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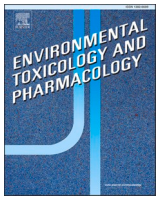

Evaluation of transcriptional biomarkers using a high-resolution regression approach: Concentration-dependence of selected transcripts in copper-exposed freshwater mussels (*Anodonta anatina*)

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ABSTRACT

We tested concentration-dependence of selected gene transcripts (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*) for evaluation as biomarkers of chemical stress. Contrary to the common approach of factorial designs and few exposure concentrations, we used regression across a high-resolution concentration series. Specifically, freshwater mussels (*Anodonta anatina*) were acutely (96 h) exposed to Cu (13 nominal concentrations, measuring 0.13–1 600 μg/L), and transcripts were measured by RT-qPCR. In digestive glands, *cat*, *hsp90* and *mt* decreased with water Cu ($p < 0.05$), but response magnitudes saturated at < 2-fold decreases. In gills, *gst*, *hsp70*, *hsp90* and *mt* increased with water Cu ($p < 0.05$). While *hsp70*, *hsp90* and *mt* exceeded 2-fold increases within the exposure range, high Cu concentrations were required (38–160 μg/L). Although gill responses were generally more robust compared to digestive glands, overall small response magnitudes and moderate sensitivity may set limit for potential application as general biomarkers of chemical stress.

1. Introduction

It has long been suggested that gene transcripts, related to specific mechanisms of action or responding to cellular stress in general, may provide important insights into biological responses to toxicants (Calzolai et al. 2007, Piña et al. 2007, Poynton & Vulpe, 2009). For instance, transcriptomic benchmark dose analysis has shown promise in toxicity screening and regulatory toxicology, for estimating chronic effect levels (Pagé-Larivière et al. 2019) and for identifying pathways sensitive to various pollutants (Martínez et al. 2019, 2020). Another approach is the use of transcriptional responses as early warnings of exposure and adverse effects in environmental risk assessment (Calzolai et al. 2007). The term *biomarker* can, in the context of ecotoxicology and environmental risk assessment, be defined as a measurable biological change in response to exposure to and/or effects from chemical pollution (van der Oost et al. 2003). Various molecular responses, such as enzyme activities and metabolites, have shown promise as biomarkers (van der Oost et al. 2003, Turja et al. 2013, Perić & Burić 2019), suggesting that selected transcriptional responses, separate or as part of integrated biomarker responses, may similarly provide a powerful tool in environmental risk assessment and biomonitoring of pollution.

Organisms have evolved various ways of coping with potentially stressful exposures, and measures commonly include regulation of cellular processes or cytoprotective proteins (Kültz 2003, Birnie-Gauvin et al. 2017). For instance, many stress proteins and enzymes involved in cellular redox homeostasis are considered to respond to general chemical stress, such as non-specific toxicity from metal stressors (Le Saux et al. 2020). Various metal and mixture exposures may induce changed transcription of e.g. metallothionein (*mt*), heat shock proteins 70 and 90 (*hsp70* and *hsp90*, respectively), catalase (*cat*), glutathione-S-transferase (*gst*) and superoxide dismutase (*sod*) (Bigot et al. 2011, Navarro et al. 2011, Liu et al. 2014, 2016; Boukadida et al. 2017). Based on mechanistic understanding, and in some cases empirical data, these and other genes of similar function are suggested to respond to a broad range of chemical stressors, and are therefore considered general biomarker candidates of chemical stress.

For practical use of any biomarker, a basic understanding of response magnitude and variation is crucial (van der Oost et al. 2003, Bahamonde et al. 2016). In order to decide appropriate application of transcriptional responses, assessment across multiple exposure concentrations (among

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other things) may therefore be required (Fent & Sumpter, 2011, Bahamonde et al. 2016). Concentration-response testing is of particular importance for biomarkers intended for quantification of pollutant exposure and/or effects, as well as biomarkers for comparative evaluation of e.g. wastewaters. Using setups of six to ten exposure concentrations, monotonic transcriptional up- and downregulations have been demonstrated in a few aquatic organisms, such as watermilfoil *(Myriophyllum spicatum*), water fleas (*Daphnia magna*), California mussels (*Mytilus californianus*) and Chinook salmon (*Oncorhynchus tshawytscha*) (Osachoff et al. 2013, Smetanová et al. 2015, Hall et al. 2020). In contrast, there is also implication of non-monotonic (for instance U-shaped) concentration-responses of various transcripts, with a seemingly strong up- or downregulation at low exposure and effects diminishing at higher toxicant concentrations (Bigot et al. 2011, Osachoff et al. 2013, Smetanová et al. 2015). Thus, non-monotonic responses may limit the exposure range under which meaningful changes can be detected, which can certainly restrict the biomarker potential. In our experience, many transcriptional studies in environmental sciences and toxicology primarily focus on specific threshold exposures, rather than adopting regression approaches across multiple exposure concentrations. By comparison, experiments covering more than four concentrations are rare even among studies that imply concentration- or dose-dependence of responses (Figure A.1). Consequently, current evaluation of transcriptional biomarker candidates may generally suffer from low resolution and narrow ranges of chemical stress.

In this study, we used regression to test concentration-dependence of six transcripts (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*) in a freshwater mussel (*Anodonta anatina*), using a high-resolution concentration-response setup of acute (96 h) copper exposures. Our hypothesis was that response magnitude, i.e. induction or inhibition relative baseline expression, increases monotonically with increasing Cu exposure via water. The aim was to assess sensitivity and robustness of each response, for evaluation of biomarker potential. For this purpose, we here define sensitivity by the exposure concentration at which a given response occurs, and robustness by the response magnitude at a given exposure or range of exposures. By our definition, a sensitive biomarker is one that responds at low and/or environmentally relevant exposure concentrations, while a robust biomarker gives a predictable, clearly distinguishable response upon exposure. For practical application, the ideal biomarker is both sensitive and robust, demonstrating clear responses to the relevant exposures of interest. Finally, we therefore qualitatively evaluated the overall biomarker potential of the selected transcripts.

2. Material and methods

2.1. Mussel collection and maintenance

We used the European freshwater duck mussel (*A. anatina*) as an environmentally relevant study organism. This generalist species occurs naturally in lakes and rivers across most of Europe and parts of Asia (Lopes-Lima, 2014), and specifically, it is the most common large freshwater mussel in Sweden (von Proschwitz & Wengström 2021). Adult mussels ($n = 40$) of similar size (86 ± 13 mm) were collected on the 10th of April 2019 in Vinne å (Southern Sweden, 56°06'45" N, 13°54'35" E). For 15 days prior to the experiment, mussels were acclimatized to laboratory conditions in continuously aerated reconstituted freshwater (ISO, 6341:2012), hereafter referred to as freshwater medium. To reduce carry-over of sediment from the field, mussels were rinsed under tap water and subsequently under deionized water. Before being transferred to aquaria for long-term acclimatization, mussels were contained for 48 h in a single 60 L aquarium with 40 L freshwater medium and no bottom substrate. During this period, freshwater medium was renewed daily. After 48 h, mussels were randomly subdivided into two 40 L aquaria, each with 30 L freshwater medium and a 5 cm layer of siliceous sand (0.2–0.7 mm) as bottom substrate. The sand was of the same origin and was washed as has been previously described (Ekelund Ugge et al. 2020). Three times weekly, all freshwater medium was renewed, upon which mussels were randomly re-distributed between aquaria to reduce any tank effects on acclimatization. The mussels were fed by additions of a *Pseudokirchneriella subcapitata* culture, corresponding to approximately 8×10^5 cells $\times$ mussel^{-1} $\times$ day^{-1}, except no food was added within 48 h of the experiment start. During acclimatization and experimental periods, water temperature was 20 ± 1 °C, and the light cycle was 16 h light: 8 h dark.

2.2. Experimental exposures

We used Cu as our model stressor, aiming to cover a large fraction of sublethal Cu stress with high resolution. A Cu stock solution was made by dissolving $CuCl_2 \bullet 2\ H_2O$ (Fisher Scientific, USA) in deionized water, to a nominal concentration of 100 mg Cu/L. A concentration series was prepared by dilution of the stock solution in freshwater medium. Using 2 000 μg Cu/L as our highest concentration and a dilution factor of 2, we prepared exposure media at nominal concentrations of 0 (control), 1, 2, 3.9, 7.8, 16, 31, 63, 125, 250, 500, 1 000 and 2 000 μg Cu/L. A total of 40 glass containers (Ø 12 cm) were prepared with 1 L of respective exposure medium 48 h prior to experimental start (four containers as control and three containers each for remaining nominal concentrations). In each container, a 5 cm layer of glass marbles (Ø 16 mm) was added as substrate, and the exposure medium was continuously aerated. At the start of experiments, mussels ($n = 40$) were randomly assigned to the exposure containers, with one individual in each. During the exposure, *P. subcapitata* was added daily at the same amount as during acclimatization. Oxygen and pH were monitored in each container at 0, 48 and 96 h (Appendix B). At the start of the experiment, oxygen saturation was 99 – 100% (9.0 – 9.2 mg/L) and pH 7.7 – 8.0 in all containers. O_2 was stable over time in most containers, but decreased slightly in some after 96 h (88 – 100%, 7.9 – 9.2 mg/L). While pH generally decreased over time ($\geq$ 6.3 at 96 h in control, 1 – 125 μg Cu/L), it increased slightly in the highest Cu exposure groups ($\leq$ 8.3 at 96 h in 250 – 2 000 μg Cu/L). After 96 h, exposure was ended, and mussels dissected to extract gills and digestive glands. Tissue aliquots from each mussel were immediately submerged in RNA-Later (Invitrogen, USA) for transcriptional analyses, and subsequently stored at − 20 °C. Remaining tissue was immediately frozen in liquid nitrogen, stored at − 80 °C, and subsequently used for chemical analysis.

2.3. Chemical analysis

Medium was sampled from each exposure container at the experimental start and frozen (−20 °C) prior to chemical analysis. Samples were acidified by addition of nitric acid (1% v/v), and total Cu concentration was measured in unfiltered samples by inductively coupled plasma sector field mass spectrometry (ICP-SFMS) (*Element*, Thermo Scientific, Germany) (ISO 17294–2:2016, U.S. EPA 1994a). Measured and nominal Cu concentrations were strongly correlated (Fig. 1), and measured concentrations were used for further analyses.

Twelve previously analyzed water samples from Vinne å (Ekelund Ugge et al., 2020, 2022) were included as background references of Cu concentrations (Appendix C). Also, one additional water sample was collected from Vinne å on the 10th of April 2019 and immediately frozen (−20 °C) upon arrival to the laboratory. After acidification by nitric acid (1% v/v), total concentrations of a number of elements (Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, P, Pb, Si, Sr, V, and Zn) were analyzed in this sample by ICP-SFMS (Element, Thermo Scientific, Germany), inductively coupled plasma atomic emission spectrometry (ICP-AES) (Agilent ICP-OES 725, Agilent, USA) and atomic fluorescence spectrometry (AFS) (PSA Millennium Merlin, P S Analytical, UK) (ISO 17852:2006, ISO 11885:2007, ISO 17294–2:2016. U.S. EPA 1994a, 1994b). This water sample was used both as a Cu background reference, and to ensure overall low metallic contamination in the sampling site. Using Swedish environmental quality standards (EQS) of As, Cd, Cr, Cu,

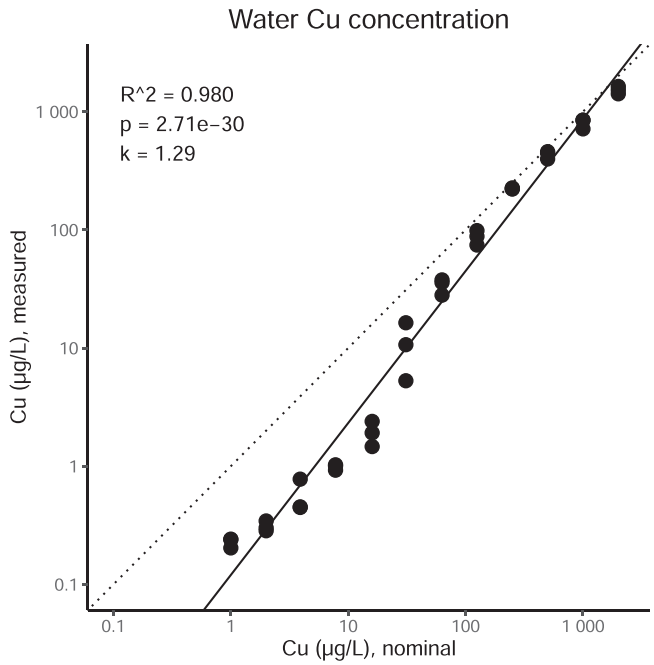

Fig. 1. Measured water concentration of total Cu relative nominal concentrations. Water was sampled at the start of experiments, 48 h after preparation of exposure medium and experimental containers. Points represent exposure containers to which Cu was added. The black line shows the fitted linear regression, while the dashed line illustrates a 1:1 relationship.

Hg, Ni, Pb and Zn (Havs- och vattenmyndigheten, 2019), background metal contamination was determined low overall. Measured total Cu slightly exceeded the EQS (although set for the bioavailable fraction only), while remaining metals measured below the respective EQS (Appendix C).

Cu concentrations were also measured in an aliquot of the snap frozen gill and digestive gland from each individual, except for one digestive gland sample that was accidentally lost (Appendix D). In addition, gill and digestive gland samples from three mussels were analyzed as biological background references for Vinne å. These tissues were dissected in the field on the 27th of April 2018, and stored at − 20 °C since. Prior to analysis, the tissue samples were digested with nitric acid/hydrogen peroxide, and metal concentrations were measured by ICP-SFMS (*Element 2*, Thermo Scientific, Germany) (ISO 17294–2:2016, U.S. EPA 1994a). In addition to Cu, tissue concentrations of Ca, Na and K were measured to give an overview of potential effects on metallic electrolytes (Appendix D). Additional trace elements and contaminants (As, Cd, Co, Cr, Fe, Hg, Mn, Ni, Pb, Zn) were also measured, to ensure low metallic contaminant loads from the sampling location (Appendix D). For each tissue, principal component analyses (PCAs) were performed to visually explore patterns of different metal content with different Cu-exposure, and to reveal potential effects on tissue electrolytes (Figure A.2).

2.4. Biomarker candidates

Six genes (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*) were selected as transcriptional biomarker candidates of general toxicity and chemical stress. Relative gene expression levels were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using the *SurePrep™ TrueTotal™ RNA Purification Kit* (Fisher Scientific, USA) or the *Norgen Total RNA Purification Kit* (Norgen, Canada) (Appendix B). A final concentration of 1% (v/v) β-mercaptoethanol (Sigma Aldrich, USA) was added to the lysis buffer to protect RNA from degradation by RNases. Tissues were homogenized using a *TissueLyser LT* (Qiagen, Germany) and 5 mm stainless steel beads (Qiagen, Germany). DNase treatment was performed using the *Norgen RNase-Free DNase I Kit* (Norgen, Canada) or *Invitrogen DNA-Free™ Kit* (Invitrogen, USA) (Appendix B). After extraction, RNA amounts were measured using the *Qubit™ RNA HS Assay Kit* on a *Qubit 4* fluorometer (Invitrogen, USA) and A260/A280 ratios were measured using a *NanoDrop 2000* spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using a *Fragment Analyzer* (Advanced Analytical, Austria). Reverse transcription was performed despite generally low RNA quality numbers (Appendix B). This measure is however likely biased towards underestimating integrity of mussel RNA, by not accounting for the invertebrate 28 S RNA hidden break (Natsidis et al. 2019, Adema, 2021). Regardless, the targeted sequences were short (<200 bp, Ekelund Ugge et al. 2020, appendix) and the objective was assessing relative rather than absolute gene expression, which reduces the risk of downstream impacts from RNA degradation (Fleige & Pfaffl, 2006). cDNA was synthesized by reverse transcription of 200 ng RNA, using the *TATAA GrandScript cDNA Synthesis Kit* (TATAA Biocenter AB, Sweden). The cDNA was diluted a 4-fold with nuclease-free water, and 2 µl was then used in each qPCR assay. qPCR was performed on an *Applied Biosystems™ QuantStudio™ 12 K Flex* (Applied Biosystems, USA), using *TATAA SYBR® GrandMaster® Mix Low Rox™* (TATAA Biocenter AB, Sweden). The assays were performed as previously described (Ekelund Ugge et al. 2020). Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), where expressions were normalized internally for each individual sample by the mean of the three reference genes *β-actin*, *18 S* rRNA and *28 S* rRNA (Figure A.3), and then by the mean of control samples of gill and digestive gland tissue, respectively.

Similar to previous transcriptional concentration-response assessment (Smetanová et al. 2015), a model selection was performed for each separate transcript from various regression models (linear, sigmoidal, exponential and hormetic). Relative expression, separated by tissue, was fitted to measured Cu concentrations in water, as well as to measured tissue Cu concentration. In order to evaluate biomarker potential, response sensitivity and robustness were evaluated from selected models. First, for all models reaching a response plateau (i.e. a maximal model response magnitude, Δ_{max}), a 50% effect concentration (EC_{50}) was determined. In this case, EC_{50} was defined as the concentration corresponding to a response magnitude of 50% relative the Δ_{max} (Hall et al. 2020). Each EC_{50} was in turn used as a measure of sensitivity and the corresponding response magnitude as a measure of robustness. Second, we assessed the sensitivity from a set response magnitude. In early transcriptional studies by microarrays, a 2-fold change in expression (100% increase relative the control) has commonly been used as a cutoff for transcriptional responses (e.g. McArthy & Smyth, 2009). Here, we set each response magnitude to a $|1|$ $\log_2$ fold-change (100% increase or 50% decrease relative the control), and in turn extracted corresponding exposure concentrations from each model. In turn, sensitivity was determined by the respective threshold concentration and robustness by the 95% confidence intervals (CIs) of the $|1|$ $\log_2$ fold-change. Finally, estimated sensitivity and robustness were used for a qualitative overall evaluation of biomarker potentials for detection of environmentally relevant Cu exposures.

2.5. Statistics

Statistical analyses were run in R version 4.0.5 (R Core Team, 2021), using the 'ggplot2' and 'ggpubr' packages (Wickham, 2016, Kassambara, 2020) for figure production. Log_2-transformed relative gene expressions, separated by tissue, were fitted to measured water and tissue concentrations of Cu by a selection of regression models. Linear models were fitted by linear regression on the linear and log_{10}-transformed concentration scale, respectively. The 'drc' package (Ritz et al. 2015) was used to fit log-logistic models, Weibull models (type I and II), asymptotic regression models, exponential decay models, and hormesis

models (Brain-Cousens hormesis models and Cedergreen-Ritz-Streibig models). For each response variable, the respective model with the lowest Akaike information criterion (AIC) score was selected as the best fit. For models fitted with the 'drc' package, the 'ED' function was used for estimation of EC_{50}. The significance ($\alpha = 0.05$) of each selected model was tested against a linear regression model with a slope of zero. Finally, we fit regression models to tissue concentrations of Cu and Na relative water Cu concentration. Tissue Cu was modeled on the basis of our experimental design to test the concentration-dependence of Cu net accumulation upon acute exposure. Tissue Na was modeled on the basis of exploratory PCAs on tissue metals (Figure A.2), to test the trend of decreasing tissue Na with increasing water Cu observed for both gills and digestive glands. As neither Cu uptake nor Na dynamics were our primary focus, we used a statistical rather than mechanistic modeling approach to test correlations. The model selection was performed by the same procedure as for transcripts, where tissue concentrations were treated as responses, analogous to relative gene expression.

3. Results

Transcripts were, in general, negatively related to water concentrations of Cu in digestive glands, and positively related in gills (Fig. 2). Three digestive gland (*cat*, *hsp90* and *mt*) and four gill transcripts (*gst*, *hsp70*, *hsp90* and *mt*) demonstrated significant monotonic concentration-response relationships ($p < 0.05$), implying transcript inhibition and induction, respectively with increasing water Cu concentration (Fig. 2, Table A.1).

According to the fitted concentration-response models, response magnitudes were in general larger in gills than in digestive glands. Three gill transcripts demonstrated robust responses by exceeding $|1|$ $\log_2$ fold-changes within the tested Cu range (*hsp70* at 160, *hsp90* at 38 and *mt* at 160 μg Cu/L, respectively) (Table 1). For *gst*, *hsp70* and *hsp90* in gills, the EC_{50} was 23, 78 and 37 μg Cu/L, respectively, with corresponding response magnitudes of 0.31, 0.67 and 0.64 $\log_2$ fold-changes, respectively. The *gst* and *hsp90* CIs however included 0 at EC_{50}. In contrast, no transcript in digestive glands was robust as to reach a $|1|$ $\log_2$ fold-change within the measured range (Table 1). Apart from *mt* (EC_{50} = 11 μg Cu/L), digestive gland EC_{50} values were < 1 μg Cu/L (Table 1). Response magnitudes in digestive gland transcripts were in general small, and CIs consistently included 0 at EC_{50}.

In gills, responses relative tissue Cu concentration were similar to responses relative water Cu, while digestive glands demonstrated mixed trends. In general, responses in gills increased monotonically with tissue Cu concentrations ($p < 0.05$ for all transcripts except *sod*) (Fig. 3, Table A.2). In digestive glands, we observed trends of both increasing and decreasing expressions, but *hsp70* and *hsp90* increased monotonically with tissue Cu concentration ($p < 0.05$) (Fig. 3, Table A.2). All gill transcripts exceeded a $|1|$ $\log_2$ fold-change under the measured tissue Cu ranges, and except for *cat*, no CI included 0 (Table 2). In digestive glands, only *sod* exceeded a $|1|$ $\log_2$ fold-change under the measured tissue Cu ranges, but the CI included 0 (Table 2). Digestive gland *hsp70* and gill *hsp90* were the only transcripts that both demonstrated significant monotonic increases and for which EC_{50} values were within the measured tissue Cu ranges (3 900 and 4 900 μg/kg WW, respectively) (Table 2). The corresponding response magnitudes (−0.34 and 0.61 $\log_2$ fold-change, respectively) were however not significantly different from 0.

Across all samples, the measured digestive gland and gill Cu concentrations ranged from 2 500–6 100 μg and 1 200–35,000 μg/kg wet weight (WW), respectively (Appendix D). Mean Cu concentrations in the background samples were 1.6 μg/L for water (ranging from 0.080 to 3.4 μg/L, Appendix C), 3 000 μg/kg in digestive glands (2 900 – 3 000 μg/kg WW, Appendix D) and 1 700 μg/kg WW in gills (1 200 – 2 100 μg/kg WW, Appendix D). In the experimental mussels, tissue concentration of Cu increased with water concentration, both in digestive glands and gills (Fig. 4, Table A.3). Digestive gland concentrations reached a plateau within the measured range of water Cu concentrations (Fig. 4A), while the Weibull model fitted to gill Cu suggested further increasing tissue Cu beyond the scope of tested water concentrations (Fig. 4B). In contrast, there was a negative correlation between water Cu and Na concentrations in both digestive gland and gill tissue, with Na declining from background levels of approximately 250 – 300 mg/kg WW down to the 100 mg/kg WW range at high exposures (Fig. 5, Table A.3). With regards to overall metal content of the tissues, measured concentrations were in general similar between tissues, except for Ca and Mn, for which concentrations were approximately two orders of magnitude higher in gills (g/kg WW) than in digestive glands (mg/kg WW) (Appendix D). Non-essential elements were present in both tissues in the low to intermediate μg/kg WW range (Cd, Hg, Pb), or approaching low mg/kg WW levels (As) (Appendix D). Remaining metals were in the intermediate μg/kg WW (Co, Cr, Ni) or mg/kg WW (Fe, K, Zn) ranges (Appendix D).

4. Discussion

4.1. Cu stress exposure

In freshwater ecosystems, Cu concentrations range from approximately a few hundreds of nanograms per liter in pristine environments to hundreds of micrograms per liter or more in polluted areas (Vukosav et al. 2014, Bhuiyan et al. 2015, Álvarez-Vázquez et al. 2017). Although toxic at high concentrations, Cu is essential to biological life and a theoretical metabolic requirement of 26 300 μg Cu/kg dry weight (DW) has been proposed for mollusks (White & Rainbow, 1985). Naturally occurring soft tissue Cu content in the range of 11 000 – 34,000 μg/kg DW has been reported for various species of freshwater mussels (Kraak et al. 1992, Le et al. 2011, Bertucci et al. 2017). Based on WW to DW conversion factors of 6.9–10.5 for bivalve soft tissue (Ricciardi & Bourget, 1998), the data would suggest a background range of approximately 1 000 – 5 000 μg Cu/kg WW, consistent with concentrations measured in our control mussels and background mussels sampled directly from Vinne å. Thus, this range appears to represent natural background Cu levels in *A. anatina* tissues, suggesting 5 000 μg/kg WW as an approximate threshold for indication of elevated tissue levels.

Tissue Cu concentrations were positively correlated to the Cu concentration in water, both for digestive glands and gills. While concentration- and time-dependent uptake into mussel soft tissues is to be expected upon water exposure to Cu (Won et al. 2016, Le et al. 2021), small increases of essential elements could potentially fall within the background noise from baseline level variation. For instance, we only observed slightly elevated digestive gland Cu levels (≤ 6 100 μg/kg WW). In contrast, multiple gill samples exceeded 5 000 μg Cu/kg WW, reaching up to 35,000 μg/kg WW. Assuming time-dependent uptake, it might not be surprising that high acute Cu exposure can result in similar accumulation as longer exposure to lower concentrations. For instance, the two highest exposure groups of our study demonstrated gill concentrations in the same range as *A. anatina* after 15 days of laboratory exposure to 120 – 360 μg Cu/L (Sohail et al. 2016). Similar gill concentrations have also been reported for the freshwater mussel *Diplodon chilensis* after long-term (30–60 days) *in situ* exposure at moderate to high Cu concentrations (Yussepone et al. 2020). For acute exposures, there are kinetic two-compartment models that, under various exposure settings, may explain higher short-term Cu uptake in the bivalve gill than in remaining soft tissues (Sánchez-Marín et al. 2016, Le et al. 2021). Similar differences might also be expected for non-essential metals, such as Cd (Cooper et al. 2010). Considering the bivalve gill is commonly the tissue in first contact with foreign compounds, metal uptake into gills, and subsequent molecular responses, may simply reflect a first line defense against acute stressors (Won et al. 2016). In contrast, digestive gland metal accumulation can on the short term be more prominent upon e.g. dietary exposure (Cooper et al. 2010, Sánchez-Marín et al. 2016). Overall, while observed differences in digestive gland Cu were

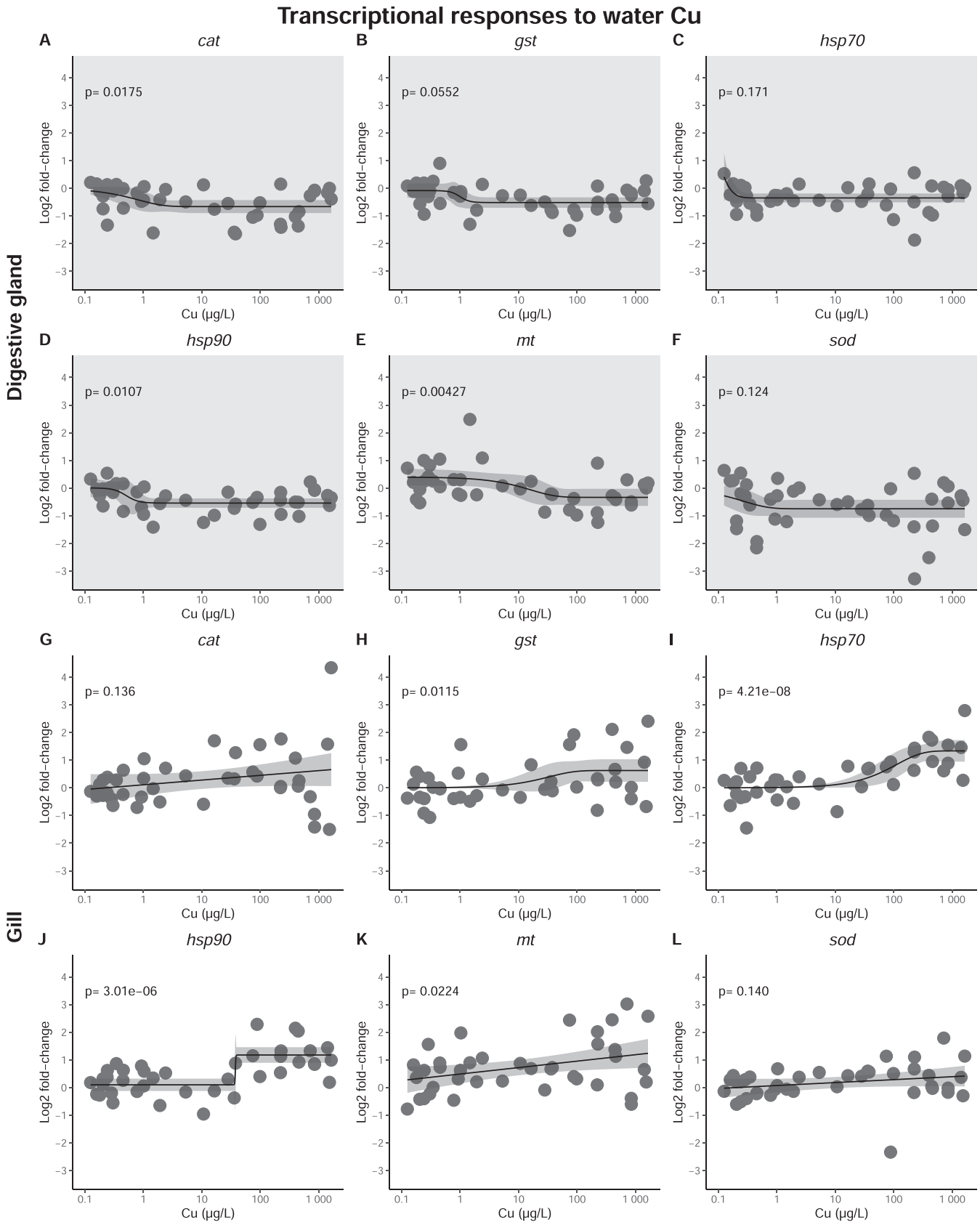

Fig. 2. Concentration-response relationships of transcriptional biomarker candidates in digestive glands (A-F) and gills (G-L) of *A. anatina* ($n = 40$) acutely exposed to Cu via water (96 h). Black lines correspond to fitted regression models and shaded areas represent model 95% confidence intervals. In digestive glands, asymptotic regression models were fitted for *cat* (A), *hsp70* (C) and *sod* (F), log-logistic models for *gst* (B) and *hsp90* (D), and an exponential decay model for *mt* (E). In gills, linear-log models were fitted for *cat* (G), *mt* (K) and *sod* (L), asymptotic regression models for *gst* (H) and *hsp70* (I), and a log-logistic model for *hsp90* (J). Model details and parameters are presented in Table A.1.

Table 1
Fitted transcriptional responses in *A. anatina* to acute (96 h) Cu exposure via water. Modeled EC_{50} values are presented with corresponding response magnitudes (log_2 fold-change). Also presented is the modeled threshold concentration for exerting a response of $\geq |1|$ (log_2 fold-change; i.e. changes corresponding to ≥100% increase or ≥50% reduction) and response confidence intervals (CIs) at this concentration.

Tissue	Transcript	EC_{50} (μg/L)	Response magnitude (log_2 fold-change) (95% CI)	Threshold for log_2 fold-change $\geq |1|$ (μg/L)	CI (95%) at log_2 fold-change $= |1|$
Digestive gland	*cat*	0.56	−0.33 (−0.68 to 0.024)	–[c]	–[c]
	gst	0.99	−0.30 (−0.68 to 0.089)	–[c]	–[c]
	hsp70	0.028[a]	8.4 (−28 to 45)	0.10[a]	−1.2 – 3.2
	hsp90	0.51	−0.27 (−0.81 to 0.28)	–[c]	–[c]
	mt	11	0.031 (−0.40 to 0.46)	–[c]	–[c]
	sod	0.185	−0.37 (−0.79 to 0.040)	–[c]	–[c]
Gill	*cat*	–[b]	–[b]	170,000[a]	−0.0098 – 2.0
	gst	23	0.31 (−0.12 to 0.75)	–[c]	–[c]
	hsp70	78	0.67 (0.22 to 1.1)	160	0.56 – 1.4
	hsp90	37	0.64 (−0.58 to 1.9)	38	0.13 – 1.9
	mt	–[b]	–[b]	160	0.63 – 1.4
	sod	–[b]	–[b]	520,000,000[a]	−0.12 – 2.1

[a] Outside the measured range.
[b] Not applicable for linear models.
[c] Maximum modeled response magnitude $< |1|$ log_2 fold-change.

small, our results imply concentration-dependent Cu accumulation in gills under current water exposure.

Generally, our study demonstrated little overall effects from Cu on tissue metal content in *A. anatina*. In addition to tissue Cu, there were however also exposure-dependent differences in tissue Na. Interestingly, Na in both gills and digestive glands was negatively correlated to measured water Cu. It has been demonstrated that Cu uptake can partially occur by Na-dependent pathways (Grosell & Wood, 2002, Nadella et al. 2007), and the external Na concentrations may affect Cu uptake by mussels (Le et al. 2021). Contrary, interference with Na uptake, for instance via inhibited Na^+/K^+-ATPase activity, could in itself occur as an effect from Cu exposure (Pelgrom et al. 1995, Giacomin et al. 2013, Zimmer et al. 2014). Exposure to Cu has previously been linked to decreasing soft-tissue Na concentrations in freshwater mussels (Jorge et al. 2013), and it has been proposed that Na turnover rate is an important predictor for Cu toxicity in freshwater animals (Grosell et al. 2002). Despite lower tissue Cu and overall smaller transcriptional responses in digestive glands than in gills, our study revealed similar relationships between tissue Na and water Cu for both tissues. This implies that Cu exposure in gills could eventually translate to downstream effects on whole-organism Na balance. While further investigation of Cu and Na fluxes would be required for mechanistic conclusions, our observations are overall consistent with previous findings, suggesting concentration-dependent osmoregulatory stress from Cu exposure.

4.2. Transcriptional concentration-response relationships

We assessed concentration-response relationships for six transcriptional biomarker candidates in *A. anatina*, and found monotonic responses for three transcripts in digestive glands and four in gills. A key aspect of practical biomarker application is to establish the relationship between an exposure and corresponding response (van der Oost et al. 2003). Consequently, it has been argued that concentration-response testing is crucial for assessing transcriptional responses to pollution (Fent & Sumpter, 2011). Many transcriptional biomarker candidates might not respond to given exposures of interest, while others may display for instance U-shaped response curves across exposure concentrations (Osachoff et al. 2013, Smetanová et al. 2015). While different response types could potentially be considered for biomarker use, monotonic relationships (e.g. linear, sigmoidal or exponential) may facilitate response prediction, and hence, practical application. Thus, establishing concentration-response relationships is necessary to determine potential applications, as well as limitations, of transcriptional biomarker candidates.

Transcripts in gills and digestive glands consistently showed opposite response directions relative water Cu. Most levels decreased in digestive glands with increasing water exposure, and corresponding transcripts increased in gills. Furthermore, some digestive gland responses showed opposite trends relative water and tissue Cu concentration. For instance, digestive gland *hsp90* increased with tissue Cu despite decreasing with water Cu. Since measured concentrations suggested only minor Cu accumulation in digestive glands, response patterns may behave differently under e.g. dietary or long-term water exposure. As compared to digestive glands, gills displayed a wider range of tissue Cu concentrations, and response trends were consistent relative both water and tissue Cu concentration for all transcripts. In general, results therefore imply that molecular responses upon acute Cu exposure, and perhaps metal stress in general, are more predictable and easier to interpret in gills.

Using RNASeq, a range of monotonic responses has been demonstrated in the marine California blue mussel, *M. californianus*, upon acute water exposure to Cu (Hall et al. 2020). Although transcripts involved in e.g. the cell cycle were proposed as the most sensitive biomarkers to acute Cu stress, there was evidence of concentration-dependent induction of genes related to oxidative stress and protein chaperoning (Hall et al. 2020), similar to our observations in *A. anatina*. For instance, *hsp70* and *gst* isoforms (GST A2, GST Mu4, GST omega-1, Hsp70B2) were upregulated in gills of adult *M. californianus*, with EC_{50} values in the range of 69 – 93 μg/L (Hall et al. 2020). These response patterns are comparable to *gst*, *hsp70* and *hsp90* in *A. anatina* gills. Therefore, although other pathways may prove more sensitive to Cu exposure and metal stress, observed gill responses in exposed bivalves do imply monotonically increasing expression of various genes involved in oxidative stress and general cytoprotection.

4.3. Biomarker application

All biomarker candidates selected for this study are related to general toxicity (oxidative stress and cellular disruption), suggesting that altered transcription can reflect coping measures against chemical stress. It has been proposed that integration into mechanistic models might be required for practically useful prediction of adverse effects (Forbes et al. 2006). In practice, incorporation into predictive models would assume that sensitivity and robustness of the potential biomarker are established and validated, which is often not the case. Hence, our main focus at this stage was response sensitivity and robustness to exposure, rather than effect prediction or further mechanistic elucidation. In our study, three gill transcripts (*gst*, *hsp70* and *hsp90*) were fitted to non-linear functions, demonstrating EC_{50} values at relatively high Cu concentrations (23–78 μg/L), indicating only moderate sensitivity. For digestive glands, we could extract EC_{50} values for all responses. Except for *mt*, EC_{50} values

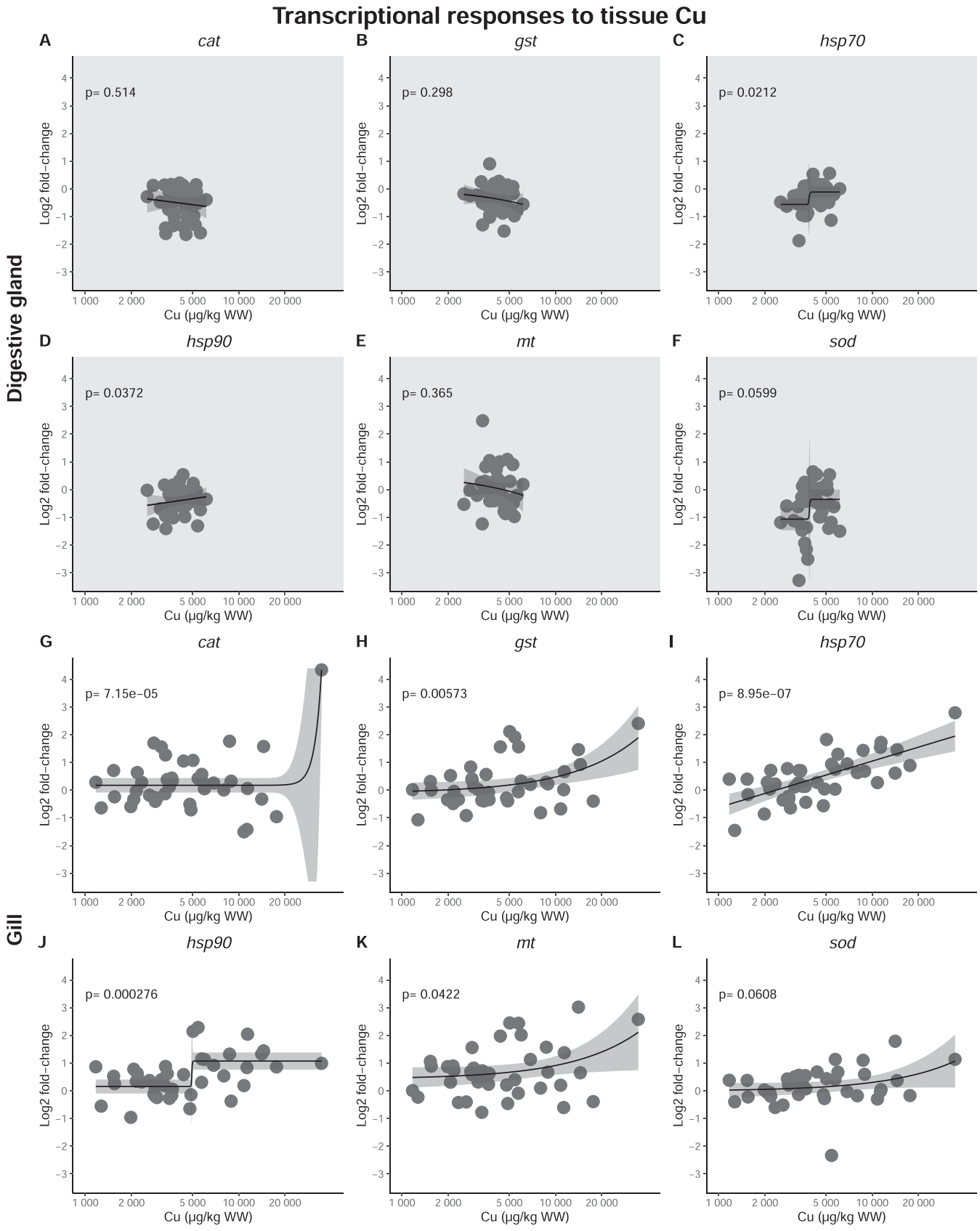

Fig. 3. Transcriptional responses in digestive glands ($n = 39$) (A-F) and gills ($n = 40$) (G-L) of Cu-exposed (96 h) *A. anatina* relative tissue Cu concentration. Black lines correspond to fitted regression models and shaded areas represent model 95% confidence intervals. In digestive glands, linear-log models were fitted for *cat* (A) and *hsp90* (D), linear models for *gst* (B) and *mt* (E), a Weibull type 1 model for *hsp70* (C) and a log-logistic model for *sod* (F). In gills, a log-logistic model was fitted for *cat* (G) and *hsp90* (J), linear models for *gst* (H), *mt* (K) and *sod* (L), and a linear-log model for *hsp70* (I). Model details and parameters are presented in Table A.2.

Table 2
Fitted transcriptional responses in *A. anatina* relative internal tissue Cu concentrations. Modeled EC_{50} values are presented with corresponding response magnitudes (log_2 fold-change). Also presented is the modeled threshold concentration for exerting a response of $\geq |1|$ (log_2 fold-change; i.e. changes corresponding to ≥100% increase or ≥50% reduction) and response confidence intervals (CIs) at this concentration.

Tissue	Transcript	EC_{50} (µg/kg WW)	Response magnitude (log_2 fold-change) (95% CI)	Threshold for log_2 fold-change $\geq \|1\|$ (µg/kg WW)	CI (95%) at log_2 fold-change $= \|1\|$
Digestive gland	*cat*	–[a]	–[a]	20,000[b]	−2.5 – 0.52
	gst	–[a]	–[a]	11,000[b]	−2.2 – 0.23
	hsp70	3 900	−0.34 (−1.6 to 0.94)	–[c]	–[c]
	hsp90	–[a]	–[a]	230,000[b]	−2.1 – 4.1
	mt	–[a]	–[a]	12,000[b]	−3.3 – 1.3
	sod	3 900	−0.71 (−3.3 to 1.9)	3 900	−2.0 – 0.036
Gill	*cat*	49,000[b]	65 (−1 300 to 1 500)	29,000	−3.8 – 5.8
	gst	–[a]	–[a]	19,000	0.43 – 1.6
	hsp70	–[a]	–[a]	9 300	0.73 – 1.3
	hsp90	4 900	0.61 (−1.2 to 2.4)	5 000	0.34 – 1.7
	mt	–[a]	–[a]	12,000	0.61 – 1.4
	sod	–[a]	–[a]	32,000	0.12 – 1.9

[a] Not applicable for linear models.
[b] Outside the measured range.
[c] Maximum modeled response magnitude $< |1|$ log_2 fold-change.

were consistently < 1 µg/L in this tissue, suggesting higher sensitivity than in the gill. On the other hand, response magnitudes were generally larger for gill than for digestive gland transcripts, implying higher robustness. Importantly, all confidence intervals of digestive gland responses at EC_{50} included 0, while *hsp70* in gills did not. Therefore, gill *hsp70* was the only biomarker giving a significant, robust response at its EC_{50}, while digestive gland responses in general appeared to saturate at background, or slightly elevated, water Cu levels.

Within the tested concentration range, all digestive gland transcripts reached their respective maximal response magnitudes at $< |1|$ log_2 fold-change. Again, this suggests that concentration-dependent responses were not very robust in this tissue. Some digestive gland transcripts could potentially be applied for detection of low concentration Cu exposure (or moderate in the case of *mt*), but this would likely require large sample sizes considering the generally small response magnitudes relative baseline variation (Ekelund Ugge et al. 2020). With regards to the separate digestive gland transcripts, practical applicability, if any, could ultimately be limited to 'on/off' response detection at selected, low water concentrations.

In gills, *hsp70*, *hsp90*, and *mt* demonstrated robust responses of ≥ 1 log_2 fold-changes, i.e. 2-fold increases, at the high end of environmentally relevant Cu concentrations (38–160 µg/L). In contrast, *cat* and *sod* would, based on extrapolation from the respective linear models, require extremely high Cu levels (170 mg/L – 520 g/L) for $\geq |1|$ log_2 fold-changes to occur. Overall, this would suggest higher importance of general cytoprotection when coping with Cu stress, as compared to pathways involved in protection against oxidative damage. In fact, considering that concentrations in the mg/L range are often lethal to bivalves (Okazaki, 1976, Ong & Din, 2001, Watters et al. 2013), the results suggest that even mussel mortality could be a more sensitive endpoint than a $\geq |1|$ log_2 fold-change of *cat* or *sod*. Furthermore, no response magnitudes reached $|2|$ log_2 fold-changes within the tested Cu range. By comparison, maximal observed response magnitudes in *M. californianus* gills ranged from $< |1|$ up to $\geq |4|$ log_2 fold-changes, despite exposures up to only 120 µg/L (Hall et al. 2020). Although potentially robust enough for detection of acute exposure to high Cu concentrations, gill *hsp70*, *hsp90* and *mt* in *A. anatina* were limited by high variation, small response magnitudes (considering the wide range of tested Cu concentrations) and a moderate sensitivity. Whether or not sufficient for practical use as biomarkers will ultimately depend on the intended application.

4.4. Conclusions

We have demonstrated concentration-dependent, monotonic responses in a range of transcriptional stress biomarker candidates in *A. anatina*. In gills, both Cu levels and most of the tested transcripts increased monotonically. In contrast, both Cu and transcripts in digestive glands generally demonstrated a substantial overlap with the background variation, even at high water Cu concentrations. Based on

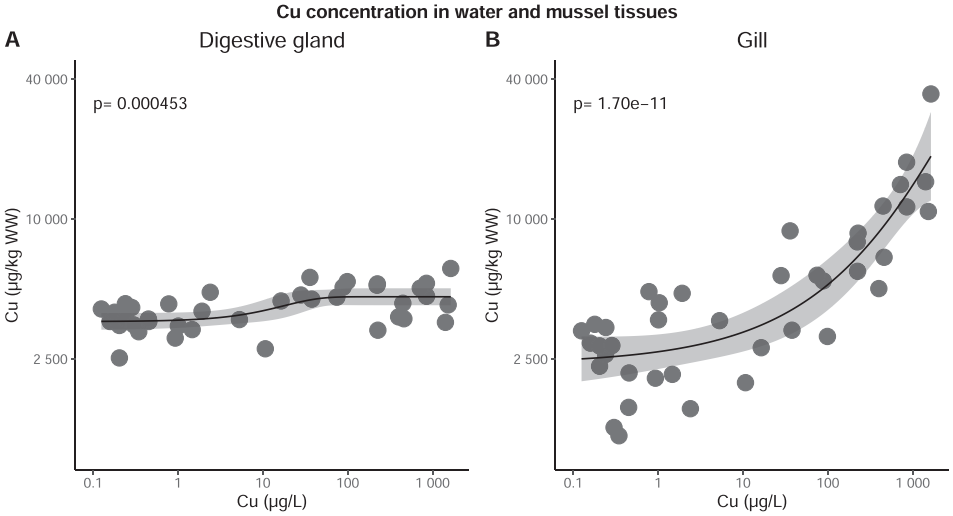

Fig. 4. Tissue Cu concentration relative water Cu in digestive glands ($n = 39$) (A) and gills ($n = 40$) (B) of mussels (*Anodonta anatina*). Black lines correspond to the fitted regression models and shaded areas represent model 95% confidence intervals. An asymptotic regression model was fitted for digestive glands (A), and a Weibull type 2 model for gills (B). Model details and parameters are presented in Table A.3.

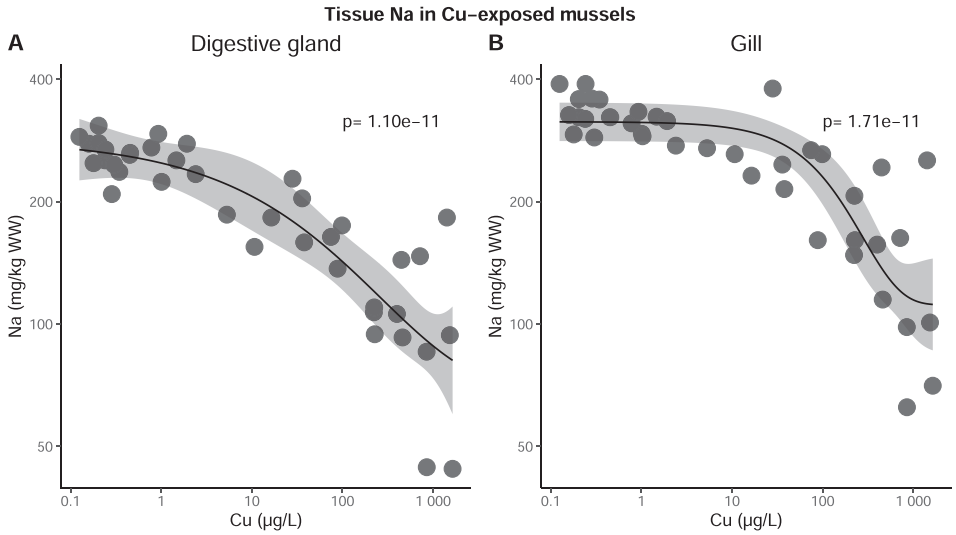

Fig. 5. Digestive gland ($n = 39$) (A) and gill ($n = 40$) (B) concentration of Na in mussels (*Anodonta anatina*) acutely exposed to Cu via water (96 h). Black lines correspond to the fitted regression models and shaded areas represent model 95% confidence intervals. A Weibull type 1 model was fitted for digestive gland (A), and an asymptotic regression model for gills (B). Model details and parameters are presented in Table A.3.

response sensitivity and robustness, we suggest that biomarker potential is overall higher for gill than for digestive gland transcripts. Still, even in gills, robust responses were limited to Cu exposures at relatively high concentrations. While biomarker predictions of adverse effects remain subject for future research, an important first step has been to quantitatively describe concentration-response relationships. Whether intended for use as simple biomarkers of exposure or for incorporation into complex mechanism-based models, effective application will ultimately require critical evaluation of biomarker potential, for which concentration-response testing should be considered standard.

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CRediT authorship contribution statement

Gustaf Ekelund Ugge: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Annie Jonsson**: Project administration, Funding acquisition, Supervision, Writing – review & editing. **Anders Walstad**: Investigation. **Olof Berglund**: Supervision, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gustaf Ekelund Ugge and Anders Walstad are employed by ALS Scandinavia Toxicon AB. Gustaf Ekelund Ugge is on a leave of absence for pursuing a PhD.

Data statement

Data are available on Mendeley (Ekelund Ugge, Gustaf Magnus Oskar; Jonsson, Annie; Walstad, Anders; Berglund, Olof (2021), "Data for: Evaluation of transcriptional biomarkers using a high-resolution regression approach: Concentration-dependence of selected transcripts in copper-exposed freshwater mussels (*Anodonta anatina*)", Mendeley Data, V1, doi: 10.17632/mkd8fw935c.1).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2021.103795.

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