges, particularly from agricultural activities (Loague et al. 1998; Ministry for the Environment & Stats NZ 2020). Agricultural practices can result in the release of chemicals (e.g. nutrients, pesticides and endocrine active chemicals) which can have profound and increasing impacts on the health and well-being of riverine ecosystems as the pressure of these stressors is compounded as waterways travel towards the ocean (Rasmussen et al. 2013). In the context of New Zealand, there is a paucity of information

regarding the effects of chemical contaminants on the native species populating affected aquatic ecosystems (Champeau et al. 2020).

Many monitoring frameworks focus on measuring ecological endpoints such as species abundance, density and biodiversity which are effective at characterising sites that have been adversely impacted by anthropogenic stressors, but often lack predictive capabilities (Dallas and Jha 2015). Biomarkers are functional measures of contaminant exposure providing insights into the biological effects of anthropogenic contaminants in exposed receptor species (Adams et al. 2001; Hook et al. 2014). Biomarkers are relatively well-established tools for investigating whether contaminants are biologically available (Handy et al. 2003), and have been used to assess the impacts of multiple stressors to exposed biota (e.g. from municipal effluent (Jasinska et al. 2015)). Commonly used biomarkers include metallothionein (*mt*) as an indicator of exposure to metals (Tremblay et al. 2021), Cytochrome P450 1A1 (*cyp1a*) as an indicator of exposure to aromatic hydrocarbons (Fent 2001; Jasinska et al. 2015), and vitellogenin (*Vtg*), an established biomarker of exposure to endocrine disruption (Jones et al. 2000). These biomarkers are often measured using real time qPCR (rt-PCR), which has been demonstrated to be an effective method for measuring changes in mRNA abundance in response to contaminant exposure (Jasinska et al. 2015).

The selection of an appropriate test species that responds to anthropogenic activity is an essential prerequisite to assessing the *in situ* impacts of exposure to anthropogenic contaminants (Adams et al. 2001). The New Zealand common bully (*Gobiomorphus cotidianus*) has shown potential as a sentinel species in previous studies because of its wide geographic distribution, variety of natural and impacted habitats that it populates, and demonstrated high site-fidelity (Tremblay et al. 2021; van den Heuvel et al. 2007). The site-fidelity of the common bully has been confirmed through stable isotope analysis and suggests that they reflect the ecology of the site they populate (Hicks 1997; van den Heuvel et al. 2007). This fish has previously been demonstrated to be a useful native species to monitor population impacts of pulp and paper mill effluent (van den Heuvel et al. 2007).

The Society of Environmental Toxicology & Chemistry (SETAC) Global Horizon Scanning Project aimed to identify, prioritise, and advance environmental quality research needs from an Australasian perspective. Many priority research questions were identified including: “What are the effects of short magnitude, frequency, and duration (e.g. intermittent, episodic) exposures to contaminants and other stressors, and how can these scenarios be effectively incorporated into water quality guidelines?” as a priority research question within the region (Gaw et al. 2019). The Mataura River provides a model study site for addressing this question as it represents a significant catchment within the Southland Region of the South Island of New Zealand (Ryder 1995). Historically the Mataura River has been subjected to major physical alterations and anthropogenic impacts resulting from animal processing industries, pulp and paper production, urbanisation, agricultural and farming activities, and industrial dairy processing industries, all of which contribute sources of multiple stressors into the catchment. The recognition of these cumulative and escalating impacts has led to expressions of growing concern for the degradation of the health of the Mataura River by local communities and Māori, the indigenous people of New Zealand.

The aim of this study was to measure *mt*, *cyp1a* and *Vtg* responses in *G. cotidianus* collected from sites along a pollution gradient on the Mataura River to assess their effectiveness as biomarkers of exposure to multiple stressors. As part of the study, preliminary work was also conducted to develop the primers for selected biomarkers and demonstrate their efficacy.

# MATERIALS AND METHODS

## Study site and fish sampling

The Mataura River flows in a southerly direction through the Southland Region of the South Island of New Zealand. It is the sixth longest river in New Zealand, spanning a total length of 240 km and is the second largest riverine catchment in Southland with a coverage area of 5360 km$^2$ (Ryder 1995). Three sampling sites within the Mataura River were selected for the field assessment (Figure 1):

i) Piano Flat (45°33.075'S, 169°01.796'E) the most upstream site located in a natural reserve in the headwaters of the Waikaia River, a major tributary of the Mataura River.

ii) Near Gore on the Mataura River downstream of the municipal sewage outfall and upstream from diffuse agricultural inputs (46°07.441'S, 168°56.426'E).

iii) Downstream from an industrial park within the township of Mataura (46°11.777'S, 168°51.918'E) (Champeau et al. 2020).

In October 2008, 20 bully, between 35 and 65 mm in size, were collected from each site using a Smith-Root (LR24) back-pack electric fishing machine. Upon collection, the fish were anaesthetised using 150 µg/mL ethyl-4-amino benzoate solution and sacrificed by severing the spinal column. The liver was excised and transferred into RNA*later*® solution (Qiagen) for preservation and stored at 4°C until processing. Condition (K) index (West et al. 2021), hepato-somatic index and gonado-somatic index were all calculated using the following formulas:

- Fulton's condition factor (K): (fish weight (g)/(total length (cm)$^3$)) x 100
- Hepato/gonado-somatic indices (HSI/GSI) = (liver/gonad weight (g)/(fish weight - liver/gonad weight)) x 100

## Development, validation and field assessment of qPCR primers

The design and optimisation of the PCR assays for *G. cotidianus* are summarised in the Supplementary Material and the resulting primers are presented in Table 1. In addition to 18S, ß-actin (Accession Number JF742980) and GAPDH (Accession Number JF742981) were sequenced as potential reference genes. However, we only used 18S as a reference gene as it showed constant expression over a range of conditions in *G. cotidianus* and displayed strong inter-seasonal stability when compared to the other potential reference genes (Bustin et al. 2009; Cubero-Leon et al. 2012; Tremblay et al. 2021).

RNA was extracted from the liver tissue using the Total RNA easy-spin™ column kit (iNtRON

**Table 1. qPCR primers for target genes used in the study.**

| Gene | Short name | Forward Primer (5'–3') | Reverse Primer (3' – 5') | Expected product size (bp) | Accession Number |
|---|---|---|---|---|---|
| 18S Ribosomal RNA | *18S* | GATTCTGTGGGTGGTGGTG | TGCCGGAGTCTCGTTCGTTA | 84 | JF715064 |
| Metallothionein | *mt* | CAGAAAGCCGCCTATTTGAC | AGCTGGTGTCACAAGTCTTC | 194 | JF742982 |
| Cytochrome P450 1A1 | *cyp1a* | CCTGGTTGCTTACCCAGA GGTACAGGAGA | GCATGGCGGAGAACC TCCAGGATG | 140 | JF742983 |
| Vitellogenin | *vtg* | CACCCTGGTGGCTAAACACT | GCAGTGCCAGAGAATGACAA | 191 | JF742984 |

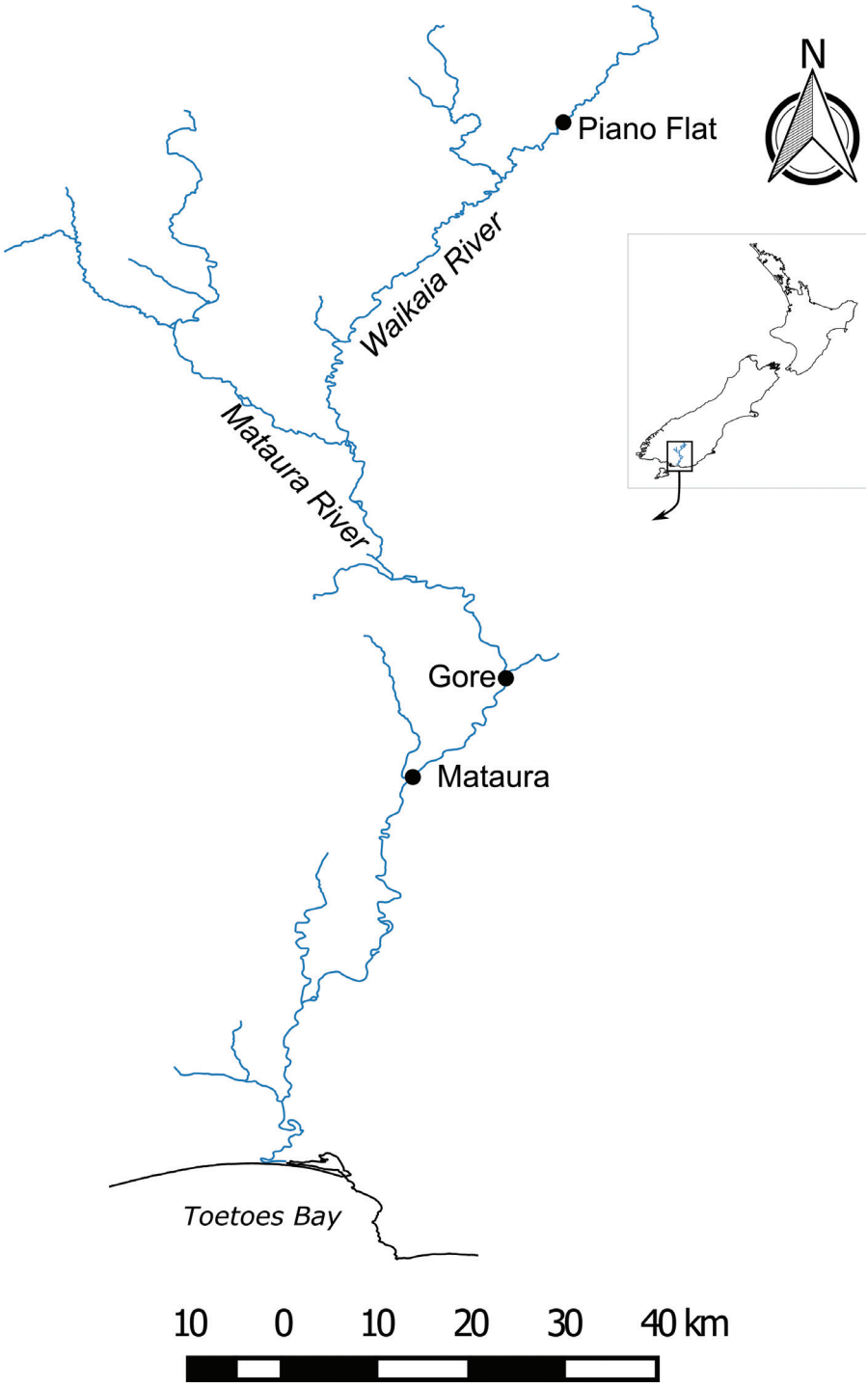


**Figure 1. Locations of the three sample sites on the Mataura River used in the study.**

#17221) following the manufacturer's protocol. After RNA was collected the purity and integrity of the RNA was checked using a NanoDrop™ spectrophotometer. RNA was diluted to 50 ng/µL and cDNA was synthesised using an M-MVL Reverse Transcriptase kit (Invitrogen) following the manufacturer's protocol using random hexamers (Promega) and 10 mM dNTPs (Bioline). The processed samples were stored at -80°C until analysis.

Results for the *mt*, *cyp1a* and *Vtg* target genes were corrected using 18S as the reference gene and data were assessed using the ΔΔct method. Fold gene expression was then determined relative to the mean value measured in bully captured at Piano Flat as this is the most upstream site and the least likely to be exposed to anthropogenic stressors.

### Statistical analysis

For somatic indices, significance testing of differences between sites and sex was carried out by analysis of covariance with site as factor and adjusted body weight (fish weight - organ weight) as covariate. Variables compared using analysis of variance (ANOVA) or analysis of covariance (ANCOVA) were tested for normality and homogeneity of variances, using Shapiro-Wilk W and Hartley F-max statistic, Cochran C statistic, and the Bartlett Chi-square tests, respectively (RStudio 2015). For the qPCR data, R (R Core Team 2015) with "R Studio 3.6.2" (RStudio 2015) was used to conduct analysis using a Shapiro-Wilk test to demonstrate that PCR data were not normally distributed. Nonparametric analysis was conducted through Kruskall-Wallis and Dunn's tests using a Bonferroni correction for multiple comparisons using the R package dunn.test with a P value of $<0.05$ indicating statistical significance. To account for sex as a potentially confounding factor, each gene was investigated to determine if statistical differences occurred between male and females. This analysis was conducted within and between sites.

## RESULTS

### Validation of the RT-qPCR assays

One way ANOVA shows no significant differences between treatments for the 18S reference gene (F value = 0.555). This indicates that the treatment does not affect the reference gene and 18S is suitable for use as a reference gene. The expressions of *mt*, *vtg* and *cyp1a* in fish exposed to copper, 17ß-estradiol, and ß-naphthoflavone were all significantly induced when compared to the control and solvent (water, ethanol or DMSO) control fish (Figures S1, S2, S3).

Indices for graphical comparisons of all sampled fish between sites were calculated and there were differences between sex at some sites but only the males from the Mataura site had a condition factor higher than males from the other sites (Figure 2).

No statistical differences were observed between the qPCR data obtained for males and females *cyp1a* and *mt* at each sampling site and results obtained from both sexes were therefore combined for subsequent analysis. Bully from the Gore site displayed a significant increase in *mt* mRNA in comparison to those from the Piano Flat reference and Mataura sites (Figure 3A). Bully sampled from the Gore and Mataura sites displayed a high degree of variation in *cyp1a* expression and both were significantly elevated compared to the fish from Piano Flat (Figure 3B). Vitellogenin demonstrated intersex variation with limited expression occurring in males at all three sites and, as a result, *vtg* was analysed only in females with no statistically significant effects observed between the three sites (Figure 3C).

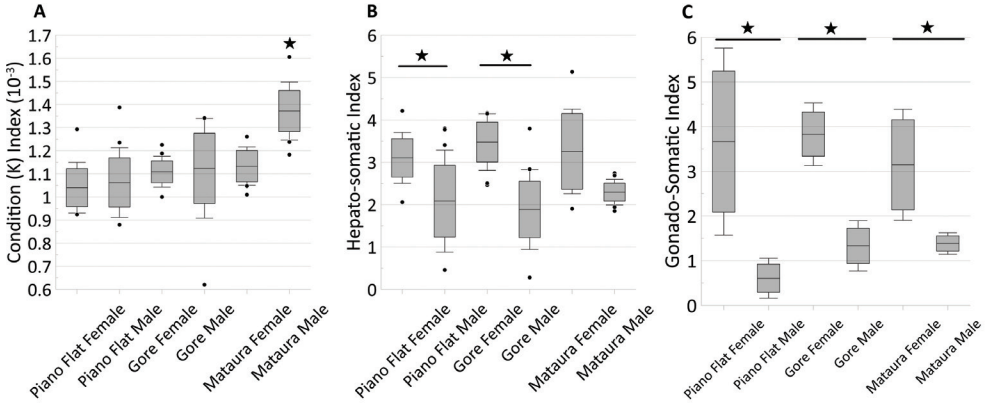


**Figure 2. Somatic indices of the common bully. Box and whisker plot indicates median percentiles and quartiles. Dots indicate outliers in the data. Asterisks indicate pairs of values significantly different at *p<0.05.**

## DISCUSSION

The water flow in the Mataura River is relatively high with median rates in the tens of $m^3$/second, which can effectively dilute and disperse urban and industrial discharges and limit the accumulation of contaminants. This is further compounded by the specific characteristics of the riverbed which consists of a solid rock base overlain by weakly weathered gravel and schistose sediment. This type of substrate exhibits poor absorption and retention of semi-volatile organic contaminants (SVOCs) and promotes the transport of chemicals out of the ecosystem (McIntosh et al. 1990). Frequent and intense rainfall events within the Mataura River catchment can lead to flash flood conditions resulting in the transport of potential anthropogenic stressors downstream to the estuary. A previous study conducted in the Mataura River demonstrated that the concentration of selected metals in sediment at the sampling sites were found at levels below ANZECC interim sediment quality guidelines (Champeau et al. 2020; Simpson et al. 2013). The concentrations of targeted SVOCs (DDT, chlordane, PCBs, chlorophenols and PAHs) in these same sediment samples also fell

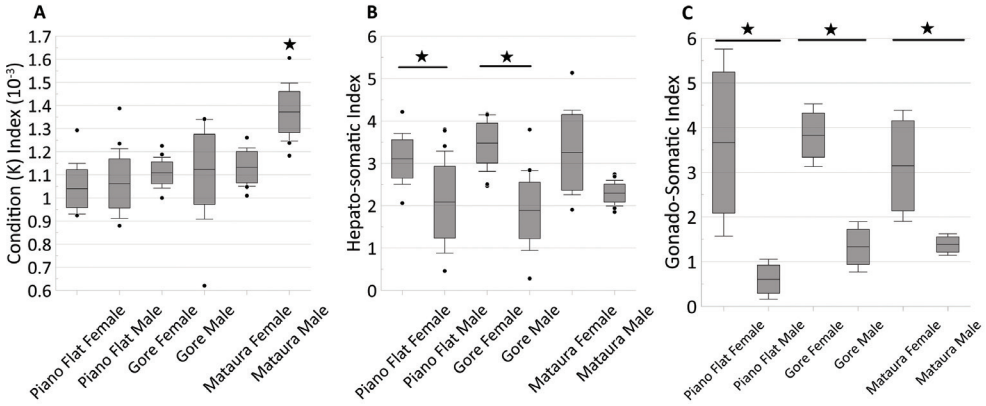


**Figure 3. Expression of metallothionein (A), cytochrome P450 1A1 (B), and vitellogenin (C) in *G. cotidianus* liver collected at the three sampling sites. Box and whisker plot indicates median percentiles and quartiles. * indicates a statistically significant difference from the control group (p<0.05).**

below their respective methods of detection limit (µg/kg dry weight; data not shown), suggesting that these organic contaminants do not persist in sediment at the sampling locations.

When considering the biological indices, the results suggest that fish at all three sites are of equivalent health and sexual development (Figure 2). These results agree with the contaminant data from previous studies that indicate that the concentrations of contaminants in this section of the Mataura River are too low to pose a risk at this level of biological organisation. When considering molecular responses however, qPCR results indicate that fish at Gore and Mataura sites are exposed to anthropogenic stressors. Inductions of *mt* at Gore and *cyp1a* in *G. cotidianus* at both Gore and Mataura sites demonstrate that these sublethal endpoints were expressed in response to the presence of low concentrations of bioavailable metal and organic contaminants at these sites (Andersson and Förlin 1992). The activation of these genes at these sites suggests adverse impacts at higher levels of biological organisation could develop over time if exposure to causative xenobiotic contaminants continues (Lacorn et al. 2001; Sleiderink et al. 1995; Zapata-Perez et al. 2002). It has been documented that anthropogenic contaminants at concentrations lower than recommended guideline values for the protection of aquatic organisms can still lead to adverse effects on receiving ecosystems (Tremblay et al. 2017). Species like *G. cotidianus* that can live for over four years (van den Heuvel et al. 2007), will also be exposed to intermittent pulses of contaminants that are not necessarily captured by periodic monitoring programs.

These results need further validation supported by appropriate chemical data, but they confirm that biomarkers are a valuable tool to complement current environmental risk assessment frameworks for aquatic ecosystem health in New Zealand.

Future assessments of ecosystem health using the common bully as a sentinel species would benefit from integrating additional biomarkers at multiple levels of biological organisation, to assess other mechanisms of toxicity (e.g. energetic reserves, oxidative stress and membrane transporters). Linking these results to population endpoints would provide a better understanding of how biota respond to mixtures of anthropogenic stressors that are present in riverine catchments like the Mataura River (Sokolova 2013; Tremblay et al. 2005).

The distances between the sampled sites on the Mataura River were relatively short (e.g. about 12 km between the Gore and Mataura sites) but biomarker responses were consistent with previous findings which indicated that the common bully display strong site fidelity (van den Heuvel et al. 2007). Piano Flat resides within a nature preserve upstream of the other sampling sites. In the context of a mountain-to-sea approach (where it is hypothesised that contaminants accumulate as they move down the river system), this site was considered to be of relatively lower impact for the purpose of this study. The biomarker responses in bully from the Piano Flat reference site displayed less *mt* and *cyp1a* expression, which support this hypothesis. The selection of suitable reference sites in field-based ecotoxicological studies remain a challenge but the concept of the mountain-to-sea approach provides the opportunity to sample across a pollution gradient.

Additional research is needed to assess the influence of other factors on biomarker expression in *G. cotidianus* including seasonality, fish sex and age (Eggens et al. 1996; Kopko and Dabrowska 2018; Lacorn et al. 2001). Future research directions will need to focus on identification of causative agents, their sources, mass loads throughout the year and potential links between gene expression and potential adverse outcome pathways on exposed biota (Ankley et al. 2010).

Validation of *G. cotidianus* as a bioindicator of ecosystem health in New Zealand requires further investigation to determine if it is predictive of environmental impacts to other receptor species (Tremblay et al. 2021; van den Heuvel et al. 2007). In addition, the inclusion of biomarkers into

monitoring frameworks depends on their ability to establish cause and effect relationships against confounding factors like climate and seasonal variations (Adams et al. 2001; Handy et al. 2003; Sanchez and Porcher 2009).

## CONCLUSION

The Gore and Mataura sites were selected to integrate urban, agricultural and industrial anthropogenic pressures within the Mataura River catchment. The *cyp1a* and to some extent the *mt* biomarker results suggest that *G. cotidianus* within the Mataura River catchment are responding to dioxin-like and metal stressors downstream from the reference site. The evidence of biological effects occurring within the Mataura River warrants additional research integrating other lines of evidence like chemical analysis to identify the causative agents and their sources to determine whether remediation actions are required to reduce and minimise effects. The relatively low biomarker responses can be attributed in part to the high energy level in the Mataura River creating a flushing effect of contaminants and sediment downstream following the regular intense flood events in the region.

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*Supplementary material*

# ANALYSIS OF BIOMARKERS IN THE COMMON BULLY (*GOBIOMORPHUS COTIDIANUS*) TO ASSESS THE HEALTH OF A LARGE RIVER EXPOSED TO MULTIPLE STRESSORS

**Louis A. Tremblay, James M. Ataria, Gen Kume, Olivier Champeau, Katherine Trought, Grant L. Northcott and Andrew Barrick**

## CONTROLLED EXPOSURE TO REFERENCE CHEMICALS

For the initial development of the qPCR primers, *G. cotidianus* were collected from the Ashley River (43°16'S, 172° 41'E) near North Canterbury, New Zealand. This site was selected for the development of the assays as previous studies have collected individuals at this site for the development of rt-qPCR assays (Laurie 2004). Individuals between 35 and 65 mm were gently collected using nets and transported to the lab where they were acclimatised for one week prior to testing.

After acclimation, fish (n=10) were anaesthetised with 150 µg/mL ethyl-4-amino benzoate solution (Sigma, St. Louis, MO) and dosed with the reference chemicals using a syringe, 20 µL/g live weight (Table S1). After 48 h, the fish (n=5) were anaesthetised using 150 µg/mL ethyl-4-amino benzoate solution and killed by severing the spinal column. The liver was excised, transferred into RNA*later*® solution (Qiagen) for preservation and stored at 4°C until processing. RNA was extracted from the liver tissue using the Total RNA Easy Spin column kit (iNtRON #17221) following the manufacturer's protocol. The concentration of RNA and purity (>1.9) was checked using a Nanodrop spectrophotometer (ND-2000). RNA was diluted to 50 ng/µL and cDNA was synthesised using an MMVL RT kit (Invitrogen) following the manufacturer's protocol using random hexamers (Promega) and 10 mM dNTPs (Bioline).

**Table S1**. Contaminants used to verify induction of target genes

| Reference chemical | Dose (mg/kg) | Solvent Used | Target Gene |
|---|---|---|---|
| Zinc ($ZnSO_4.7H_2O$ AnalaR, VWR) | 10 | Ultra-pure water | Metallothionein |
| Copper ($CuSO_4$ Sigma) | 10 | Ultra-pure water | Metallothionein |
| Cadmium ($CdCl_2$ Sigma) | 10 | Ultra-pure water | Metallothionein |
| 17ß-estradiol (Sigma) | 10 | Ethanol | Vitellogenin |
| ß-naphthoflavone (BDH) | 50 | Dimethyl sulfoxide (DMSO) | Cytochrome P450 1A1 |

Target genes sequences were determined by aligning sequences from other fish species (*Perciformes* sp.) and regions of homology were identified. cDNA was synthesised using the MMLV-RT enzyme (Invitrogen, Life Technologies). The target genes were amplified using a Stratagene MX3000P qPCR machine and SybrGreen PCR mastermix (Applied Biosystems). Thermocycling was conducted using polymerase activation at 95°C for 10 minutes with an amplification and quantification cycle repeated for 40 cycles (95°C for 30 sec, 55°C for 1 min and 72°C for 1 min). For the development of the

qPCR assays, the 18S gene was analysed to determine if it can be used as a suitable reference gene. After confirmation that no alterations were occurring due to exposure to the reference contaminants, all other genes were normalised to the 18S and expression relative to the control was determined.

## EXPOSURE TO REFERENCE CONTAMINANTS

The expression of *mt* following exposure to copper and cadmium were both significantly different from the control (Figure S1). The expression of *mt* following exposure to zinc, however, was significantly different from Cd, but not from the control. Estradiol caused a statistically significant increase in vtg expression compared with the control (Figure S2). The expression of *cyp1a* also displayed statistical significance in samples exposed to ß-naphthoflavone when compared to the control prepared with DMSO (Figure S3).

## REFERENCE

Laurie AD. (2004) Quantitation of metallothionein mRNA from the New Zealand common bully (*Gobiomorphus cotidianus*) and its implications for biomonitoring. *New Zealand Journal of Marine and Freshwater Research* **38**, 869-877.

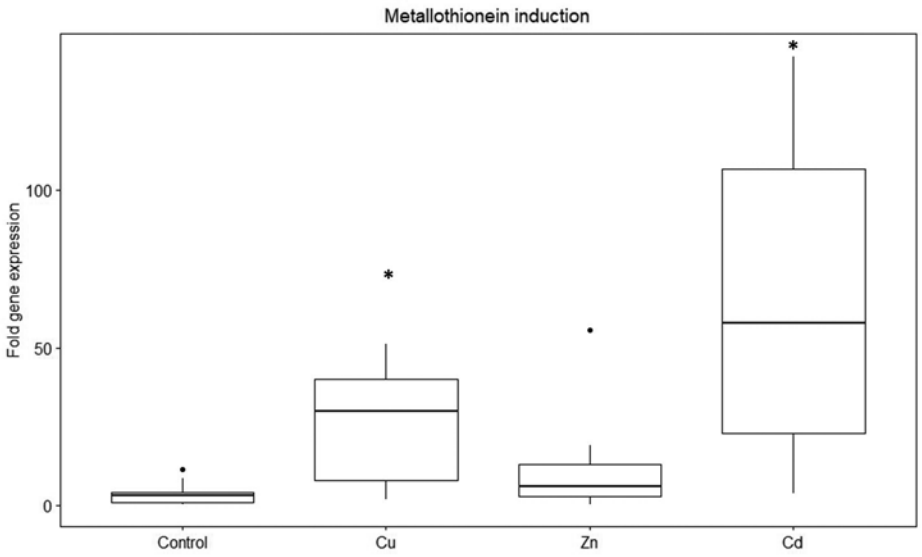


**Figure S1**. Expression of *mt* in common bully exposed to several reference metals. Stars indicate a statistically significant difference from the control ($p<0.05$).

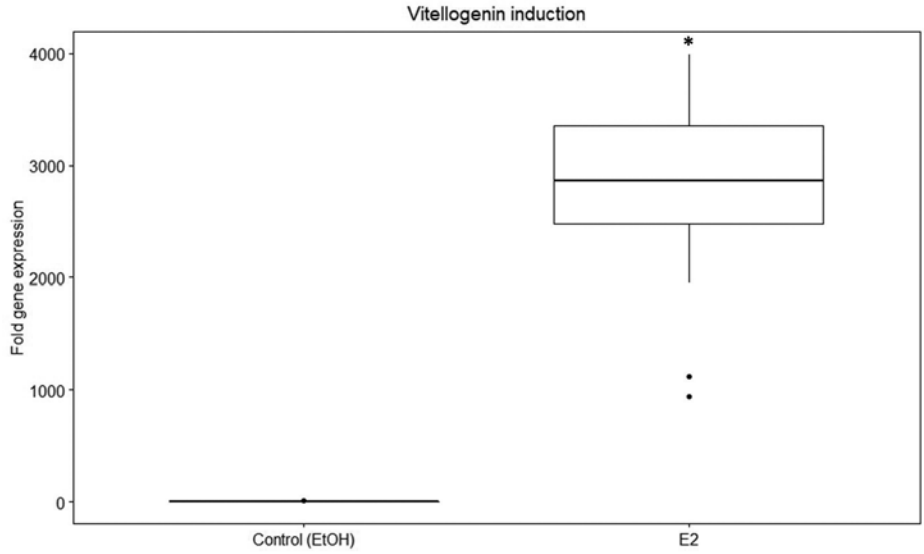


**Figure S2**. Expression of *vtg* in common bully exposed to estradiol compared to an ethanol control. Stars indicate statistical significance ($p<0.05$).

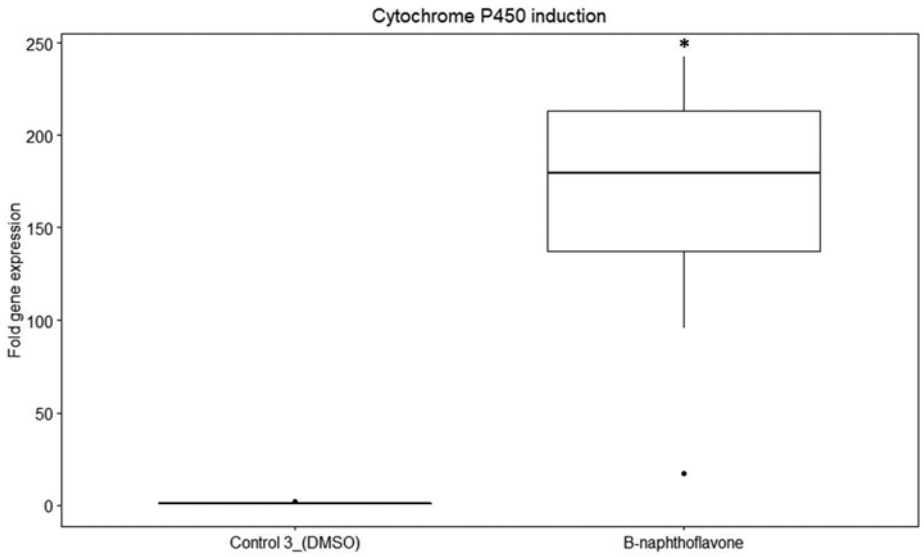


**Figure S3**. Expression of *cyp1a* in common bully exposed to ß-naphthoflavone compared to a DMSO control. Stars indicate statistical significance ($p<0.05$).