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Thyroid hormone related gene transcription in southern sand flathead (*Platycephalus bassensis*) is associated with environmental mercury and arsenic exposure

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Abstract Arsenic (As) and mercury (Hg) are ubiquitous elements known to disrupt thyroid function in vertebrates. To explore the underlying mechanisms of Hg and As on the fish thyroid system, we investigated the associations between muscle concentrations of Hg and As with thyroid-related gene transcription in flathead (*Platycephalus bassensis*) from a contaminated estuary. We sampled fish at several sites to determine the hepatic expression of genes including deiodinases (D1 and D2), transthyretin (TTR), thyroid hormone receptors (TRα and TRβ) and related them to Hg and As levels in the same individuals. Negative correlations were observed between Hg levels and D2, TTR, TRα and TRβ, whereas positive associations were found between As concentrations and TTR and TRβ. These results suggest that Hg and As exposures from environmental pollution affect the regulation of genes important for normal thyroid function in fish. These thyroid-related genes could be used as biomarkers for monitoring environmental thyroid-hormone disrupting chemicals.

Keywords Thyroid-related genes · Gene expression · Mercury · Arsenic · Biomarker · Fish

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Introduction

Arsenic (As) and mercury (Hg) are environmental contaminants commonly associated with industrial pollution, which can be bioaccumulated and biomagnified and pose adverse effects on humans and wildlife (Abernathy et al. 2003; Eisler 2004; Mason et al. 1996; Ward et al. 2010). In recent years, there has been substantial evidence suggesting that both Hg and As can disrupt the thyroid system of vertebrates (Ciarrocca et al. 2012; Iavicoli et al. 2009; Tan et al. 2009). The thyroid system is highly conserved throughout vertebrates and plays a crucial role in many bioactivities such as normal metabolism, reproduction and development (Power et al. 2001; Zoeller et al. 2007). Therefore, the impairment of the thyroid system by an exposure to Hg and As may exert profound effects on the health of vertebrates. Previous studies reported Hg concentrations were positively associated with the circulating free thyroxine (T4)/free triiodothyronine (T3) ratio in humans (Barregard et al. 1994; Ellingsen et al. 2000). In mammals, low level As exposure altered thyroid hormone receptor-mediated gene expression in pituitary cells (Davey et al. 2008). Likewise, associations between Hg exposure and thyroid hormone concentrations have been observed in birds (Wada et al. 2009) and exposure to As affected the thyroid hormone receptor dependent developmental processes of amphibians (Davey et al. 2008). In fish, chronic As exposure decreased the plasma T4 concentration in coho salmon (*Oncorhynchus kisutch*) (see Nichols et al. 1984), while in juvenile rainbow trout (*Oncorhynchus mykiss*), both plasma T4 and T3 concentrations were increased when exposed to Hg (Bleau et al. 1996). Although Hg and As have been proven to be associated with thyroid parameters in different vertebrate species, the relationships between Hg, As, and thyroid related genes are poorly understood. In

particular, the synergistic effects of Hg and As on these genes are still unknown (Iavicoli et al. 2009).

Fish can be used to provide valuable information on potential thyroid disrupting effects of environmental factors in fish or other animals that prey upon fish (Brown et al. 2004; Leatherland 2000). Thyroid hormone endocrine disruptors affect thyroid hormone synthesis, thyroid hormone metabolism, thyroid hormone transport and thyroid hormone receptors (Patrick 2009). In teleost fish, two types of thyroid hormone (T4 and T3) are synthesized in the thyroid follicles and then released into the circulation (Brown et al. 2004). Transthyretin (TTR) is one of the major transporter proteins that carry thyroid hormone into different target tissues in fish (Power et al. 2009). Thus, TTR has been suggested as one of the major targets for endocrine disrupting chemicals (Lans et al. 1994). In the target tissues, the majority of T4 is converted into T3, a more biologically active form of hormone by deiodinases (D1 and D2) and this is a crucial step in regulating the peripheral thyroid status (Orozco and Valverde-R 2005). In target cells, T3 binds to thyroid hormone receptors (TRs) as a transcription factor to regulate gene expression (Yamano 2005). Therefore, the transthyretin (TTR), deiodinases (D1 and D2) and thyroid hormone receptors (TRs) are important for thyroid hormone disruption.

Southern sand flathead (*Platycephalus bassensis*) have been used as an indicator species to assess the health of contaminant-exposed fish populations (Ayling et al. 1975). *P. bassensis* are demersal, non-migratory and they accumulate heavy metals from their diet consisting of benthic organisms (Parry and Scientific 1995). The Derwent estuary in Tasmania has been recognized as a river polluted with both Hg and As (Bloom and Ayling 1977; Green and Coughanowr 2003) and flathead in the Derwent River have been monitored for heavy metal residues since the 1970s (http://www.derwentestuary.org.au/). Metallurgical liquid effluent has been discharged into the estuary for decades (Bloom and Ayling 1977; Langlois et al. 1987). The bioaccumulations of heavy metals in fish and shellfish from the Derwent estuary are much higher than elsewhere in Tasmanian waters (Eustace 1974; Ratkowsky et al. 1975; Verdouw et al. 2011).

The Derwent estuary is located in the southern part of Tasmania and is close to the capital city Hobart, which has a high level of industrial and urban activities, suggesting the accumulation of not only heavy metals but organic pollutants in flathead is high. Therefore, cytochrome P4501A (CYP1A) gene, a commonly used molecular marker for monitoring organic pollutants such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), furans and dioxins (Rees et al. 2003), was employed as an additional marker. The metallothionein (MT) gene, the most commonly used molecular marker of metal pollution in fish, was also assessed in this study. However, previous studies have suggested that the use of the MT gene as a biomarker in fish may be inappropriate as it can be influenced by a variety of abiotic and biotic factors such as salinity, temperature, pH, fish species and tissues (Chen et al. 2004; Cho et al. 2008). Nonetheless, the use of the MT gene as a pollution biomarker has been widely adopted in a range of fish species even though most of the studies assessing the validity of MT as a biomarker were conducted under laboratory conditions (Knapen et al. 2007). The suitability of the MT gene as a molecular marker for metals exposure under field conditions in sand flathead is unknown.

In the present study, *P. bassensis* from the historically polluted Derwent estuary were used as to explore the relationships between As, Hg and thyroid-related gene expression levels. Thyroid related genes (D1, D2, TTR, TRα and TRβ), CYP1A and MT were cloned and examined from the flathead liver. The hepatic mRNA levels of these genes were compared in flathead from different sites to investigate the effects of heavy metal exposure on thyroid related genes.

Materials and methods

Site descriptions

Three sites were chosen in Derwent River based on historical data showing different levels of As and Hg residues in flathead. The sites are as follows: Cornelian Bay (CB), Kingston Beach North (KBN) and Ralphs Bay (RB) (Fig. 1). Cornelian Bay is located in the upstream of Derwent River and is a graduating area of salt water to fresh water. This site is close to the main industrial inputs, but the previous data have indicated that As and Hg in sand flathead are lower than in flathead of Kingston Beach North and Ralphs Bay respectively (Unpublished data from Zinifex monitoring program, 2011). Kingston Beach North and Ralphs Bay are located on the western shore and eastern shore respectively. These two sites are primarily sea water and are approximately 20 km from the major pollution sources such as Hobart city and Nyrstar. However, the previous studies have shown that sand flathead from Kingston Beach North have the highest As level and the fish from Ralphs Bay have the highest Hg level among a number of sampling sites in Derwent River (Unpublished data from Zinifex monitoring program, 2011).

Sample collection

Juvenile *P. bassensis* were caught from Kingston Beach North ($n = 8$), Ralphs Bay ($n = 8$) and Cornelian Bay ($n = 9$) between October and November, 2012 (Table 1). Only

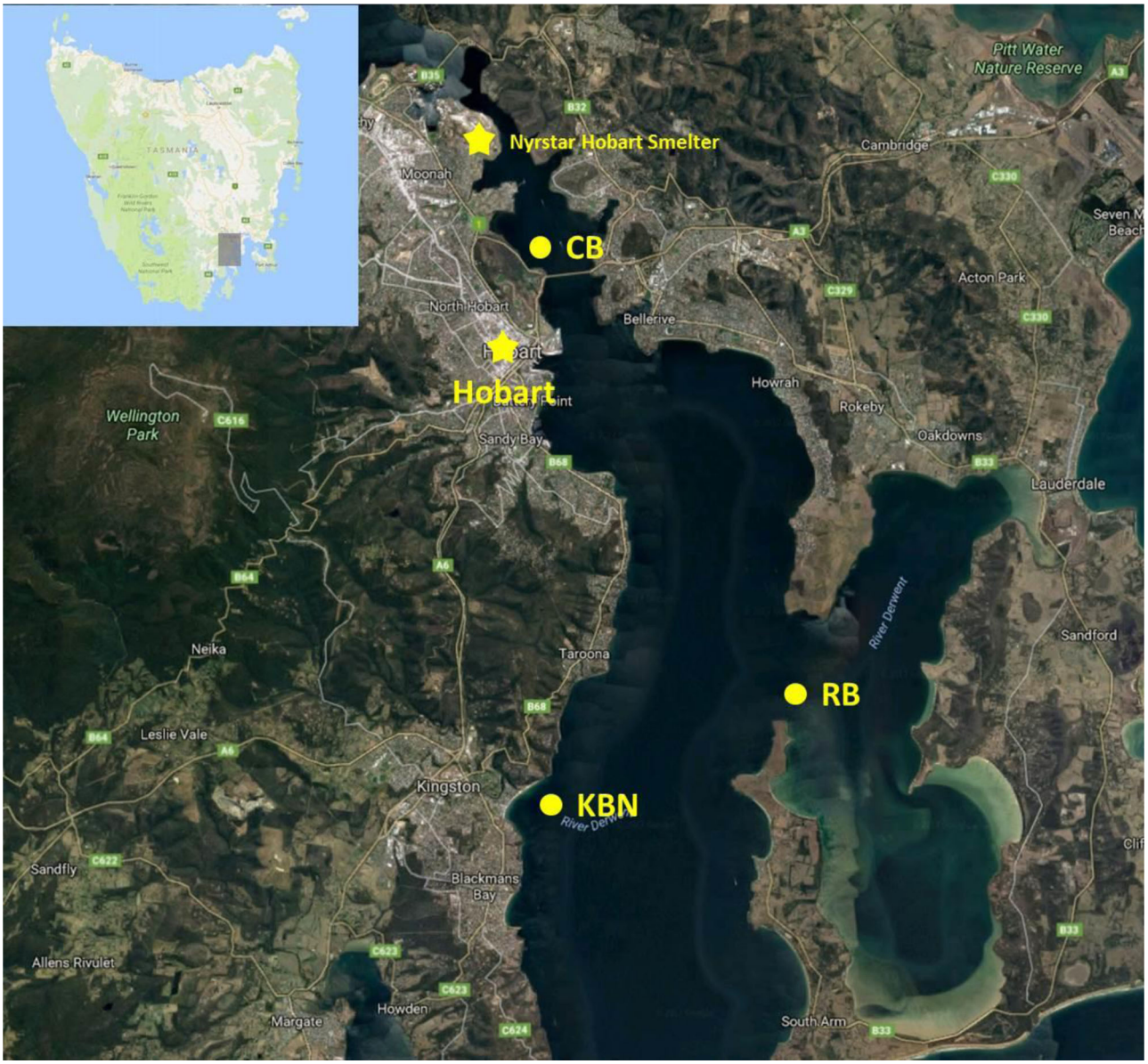

Fig. 1 Map of Derwent River estuary sampling sites: Kingston Beach North (KBN), Ralphs Bay (RB) and Cornelian Bay (CB)

Table 1 Morphometric measurements of 25 flathead were used for gene expression analysis and heavy metal determination

Site	*n*	Length (cm)	Weight (g)	*K*
Cornelian Bay	9	26.6 ± 1.7	177.9 ± 47.8	0.92 ± 0.1
Kingston Beach North	8	23.6 ± 2.4	113.4 ± 48.5	0.82 ± 0.1
Ralphs Bay	8	29.0 ± 1.8	237.7 ± 47.6	0.97 ± 0.1

juvenile fish were used in gene expression analysis. The gender was not identified due to these fish being sexually immature. All the fish used in this study were opportunistically sampled as part of a heavy metal monitoring project conducted by Nyrstar N. V Hobart (a mining and metals industry) under a permit issued under section 14 of the Tasmanian Governments *Living Marine Resources Management Act 1995* (2011–2012 Annual Environment Review of Nyrstar N.V). Fish were captured from a small boat using a fishing rod with baited hook. Body weight (*W*) and standard length (*L*) were measured for each individual to calculate the condition factor (*K*) using the following formula: $K = 100 \times W\ (\text{kg}) \times L\ (\text{cm})^{-3}$. For As and Hg analysis, approximately 50 g of muscle was collected from individual fish and then put in a labeled plastic bag placed on ice for transport to the laboratory. All the samples were stored at −20 °C until Hg and As analyses. A sample of liver was collected from a small ventral incision in each fish and immediately stored in a RNA preservation reagent

(25 mM sodium citrate, 10 mM EDTA, 4 M ammonium sulphate, pH 5.2). The samples were held on ice until transport to the laboratory and stored at −80 °C until further processing.

Metals determination

All samples were analysed by Analytical Services Tasmania that is accredited by the National Association of Testing Authorities (NATA). The determination of total Hg in each sample was used as a proxy of methylmercury (MeHg) due to the assumption that MeHg is the primary form of Hg in fish muscle (Harris et al. 2003). Samples were digested and analysed using cold vapour atomic fluorescence spectrometry conducted on an atomic fluorescence analyser as described by (Verdouw et al. 2011). For the analysis of other metals including, As, Cd, Co, Cr, Cu, Fe, Zn, Se, Pb, Ni and Mn, approximately 12 g of skinless and boneless muscle samples from individuals were dried and ground into powder. 1 g of each sample was digested and analyzed using an inductively coupled plasma atomic emission spectrophotometer. All the data of residues were given as milligrams per wet kilogram of sample with a minimum detection level of 0.02 mg/kg.

RT-qPCR analysis

Total RNA was isolated from the liver of *P. bassensis* using TRI Reagent (Molecular Research Centre, OH, USA) and treated with DNAse (Turbo DNase, Ambion, TX, USA) to remove any contaminating genomic DNA. RNA quality was assessed by electrophoresis in a 1.0% agarose gel. The RNA concentrations were determined using an Invitrogen Qubit fluorometer and Quant-iT RNA assay kit (Invitrogen, VIC, Australia). Total RNA (1 µg) was used for the first-strand cDNA synthesis using BioScript reverse transcriptase (Bioline, NSW, Australia) with Oligo (dT) 18 priming as per manufacturers instructions.

Degenerate oligonucleotide primers (Macrogen, South Korea) were designed from the conserved regions of other teleost sequences retrieved from Genbank (Table 2) and used to obtain nine target partial genes by PCR amplification of *P. bassensi* hepatic cDNA; deiodinase type I (D1), deiodinase type II (D2), transthyretin (TTR) thyroid hormone receptor α (TRα), hormone receptor β (TRβ), cytochrome P4501A (CYP1A), metallothionein (MT), β-actin and elongation factor 1-alpha (EF1α). PCR amplification of each gene was conducted in a volume of 20 µl containing 2× PCR mixtures (Bioline, NSW, Australia), 0.5–0.75 µM of each primer and approximate 50 ng template cDNA. Amplification was carried out on an Eppendorf thermal cycler using the following PCR profile: 2 min at 94 C, then 32 cycles of 94 °C for 30 s, 50–52 °C for 45 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were separated via 1.5% agarose gel and purified from gel by using a gel extraction kit (Qiagen, NSW, Australia). Purified PCR products were Sanger sequenced directly in the forward direction (Macrogen). Homologs of these genes were confirmed with published sequences by BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the gene sequences deposited in Genbank (Table 2). The sequence identity of thyroid related genes was analysed by comparing with other known teleost homologues (Table 3).

The deiodinases (D1 and D2), transthyretin (TTR), thyroid hormone receptors (TRα and TRβ), metallothionein (MT), and cytochrome P4501A (*CYP1A*) mRNA expression were determined by quantitative real-time RT-PCR using gene-specific primers (Table 1). Real-time PCR was performed on an iQ5 Real-time PCR Detection System (Bio-Rad, NSW, Australia) in a volume of 10 µl containing 0.1 µM of each primer, 5 µl 2× SensiMixPlus SYBR & Fluorescein PCR master mix (Bioline, NSW, Australia), and 1 µl of cDNA. The PCR cycling consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 20 s with signal collection in each cycle. To assess the specificity of the PCR amplification, a melting curve was obtained at the end of the cycling and a single peak for each target gene was observed. The data analysis of the qPCR results was performed using qBasePlus Biogazelle software (Hellemans et al. 2007). The target genes expression in different samples was normalized to β-actin and EF1α quantities. The geometric mean was used to normalize relative quantity (MNRQ) and was calculated for each gene of grouped replicates from the site. The heavy metal determination data have indicated that fish from CB have the relatively lower As level than those from KBN and have the lower Hg level than fish from RB. Therefore, the expression level of all the genes was relative to that of expression in fish from CB.

Statistical analysis

The Bartlett's test was used to assess homogeneity of variance for heavy metal data prior to statistical analysis. Spatial differences in Hg and As concentrations and hepatic gene expression were performed using one-way analysis of variance (ANOVA) followed by the Tukey HSD test to determine differences among the sites. To investigate the relationship between gene expression and heavy metal levels, correlation analyses were performed on As, Hg and hepatic genes expression from all fish. The Pearson's correlation coefficient was used to assess the relationships between parameters (heavy metal levels and relative genes expression after all samples pooled together). All statistical analyses were carried out using GraphPad Prism version 6.0 (GraphPad software Inc) with statistical significance at $P < 0.05$.

Table 2 Primers used for cDNA sequence and quantification of the mRNA expression by Real-time PCR

Gene (accession no.)	Oligo name	Primer DNA sequence (5′→3′)
cDNA sequence		
D1	D1F	ACBATGACCCAGAAYC
	D1R	TCCACMACCACWGGRCACAGGGG
D2	D2F	GGYTTCTTYTCSAACTG
	D2R	GGCTKATRAAGGGGGGTCA
TTR	TTRF	TCCTGGTCCTCGAAGACCTCCA
	TTRR	TCAAACTCMCGATACACKCC
TRα	TRαF	ATGTGCCGAAGAGAAAGA
	TRαR	TCCTGGTCCTCGAAGACCTCCA
TRβ	TRβF	GTGTGYGGGGACAAAGC
	TRβR	AGCAGCTTDGGGCCAGAA
MT	MTF	ATGGAYCCTTGYGMMTGC
	MTR	TCACTGACAGCAGSTBGTGTC
CYP1A	CYP1AF	ATYGATCACTGYGARGAC
	CYP1AR	TTGTGYTTCATKGTGAGRCC
Real-time RT-PCR		
D1 (KP893709)	qD1F	ACAGATCCTGGTTCAGAA
	qD1R	ATACTTCACGGCAGACAT
D2 (KP893710)	qD2F	GCACTCAACTCCAAAGTAG
	qD2R	ACCAGGTGACACATTAGT
TTR (KP893711)	qTTRF	AGGTCCATAATCTCATCAC
	qTTRR	CTCATCTTCCCAGTTAGC
TRα (KP893714)	qTRαF	CCATCCAGAAGAACCTCCA
	qTRαR	GTTGCGGGTGATCTTGTC
TRβ(KP893715)	qTRβF	TAAGCCTGAGGATATTGG
	qTRβR	TTTGTAAACTGACTGAAGG
CYP1A (KP893713)	qCYP1AF	ATGACAAGGACAACATTC
	qCYP1AR	ATCTGACATCTGGACATT
MT (KP893712)	qMTF	ATCCGGCTGCACCAAATG
	qMTR	GTTTACTGACAGCAGGTGGT
β-actin (KR076429)	qβ-actinF	ACCTCACAGACTACCTCAT
	qβ-actinR	TTGATGTCACGCACGATT
EF1α (KP893716)	qEF1αF	TTGGAGTCAACAAGATGG
	qEF1αR	GATGTAGGTGCTCACTTC

Results

Metal concentrations

As and Hg residues in flathead muscle from Cornelian Bay, Kingston Beach North and Ralphs Bay showed significant spatial differences (Fig. 2). As residues in flathead from Kingston Beach North were significantly higher than those of Cornelian Bay and Ralphs Bay ($P < 0.0001$, Fig. 2a).The Hg concentrations in flathead from Cornelian Bay and Kingston Beach North were significantly lower than in Ralphs Bay ($P < 0.0001$, Fig. 2b). No significant differences were found for the concentrations of Zn and Fe. Other metals were below the minimum detection level (0.02 mg/kg).

Hepatic genes expression

The combined reference genes (EF1α, β-actin) were considered stable by the qBase plus software and therefore suitable for normalisation with a stability value (*M*-value) of < 0.7 and a CV of 0.25. Hepatic expression levels of D2 mRNA were significantly down-regulated in flathead caught at Ralphs Bay compared to those from Cornelian Bay (CB) ($P < 0.05$, Fig. 3b), but not significantly different from fish at Kingston Beach North. There was no significant difference among these three sites for the expression levels of D1 (Fig. 3a). For the transcripts of TTR, the highest expression level was observed in fish from Kingston Beach North (3.62-fold that of Cornelian Bay, $P < 0.0001$,

Table 3 Sequence identity of thyroid related genes with other known teleost homologues

D1 (KP893709)		D2 (KP893710)		TTR (KP893711)		TRα (KP893714)		TRβ (KP893715)	
Species (Accession no.)	Identity	Species (Accession no.)	Identity	Species (Accession no.)	Identity	Species (Accession no.)	Identity	Species (Accession no.)	Identity
Sebastiscus marmoratus (JX135096)	92%	*Gasterosteus aculeatus* (BT027288)	87%	*Sparus aurata* (AF059193)	88%	*Paralichthys olivaceus* (D16461)	91%	*Epinephelus coioides* (EF502004)	95%
Sparus aurata (AJ619717)	88%	*Siganus guttatus* (GU372962)	88%	*Lates calcarifer* (XM_018701843)	85%	*Lateolabrax japonicas* (KF285430)	93%	*Sciaenops ocellatus* (HM010954)	94%
Oreochromis niloticus (Y11109)	84%	*Paralichthys olivaceus* (AB362422)	85%	–	–	*Sciaenops ocellatus* (HM010983)	92%	*Paralichthys olivaceus* (D45245)	93%
Lates calcarifer (XM_018667354)	86%	*Chrysiptera cyanea* (GU583739)	86%	–	–	*Epinephelus coioides* (EF502002)	91%	*Sparus aurata* (AY246695)	92%

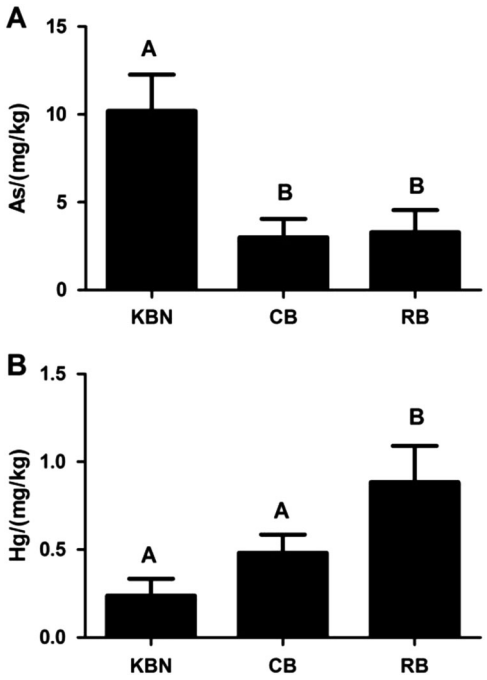

Fig. 2 The mean ± S.E As **a** and Hg **b** concentrations in flathead muscle sampled at Kingston Beach North (KBN, $n = 8$), Cornelian Bay (CB, $n = 9$) and Ralphs Bay (RB, $n = 8$). *Vertical bars* represent the means. *Different letters* indicated significant differences between sites

Fig. 3c). Similarly, TRβ mRNA levels were significantly higher in flathead from Kingston Beach North than those from Cornelian Bay and Ralphs Bay ($P < 0.0001$, Fig. 3e). In contrast, TRα hepatic mRNA levels were not significantly different in flathead sampled at different sites (Fig. 3d). There were no significant differences in hepatic mRNA levels of CYP1A in flathead among all research sites (Fig. 3f). MT mRNA expression was significantly higher in flathead from the Kingston Beach North (KBN) than in the fish from Cornelian Bay (CB) and Ralphs Bay (RB) ($P < 0.05$) (Fig. 3g).

Relationships between gene transcripts, morphometric measurements, Hg and As levels

There were statistically significant positive correlations between As levels and expression of TTR ($r = 0.6584$, $P = 0.0001$, Fig. 4a), expression of TRβ ($r = 0.6455$, $P = 0.0002$, Fig. 4b) and expression of MT ($r = 0.4834$, $P = 0.0062$, Fig. 4g). Significant negative correlations were observed between the levels of Hg and the mRNA expression of D2 ($r = -0.4021$, $P = 0.0232$, Fig. 4c), TTR ($r = -0.4953$, $P = 0.0050$, Fig. 4d), TRα ($r = -0.3663$, $P = 0.0329$, Fig. 4e), TRβ ($r = -0.6241$, $P = 0.0003$, Fig. 4f) and MT ($r = -0.4101$, $P = 0.0187$, Fig. 4h). No correlation was observed between the morphometric measurements (fork

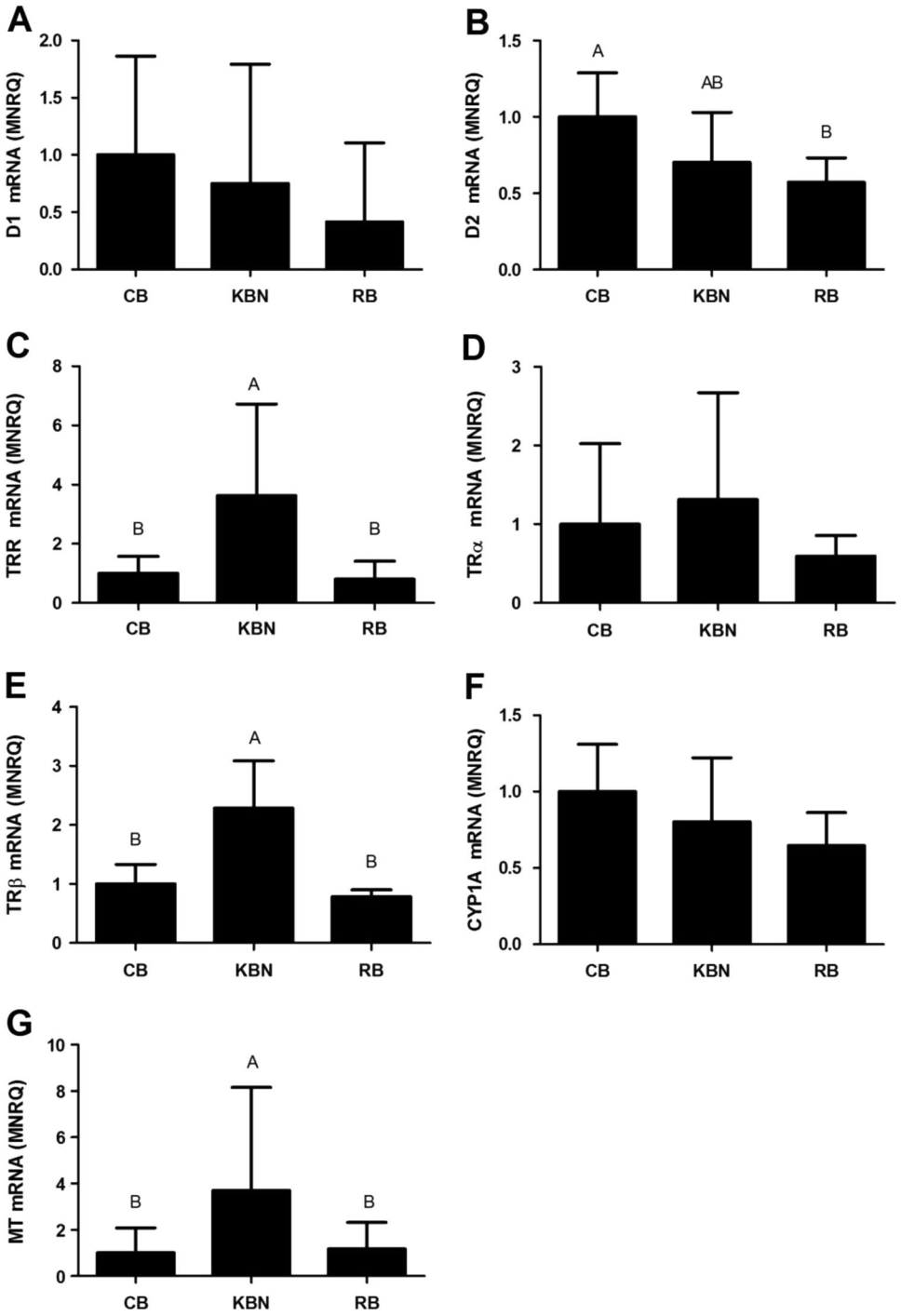

Fig. 3 Hepatic mRNA levels of D1, D2, TTR, TRα, TRβ, CYP1A and MT, relative to two reference genes (β-actin and EF1α) were analysed by qPCR in flathead captured at three sites of Derwent River estuary. The *values* are shown as geometric means of normalized relative quantity (MNRQ) ± 95% confidence interval. *Different letters* indicated significant differences between sites

length, weight and condition factor) and gene transcripts (Fig. 5).

Discussion

D2 mRNA in flathead livers was significantly lower in Ralphs Bay, where flathead had the highest Hg concentrations. In addition, a significantly negative correlation was observed between D2 transcripts and Hg levels for fish from all sites. It has been proposed that the synthesis and activity of 5′-deiodinases are generally inhibited by Hg exposure (Barregard et al. 1994; Mori et al. 2006). In previous studies, Hg exposure was associated with the decreased T4 concentrations of snakehead fish (*Channa punctatus*) (see Bhattacharya et al. 1989) and decreased both T3 and T4 in plasma of catfish (*Clarias batrachus*) (see Kirubagaran and Joy 1994). In birds, a recent study of the tree swallow

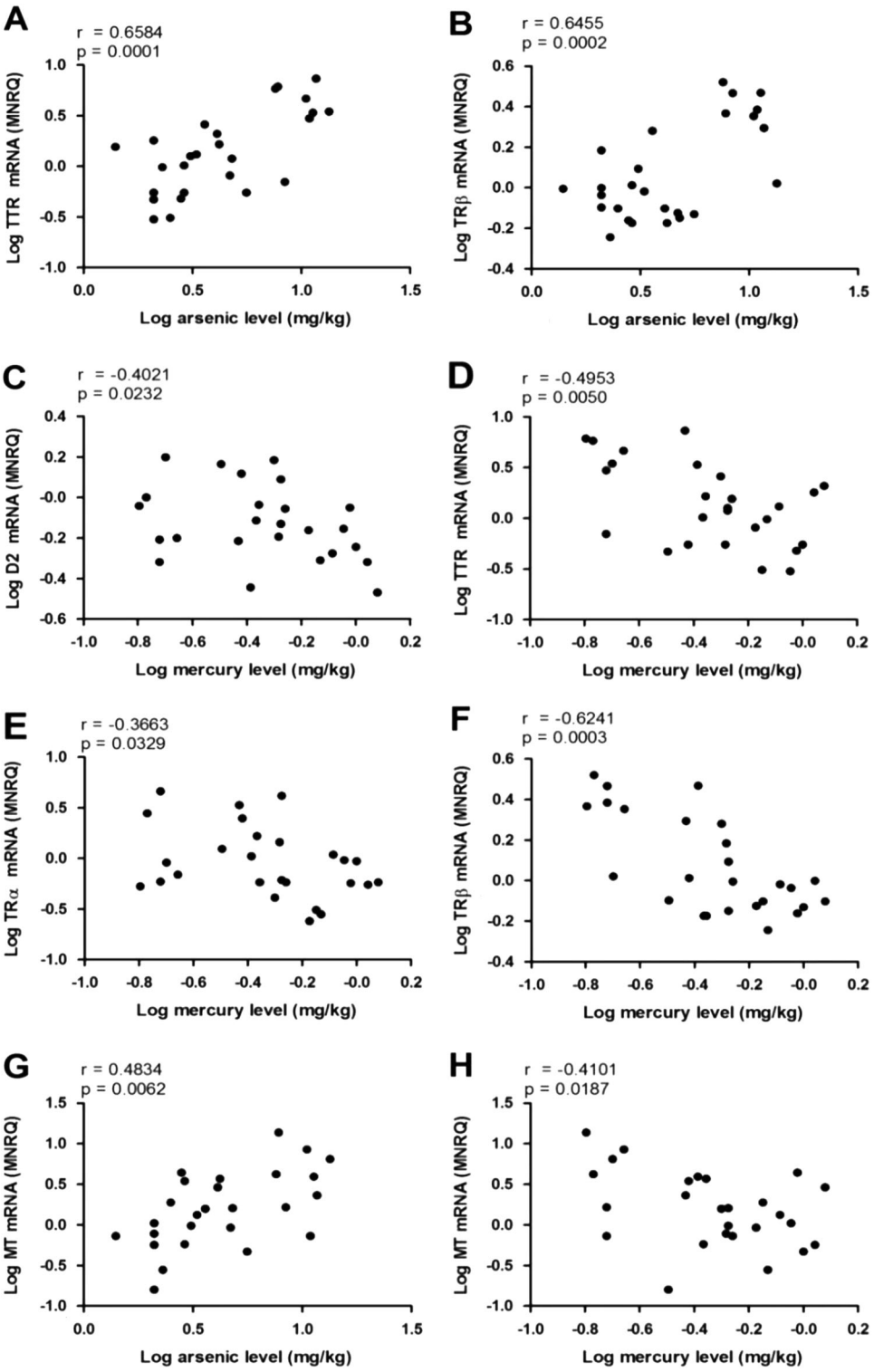

Fig. 4 Correlations between gene expression and heavy metal concentration in sampled fish. The gene expression of TTR **a**, TRβ **b** and MT **g** were positively correlated with As concentration. The significantly negative correlations were found between D2 **c**, TTR **d**, TRα **e**, TRβ **f** and MT **h** gene expressional levels and Hg concentrations

(*Tachycineta bicolor*) inhabiting an area near a Hg polluted river found that both plasma T3 and T4 were suppressed compared to those in reference sites, suggesting Hg may exert inhibitory effects on 5′-deiodinases (Wada et al. 2009). Deiodinases are selenoproteins, and selenium (Se) is the essential element required by deiodinases (Khan and Wang 2009; Orozco and Valverde-R 2005). Methylmercury (MeHg), the predominant form of Hg in the fish body, could impact on the availability of Se (Raymond and Ralston 2004). In zebrafish (*Danio rerio*), selenium protein genes could be down-regulated by elevated MeHg level, suggesting MeHg regulates the selenium protein transcripts

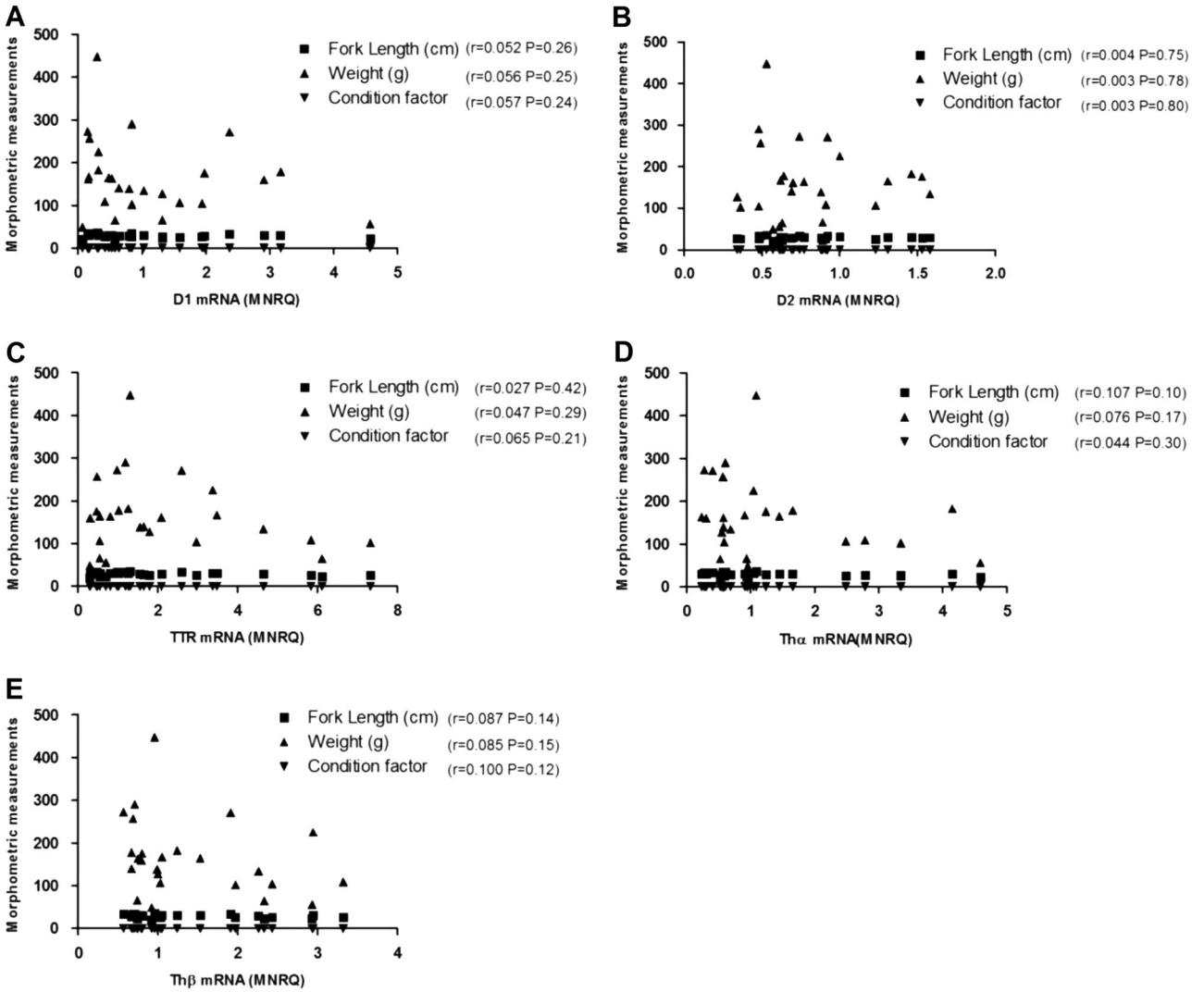

Fig. 5 Correlations between morphometric measurements and thyroid-related gene transcripts in sampled fish

may be affected through low Se availability (Penglase et al. 2014). More recently, it has been confirmed that low levels of Se in sediments were associated with elevated MeHg bioaccumulation in flathead from the Derwent estuary (Jones et al. 2014). Hence, it is reasonable to hypothesise that the interaction between MeHg and Se may result in decreasing the bioavailability of Se thus contributing to down-regulation of the deiodinase gene. The D1 gene showed a similar spatial expression pattern to D2 but there was no significant effect of site and it was not significantly correlated with Hg levels when all the samples were pooled together. It has been suggested that the teleost D1 is resistant to 5′-deiodinase inhibitors (Orozco and Valverde-R 2005). A field study on walleye (*Sander vitreus*) has shown that the D1 gene was not as sensitive as the D2 gene as a molecular marker for environmental pollutants (Picard-Aitken et al. 2007). The data in our study suggest D2 gene was sensitive to Hg exposure.

In this study, TTR gene expression levels were negatively correlated with Hg residues in flathead. In teleost fish, the TTR gene has been cloned from few species (Richardson 2009) and therefore the transcriptional regulation of TTR by thyroid endocrine disrupting chemicals is largely unknown. In the rat, it has been reported that hepatic mRNA levels of TTR were significantly decreased due to selenium deficiency (Kendall and Christensen 1997). As stated above, the accumulation of MeHg could cause Se deficiency which results in down-regulation of transcripts of deiodinases. Thus, one possible explanation for the negative correlation between TTR mRNA levels and Hg residues is that Se deficiency in the fish liver may result in a decrease of TTR transcripts. However, further investigations are needed to confirm this and explain the detailed mechanisms.

The expression levels of two TRs genes, TRα and TRβ, were both negatively correlated with Hg concentrations in the flathead. To the best of our knowledge, no studies on the

effects of Hg on TRs genes regulations in fish have been reported. Previously, it has been demonstrated that the TRs genes could be regulated by thyroid hormones levels. For instance, a study in frog (*Xenopus laevis*) reported that expression levels of TRα and TRβ were up-regulated by T3 at 2-fold and 20-fold, respectively (Yaoita and Brown 1990). Similar results were observed in zebrafish (*Danio rerio*) and striped parrotfish (*Scarus iseri*), TRα and TRβ transcripts were increased by T3 (Johnson and Lema 2011; Liu et al. 2000). In this context, it can be hypothesized that the down-regulations of these two TRs might be due to the decrease of T3 in the fish livers. Consistent with this, in flathead, the expression levels of D2, the predominant 5′-deiodinase in the fish liver that is responsible for the synthesis of T3, was also negatively correlated with Hg concentrations. However, an additional endpoint, such as measurement of T3 levels in fish liver is required to support this hypothesis.

The mRNA levels of TTR in fish liver were higher in Kingston Beach North and positively correlated with As concentrations in fish body. The precise action of As as a toxin is not known, but it is believed to be a result of As binding to cellular proteins (Yan et al. 2009). Proteins such as tubulin, PARP-1, thioredoxin reductase and estrogen receptor-alpha could be the As binding targets (Kitchin and Wallace 2008). In fish, it has been reported that sea bream (*Sparus auratus*) TTR has high affinity with a number of common pollutants including loxinyl and several polybrominated diphenyl ethers (PBDEs) (Morgado et al. 2007). In other vertebrates, several environmental pollutants, such as bisphenol A (BPA), nonylphenol and polychlorinated biphenyls (PCBs), could bind to TTR and disrupt the thyroid hormones homeostasis (Simon et al. 2011; Yamauchi et al. 2003). Based on this observation, it could be assumed that As may bind to TTR and up-regulation of TTR mRNA levels could be a requirement of keeping hormone homeostasis in the fish liver.

Similarly, the thyroid hormone receptor gene TRβ was up-regulated in fish from Kingston Beach North as compared to flathead from Cornelian Bay and its transcripts were positively correlated with As concentrations. It has been reported that As has strong disruptive effects on thyroid hormones by altering gene regulation through the thyroid hormone receptors (Davey et al. 2008; Freitas et al. 2011), but the precise mechanism of As interfering with thyroid hormone receptors is still unclear. In the present study, the high expression of TRβ could contribute to maintaining the normal biological processes when TRs were inhibited by As.

No significant differences were found for TRα mRNA expression among sampling sites and there was no correlation with As concentrations. Previous study reported that mRNA of TRα was not changed in the Chinese rare minnow (*Gobiocypris rarus*) liver but decreased in fish brain when exposed to amitrole (Li et al. 2009). A similar phenomenon was observed in the frog (*Xenopus laevis*) when TRα was down-regulated in the brain but there was no effect on the transcription of this gene in the tail after MMI treatment (Zhang et al. 2006). These results indicated that TRα expression was organ or tissue-specific (Li et al. 2009). The observation in the present study indicates TRα in flathead liver is not sensitive to As exposure. Therefore, these results may imply that thyroid hormone transporter proteins and their receptors could be the target sites for fish exposed to As and that both TTR and TRβ could be regulated by high levels of As in fish.

The highest expression level of MT in flathead was at Kingston Beach North and its transcript was positively correlated with the concentrations of As in flathead muscles. The toxicity of As has been associated with the generation of reactive oxygen species (ROS) (Kondoh et al. 2001; Thornalley and Vasak 1985). Therefore, high levels of ROS could result in lipid peroxidation and cellular damage. In previous studies, it has been proposed that MTs acting as scavengers of ROS could be induced by cumulative ROS. However, a study on the effects of As compounds on hepatic MT expression in catfish liver, showed the induction of hepatic MT by As was not through an oxidative stress mechanism (Schlenk et al. 1997). Additionally, a recent study in mammals suggested that the metal-activated transcription factor 1 (MTF1) was involved in As induction of metallothioneins (Shen et al. 2013). It has been demonstrated that As covalently bound to the C-terminal cysteine cluster of MTF1, which results in inducing the binding of MTF1 to the metal response elements of metallothioneins (He and Ma 2009). In the present study, the up-regulation of MT was present in flathead from Kingston Beach North where the fish had the highest concentrations of As, suggesting MT in flathead could be induced by As exposure. The positive correlation between As and MT transcripts strongly suggests that MT plays a critical role in protecting the liver cells from arsenic-mediated toxicity. These lines of evidence indicate MT could be used as a molecular marker to monitor the As exposure in the Derwent River.

The failure of MT mRNA expression to reflect Hg bioaccumulation in flathead is consistent with previous studies. For example, none of the investigated organs displayed significant correlations between total Hg and MT levels for two fish species, *Dicentrarchus labrax* and *Liza aurata* (Mieiro et al. 2011). Although several investigations have associated an increased MT expression with Hg exposure (Schlenk et al. 1995), the use of fish MTs gene as biomarkers of exposure to Hg is problematic and has been questioned (Mieiro et al. 2011). It is well documented that metallic Hg (II) could induce MT in the injected mouse, while MeHg and Hg (0) are inert in inducing MT(Yasutake and Nakamura 2011). MeHg is considered to be the primary

form of Hg in fish muscle (Harris et al. 2003). In our study, MT expression was not regulated at Ralphs Bay where the fish had the highest levels of Hg accumulation, suggesting the mRNA expression of MT is not sensitive for Hg exposure in flathead. Interestingly, a significant negative correlation was observed between the MT mRNA expression levels and the Hg concentrations in flathead. These results suggest that MT mRNA expression is not suitable for monitoring Hg levels in flathead.

In the present study, there were no significant differences among the relative expressions of CYP1A at different sites. The hepatic mRNA expression of the CYP1A gene in fish has been used to reflect the degree of organic pollution, such as PCBs, PAHs and PBDEs (An et al. 2011; Ibhazehiebo et al. 2011; Shirey et al. 2006). The expression profile of CYP1A in the present study supports the previous report of the Derwent Estuary Program (DEP) that organic pollutants were at low levels in the sediments of the Derwent estuary (http://www.derwentestuary.org.au/).

Conclusion

Flathead from sites contaminated with As and Hg showed changes in hepatic thyroid hormone-related gene expression. The transcription of TTR and TRβ genes were positively correlated with As concentration, suggesting they were sensitive to As exposure, while D2, TTR, TRα and TRβ gene expression were suppressed with increased Hg levels. Whether the relationships between these pollutants and thyroid-related genes transcripts are direct contaminant effects or compensatory reactions remain to be confirmed in a controlled laboratory experiment. Apart from this, further studies of the thyroid hormone levels in fish blood, and histological assessment of fish thyroid follicles are required to adequately address the biological impact of the environmental Hg and As exposure.

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Conflict of interest The authors declare that they have no competing interests.

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