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Environmental Toxicology

Transcriptional Responses as Biomarkers of General Toxicity: A Systematic Review and Meta-analysis on Metal-Exposed Bivalves

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Abstract: Through a systematic review and a series of meta-analyses, we evaluated the general responsiveness of putative transcriptional biomarkers of general toxicity and chemical stress. We targeted metal exposures performed on bivalves under controlled laboratory conditions and selected six transcripts associated with general toxicity for evaluation: catalase, glutathione-*S*-transferase, heat shock proteins 70 and 90, metallothionein, and superoxide dismutase. Transcriptional responses ($n = 396$) were extracted from published scientific articles ($k = 22$) and converted to log response ratios (lnRRs). By estimating toxic units, we normalized different metal exposures to a common scale, as a proxy of concentration. Using Bayesian hierarchical random effect models, we then tested the effects of metal exposure on lnRR, both for metal exposure in general and in meta-regressions using toxic unit and exposure time as independent variables. Corresponding analyses were also repeated with transcript and tissue as additional moderators. Observed patterns were similar for general and for transcript- and tissue-specific responses. The expected overall response to arbitrary metal exposure was an lnRR of 0.50, corresponding to a 65% increase relative to a nonexposed control. However, when accounting for publication bias, the estimated "true" response showed no such effect. Furthermore, expected response magnitude increased slightly with exposure time, but there was little support for general monotonic concentration dependence with regard to toxic unit. Altogether, the present study reveals potential limitations that need consideration prior to applying the selected transcripts as biomarkers in environmental risk assessment. *Environ Toxicol Chem* 2023;42:628–641. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Aquatic toxicology; bayesian statistics; ecotoxicology; meta-regression; mollusk toxicology; publication bias; risk assessment; toxic unit

INTRODUCTION

In ecotoxicology, a biomarker is considered a measurable biological change that can be used as an indicator of chemical exposure and/or a predictor of adverse effects (van der Oost et al., 2003). In the context of environmental risk assessment (ERA) of chemicals, understanding of both mechanistic and quantitative links between exposures and relevant biological effects is crucial to predict harm to biota and ecosystems (Martin et al., 2019; van der Oost et al., 2003). Consequently, it is of great importance that ERAs are supported by robust scientific evidence (Martin et al., 2019). For practical application of biomarkers, empirical support can therefore be required to show that a specific marker candidate is both sensitive, by responding at relevant exposures, and robust, by large and predictable response magnitudes.

Molecular biomarkers such as gene transcripts have been proposed to capture responses upstream of adverse effects on the organism level (Calzolai et al., 2007; Piña et al., 2007). Some transcripts may be specific to certain toxicants or biological effects, whereas others, including responses involved in toxicant metabolism, oxidative stress, and general cytoprotection, are considered potential biomarkers of general toxicity and chemical stress (Le Saux et al., 2020; Sulmon et al., 2015). Many studies on transcriptional biomarker candidates are,

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however, based on single or few exposure groups and/or pooled samples from multiple individuals. Such exposure setups may provide important understanding of the molecular mechanisms involved in the responses that may guide identifying markers likely to respond to a chemical stressor. However, substantial empirical support of response effect sizes and variability is required for successful detection and appropriate interpretation of biomarker responses. The evaluation of a putative biomarker can therefore suffer greatly if, for instance, concentration dependence and individual variation are insufficiently addressed (Bahamonde et al., 2016; Fent & Sumpter, 2011). Furthermore, experimental setups in ecotoxicological research often differ in study species, biomarker candidates (transcripts and/or tissues), and exposure conditions (chemicals, concentrations, and/or exposure durations; Martin et al., 2019). As a result, it can be difficult to put single transcriptional studies in a relevant frame of reference within the body of scientific literature. Therefore, out of context, even results standing out as highly significant may on their own offer little information on the general potential and practical applicability of a biomarker candidate in ERAs.

Bivalve mollusks are common study organisms used for studying various aspects of aquatic pollution (Beyer et al., 2017; Binelli et al., 2015; Zhou et al., 2008). One important feature among bivalves is that they are sessile, which greatly facilitates both site-specific in situ assessments and field collection for laboratory studies (Beyer et al., 2017; Binelli et al., 2015; Zhou et al., 2008). As filter-feeders, they are continuously exposed to large volumes of water and, consequently, pollutants present in the water column (Beyer et al., 2017; Binelli et al., 2015). Also, because many bivalves are bottom-dwellers (Kraan et al., 2010; Zieritz et al., 2014), sediment is often an additional plausible exposure route. Furthermore, bivalves occupy various aquatic habitat types, which can allow selection of relevant study species on a case-by-case basis, rather than having to rely on laboratory model species. In general, their role as sentinel species and the high availability of ecotoxicological studies make bivalves candidates for further evaluation of transcriptional biomarkers of pollution.

In the present study, we performed a systematic literature review to synthesize published research on transcriptional responses to toxicants and subsequently a series of meta-analyses to quantify expected responses to toxicant exposure. Because of their ecological relevance and practical use in ERAs and biomonitoring, we targeted responses in bivalves. In a previous literature review, Miao et al. (2015) identified glutathione-*S*-transferase (*gst*), heat shock proteins 70 and 90 (*hsp70*, *hsp90*), metallothionein (*mt*), and superoxide dismutase (*sod*) among the genes most frequently reported in bivalves to respond to pollutant exposures in general. By addressing response trends to both general and continuous exposures (concentration and time), our objective was to evaluate the overall responsiveness of transcriptional biomarker candidates of general toxicity and chemical stress. Specifically, we selected metal exposures to represent general toxicity and a specific set of transcripts (catalase [*cat*], *gst*, *hsp70*, *hsp90*, *mt*, and *sod*) that represent common biomarkers of nonspecific chemical stress (Le Saux et al., 2020; Miao et al., 2015; Sulmon et al., 2015). To account for individual variation while also reducing the variability of experimental exposures, we limited the analysis to include controlled laboratory studies where transcriptional responses were measured on the individual level. Specifically, we asked whether available data can generally support that (1) transcript levels respond to metal exposure, (2) responses show monotonic concentration dependence, and (3) response magnitudes increase or decrease with exposure time. For each of these questions, we evaluated general responses as well as responses in transcript- and tissue-specific subsets. For transparency and reproducibility, we used the guidelines specified by O'Dea et al. (2021) as a basis for the reporting of our study (see checklist in Supporting Information).

MATERIALS AND METHODS

Systematic review

Literature searches were performed in two databases, Web of Science and Scopus (Table 1). The searches were initially performed on May 15, 2019, followed by an updated search on September 13, 2021. In the first search, we included all publications to date, whereas we excluded publications indexed before 2019 in the second search. In addition, for articles subsequently selected for inclusion in the meta-analysis, a backward citation search was performed to identify additional potentially relevant literature not captured in the database searches. For this purpose, we used the reference indexing functions in both Web of Science and Scopus.

The same screening procedure was performed for all articles, whether found directly from database searches or subsequently from the backward citation search (Figure 1; Supporting Information, Table S1). The screening and selection

TABLE 1: Databases and search terms used in the literature search for the systematic review

Database	Search terms	Search hits
Web of Science (search for "Topic" within "All databases")	(*transcript* OR *pcr OR (gene NEAR/1 expression)) AND (mollus* OR mussel* OR bivalv* OR clam*) AND (pollut* OR *toxic* OR xenobiot* OR (stress* NEAR/3 chemic*)) AND (*toxic* OR stress* OR respons* OR biomarker*) AND (aquat* OR fresh* OR limn* OR marine)	2151 (May 15, 2019) + 653 (September 13, 2021)
Scopus (search for "Title, Abstract, Keywords")	(*transcript* OR *pcr OR (gene W/1 expression)) AND (mollus* OR mussel* OR bivalv* OR clam*) AND (pollut* OR *toxic* OR xenobiot* OR (stress* W/3 chemic*)) AND (*toxic* OR stress* OR respons* OR biomarker*) AND (aquat* OR fresh* OR limn* OR marine)	478 (May 15, 2019) + 183 (September 13, 2021)

The search was initially performed on May 15, 2019, and updated on September 13, 2021.

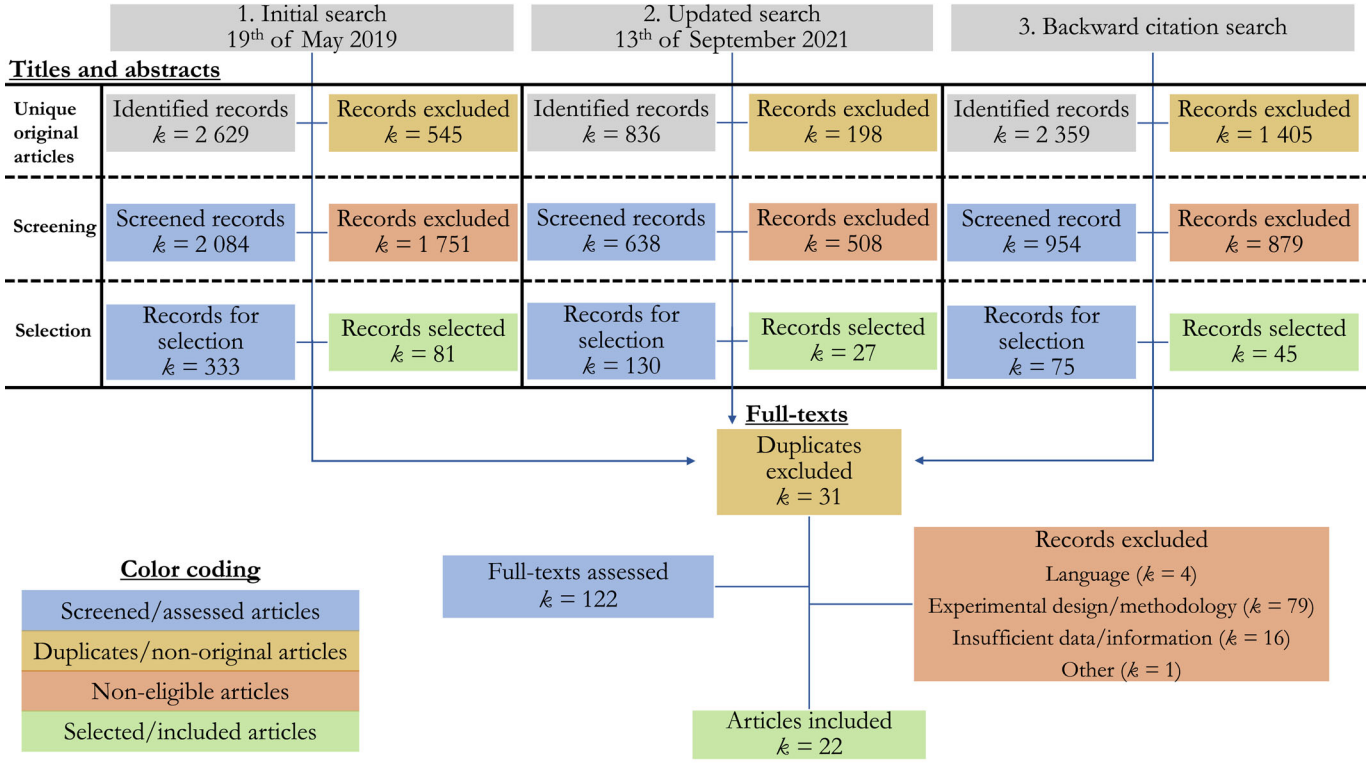

FIGURE 1: A Preferred Reporting Items for Systematic Reviews and Meta-Analyses flowchart summarizing the screening and selection processes for articles included in the final meta-analysis. The number of studies (*k*) is presented for each screening and selection step. For details on the evaluation procedure, see text and Supporting Information, Table S1.

procedures were performed by one and the same person (G. Ekelund Ugge) for all searches. Duplicates, review articles, and conference abstracts were removed, leaving original research articles, for which we screened all titles and abstracts. In the screening process, we first removed articles not based on chemical exposures and articles on irrelevant topics (e.g., parasitology, immunology, phylogenetics, and human toxicology). Second, articles were excluded if based on study organisms other than bivalves. Third, we removed articles in which other types of responses or biomarkers, but not transcripts, were mentioned in the abstract (e.g., proteins, metabolites, enzyme activity, or histopathology). Fourth, we required that exposures were performed in vivo under controlled laboratory settings and based on single compounds in water. Specifically, in situ and in vitro studies were excluded, as were experiments in which chemical exposure was performed via, for instance, diet, sediment or by injection. Similarly, we also excluded studies on nano- and microparticles or chemical mixtures and studies on environmental stressors typically considered outside of ecotoxicology (e.g., pH, nutrients, radiation). After screening for eligible ecotoxicological studies, we performed an additional selection step to narrow the range from all pollutants and transcripts to general toxicity and general stress responses. As a proxy of general toxicity, we included studies (1) based on metal exposures and (2) testing one or more biomarker candidates from the selected set of transcripts (*cat*, *gst*, *hsp70*, *hsp90*, *mt*, and *sod*), all of which are representative of general cytoprotection and oxidative stress defense.

After removal of duplicates representing overlap between literature searches ($k = 31$), a total of 122 articles were selected for full-text assessment of experimental setups. To ensure a sufficient level of understanding of experimental setups, we excluded articles not written in English ($k = 4$). Also, one article that was previously not identified as a review article was excluded for not presenting original data. When evaluating experimental designs, we required that (1) a negative control exposure had been performed parallel to metal exposures; (2) transcriptional responses were assessed on the individual level, by quantitative polymerase chain reaction (qPCR); (3) exposure setups were unambiguous and replicated; and (4) criteria specified for title and abstract screening were still met after full-text evaluation. In case an article contained multiple experiments, exposure groups, and/or transcriptional responses, all subsets fulfilling the criteria were included (see section *Data extraction*). Articles not fulfilling all criteria were excluded from the meta-analysis for noneligible experimental design/nonapplicable methodology ($k = 79$). Consequently, we excluded, for instance, studies that only used a 0-h exposure as a control group and studies that pooled tissue or RNA samples from multiple individuals prior to qPCR. For data extraction, we required that measures of response effect size, variation, and sample size were presented for each exposure group, including the negative control. In case any essential piece of information was unclear or lacking from an article at this stage, authors were contacted, initially via e-mail. If no author response was received after first contact or if the response left unclarities, requests were

clarified and repeated at least once. Repeated requests were made both via e-mail and, when possible to track the author profile, via ResearchGate. Studies for which available information remained insufficient ($k = 16$) were ultimately excluded from further analysis to avoid uncertain assumptions of missing data or unclear exposures. In the end, 22 studies were left for inclusion in the meta-analysis (see *Study characteristics* section and Supporting Information for a seperate list).

Data set

Data extraction. Data were extracted for relevant responses (transcript × tissue) in all relevant exposure groups (toxicant × concentration × exposure time), including negative control treatments. All data extractions were performed by one and the same person (G. Ekelund Ugge). In case a study included additional pollutant exposures (e.g., mixtures or nanoparticles), we included any exposure group corresponding to single-metal exposure via water but omitted remaining groups. If interactions with other environmental stressors were investigated (e.g., different temperatures or CO_2 levels), we extracted data only from exposure groups representing normal or background conditions of that stressor.

In a few cases, data sets were provided directly by the authors. Otherwise, all data were collected from original articles and supplementary materials. Unless presented in text or tables, we used the software Graph Grabber, Ver 2.0.2, for graphical data extraction. Specifically, we extracted or calculated response mean, standard deviation, and sample size for each available biomarker candidate (*cat*, *gst*, *hsp70*, *hsp90*, *mt*, and *sod*) in all analyzed tissues and species. Different isoforms of the transcripts were treated as replicates of the same biomarker candidate as a way to retain as much data as possible because transcript isoform was not specified in all studies. In case presented sample sizes were nonspecified (e.g., presented as ranges) or inconsistent (e.g., different *n* in text and figure legend), the smallest presented sample size was assumed for all exposure groups of that study, unless specific sample sizes were provided on request to the authors.

Toxic units. As a way to normalize different metals to a common scale, we used toxic units as a proxy of exposure concentration. For simplicity, the term *concentration* will hereafter be used to also include relative measures of exposure such as toxic unit. Specifically, we used the "standartox" package in R to obtain metal toxicity data from the US Environmental Protection Agency's ECOTOX Knowledgebase (Scharmüller et al., 2020). For each metal represented in the meta-analysis data set, acute toxicity data (72–96-h 50% lethal concentrations [LC50s]) for bivalve species were retrieved on December 15, 2021. The choice of using mortality as an endpoint for normalization was largely based on data availability. In addition, although not directly linked to the transcriptional responses of interest, mortality from metal exposure was considered a measure of general chemical stress. No bivalve acute toxicity data were available for As(V), Gd, Sm, and Y; and these exposures were excluded from toxic unit determination. In the downstream analyses, corresponding data points ($n = 58$) were, however, excluded only from models based on toxic units. For remaining metals, an LC50 was retrieved for every available bivalve species. In case of more than one LC50 data point for a metal × species combination, only the lowest LC50 was retained, as a conservative estimate of species sensitivity. In turn, the median $\log_{10}$(LC50) was selected across species to represent general bivalve sensitivity and for normalization of toxic unit. The reported metal concentration ($\log_{10}$-transformed) was then used to determine logTU (toxic unit) for each (applicable) entry in the meta-analysis data set, according to Equation 1.

$$\log \mathrm{TU} = \log_{10}(\text{Reported concentration}) - \text{Median}\log_{10}(\mathrm{LC50}) \quad (1)$$

Nonindependence and effect size calculation. To account for nonindependence of multiple effect measurements from the same study, we (1) split the control group sample size between exposure treatments, and (2) included a variance–covariance matrix in our models. Prior to effect size calculations, the control group sample size was in each case divided by the number of corresponding exposure groups (toxicant × concentration), which is one approach to adjust for nonindependence from multiple comparisons (Higgins et al., 2021). The adjusted control group sample size was then in each case used for calculation of response effect size. The variance–covariance matrix was generated using the R package "metaAidR" (Lagisz et al., 2021) and included in our models to account for nonindependence of multiple effect measures within the same exposure group (see section, *Meta-analyses*). We assumed a correlation factor of 0.5 for effects from the same exposure group (study × toxicant × concentration).

Because the majority of studies presented results on the linear scale, all data presented on a log scale were back-transformed prior to effect size calculations. Log response ratios (lnRRs) and corresponding variances (vLRR) were determined for each extracted response (Rosenberg et al., 2013), according to Equations 2 and 3. *Response* represents response magnitude, SD is standard deviation, and *n* is the (adjusted) sample size. Exposed and control groups are denoted by subscripts *E* and *C*, respectively.

$$\mathrm{lnRR} = \ln\frac{\mathrm{Response}_E}{\mathrm{Response}_C} \quad (2)$$

$$\mathrm{vLRR} = \frac{\mathrm{SD}_E{}^2}{n_E \times \mathrm{Response}_E{}^2} + \frac{\mathrm{SD}_C{}^2}{n_C \times \mathrm{Response}_C{}^2} \quad (3)$$

Meta-analyses

To address our questions, the response variable lnRR was assessed under different combinations of categorical (transcript and tissue) and continuous (toxic unit and exposure time) moderators, resulting in the nine models summarized in Table 2. Linear regression was used to evaluate the general trends of concentration and time dependence of response magnitudes. One tissue, visceral mass, was only included in a single study; and, because of the low replication, the

TABLE 2: Summary of model structure used for the nine meta-analyses of bivalve transcriptional responses to metal exposure

		Continuous		
	Moderator	None	Toxic unit (log_{10}TU)	Exposure time (log_2time [h])
Categorical	None	Overall response (intercept model) ~(1|group[a]) + fcor(vcv_matrix) ESS: 1660	Overall concentration-dependent response ~Log_{10}TU + (1|group[a]) + fcor(vcv_matrix) ESS: 1605–2751	Overall time-dependent response ~Log_2time + (1|group[b]) + fcor(vcv_matrix) ESS: 2332–2809
	Transcript	Transcript overall response ~Transcript + (1|group[c]) + fcor(vcv_matrix) ESS: 1306–2428	Transcript-specific concentration dependence ~Log_{10}TU × transcript + (1|group[c]) + fcor (vcv_matrix) ESS: 2217–3325	Transcript-specific time dependence ~Log_2time × transcript + (1|group[d]) + fcor (vcv_matrix) ESS: 2114–2811
	Tissue	Tissue overall response ~Tissue + (1|group[e]) + fcor (vcv_matrix) ESS: 1724–1815	Tissue-specific concentration dependence ~Log_{10}TU × tissue + (1|group[e]) + fcor (vcv_matrix) ESS: 1623–2142	Tissue-specific time dependence ~Log_2time × tissue + (1|group[f]) + fcor (vcv_matrix) ESS: 2211–2806

[a]Study × species × tissue × transcript × time.
[b]Study × species × tissue × transcript.
[c]Study × species × tissue × time.
[d]Study × species × tissue.
[e]Study × species × transcript × time.
[f]Study × species × transcript.
The group random effect represents the study × species × transcript × tissue × time combination, modified to exclude any grouping factor used as a moderator in the specific model. The same variance–covariance matrix was used for all models. Simulation effective sample sizes for effect size parameters are reported for each model. TU = toxic unit; vcv_matrix = variance–covariance matrix; ESS = effective sample size.

corresponding data points ($n = 3$) were excluded from analyses using tissue as a moderator. To account for heterogeneity between and within studies, we included a random effect that grouped measurements by the respective study × species × transcript × tissue × time combination. For each specific model, the random effect was, however, modified to omit any grouping factor also occurring as a moderator (transcript, tissue, and time). Also, I^2 was calculated for each model as a measure of heterogeneity between the groups. First, a matrix (P) was defined for each model according to Equation 4, where X denotes the model matrix for the respective model and W corresponds to the inverse of the variance–covariance matrix (see Section *Nonindependence and effect size calculation*; Viechtbauer, 2022).

$$P = W - WX(X'WX)^{-1}X'W \quad (4)$$

This was in turn used to calculate I^2, according to Equation 5. In the present study, $\hat{\tau}^2$ corresponds to the estimated between-group variance extracted from the posterior distribution, k to the number of observations, and p to the number of columns in the respective X matrix (Viechtbauer, 2022).

$$I^2 = 100\% \times \frac{\hat{\tau}^2}{\hat{\tau}^2 + \frac{k-p}{tr[P]}} \quad (5)$$

The meta-analyses were implemented as Bayesian hierarchical random effect models in the "brms" R package (Bürkner, 2017). The variance–covariance matrix was incorporated into the models using the "fcor" function (Table 2). Parameters were estimated from the posterior sample derived by Markov chain Monte Carlo sampling in "Stan" (Stan Development Team, 2021), with 2000 iterations and four chains, using a burn-in of 2000 iterations. Prediction intervals of the effect sizes were estimated for models with no or categorial moderators only, using the posterior sample and assuming normal distributions for study effects (IntHout et al., 2016). All models were checked for convergence. The "Rhat" statistic did in no case exceed the critical threshold (1.05), and simulation effective sample sizes (ESSs) for the effect parameters were judged to be sufficiently large (Table 2).

Sensitivity analysis

To test the influence of the assumed 0.5 correlation factor in the variance–covariance matrix, all models were repeated using variance–covariance matrices based on correlation factors of 0.1 and 0.9, respectively. We also assessed publication bias toward reporting positive results, using a funnel plot and meta-regressions based on ESS of the response data, as suggested for data sets with many nonindependent effects (Nakagawa et al., 2022). The ESS ($4\tilde{n}_i$) was calculated according to Equation 6, where n_E represents the sample size of the exposure group and n_C represent the control group sample size (adjusted for multiple comparisons).

$$4\tilde{n}_i = \frac{4n_{Ei}n_{Ci}}{n_{Ei} + n_{Ci}} \quad (6)$$

The meta-regressions were performed by adding ESS as an independent variable to the models without continuous moderators. The intercept from such meta-regression models has been suggested to function as an estimate of a "true" effect size, adjusted for bias at infinite sample sizes (Nakagawa et al., 2022). First, the square root of the inverted ESS was included as an independent variable. In cases where the modeled intercept overlapped 0, this intercept was used as an estimate of the "true" effect size. If the intercept did not overlap 0, the inverse

ESS was instead used for the corresponding model (Nakagawa et al., 2022). Also, the correlation between ESS and toxic unit/exposure time was assessed to estimate the potential influence from publication bias on the slopes of corresponding models (Supporting Information, Figure S1).

Software

The statistical analyses were performed using R, Ver 4.0.5. The packages "brms," Ver 2.16.1 (Bürkner, 2017); "brmstools," Ver 0.5.3 (Vuorre, 2018); "metaAidR," Ver 0.0.0.9000 (Lagisz et al., 2021); "openxlsx," Ver 4.2.4 (Schauberger & Walker, 2021); and "standartox," Ver 0.0.1 (Scharmüller et al., 2020) were used for statistical analyses and data set manipulation, whereas "dplyr," Ver 1.0.7 (Wickham et al., 2021); "ggbeeswarm," Ver 0.6.0 (Clarke & Sherrill-Mix, 2017); "ggplot2," Ver 3.3.5 (Wickham, 2016); "ggpubr," Ver 0.4.0 (Kassambra, 2020); and "tidybayes," Ver 3.0.1 (Kay, 2021) were used for producing figures.

RESULTS AND DISCUSSION

Study characteristics

A total of 396 effect sizes were extracted from the 22 included studies. The most abundant transcript was *mt* (27%), followed by *cat* (18%), *gst* (18%), *sod* (16%), *hsp90* (10%), and *hsp70* (9.3%). Most effect sizes corresponded to measurements in gills (54%), followed by digestive gland (36%), gonads (9.3%), and visceral mass (0.76%). For more detail, see sections *Transcript-specific effects* and *Tissue-specific effects*, respectively. Furthermore, 13 different bivalve species were represented in the data set: *Dreissena polymorpha* (27%, $n = 108$; Hanana et al., 2017, 2018; Louis et al., 2021; Navarro et al., 2011), *Crassostrea gigas* (20%, $n = 81$; Choi et al., 2008; Cong et al., 2012, 2013; Jo et al., 2008; Metzger et al., 2012), *Cerastoderma glaucum* (20%, $n = 80$; Karray et al., 2015), *Mytilus galloprovincialis* (8.6%, $n = 34$; Jimeno-Romero et al., 2017; Piscopo et al., 2016; Rocha et al., 2018), *Anodonta anatina* (6.1%, $n = 24$; Ekelund Ugge et al., 2020), *Geloina coaxans* (6.1%, $n = 24$; Guo et al., 2020), *Ruditapes philippinarum* (4.0%, $n = 16$; Chen et al., 2018), *Crassostrea virginica* (2.5%, $n = 10$; Götze et al., 2014; Lebordais et al., 2021), *Mytilus edulis* (1.8%, $n = 7$; Poynton et al., 2014), *Mercenaria mercenaria* (1.0%, $n = 4$; Götze et al., 2014), *Meretrix meretrix* (1.0%, $n = 4$; Gao et al., 2021), *Mactra chinensis* (0.76%, $n = 3$; Zhang et al., 2016), and *Cerastoderma edule* (0.25%, $n = 1$; Desclaux-Marchand et al., 2007). Finally, Cd was the most common metal exposure (53%, $n = 210$, $k = 14$), followed by Cu (14%, $n = 55$, $k = 5$), Cr(VI) (6.1%, $n = 24$, $k = 1$), Hg (5.1%, $n = 20$, $k = 1$), Gd (4.0%, $n = 16$, $k = 1$), Sm (4.0%, $n = 16$, $k = 1$), As(V) (3.5%, $n = 14$, $k = 2$), Y (3.0%, $n = 12$, $k = 1$), Ag (2.0%, $n = 8$, $k = 1$), As(III) (2.0%, $n = 8$, $k = 1$), Ni (1.5%, $n = 6$, $k = 1$), Pb (1.0%, $n = 4$, $k = 1$), and Zn (0.76%, $n = 3$, $k = 1$).

Overall effects

By addressing responses to general metal exposure and by using concentration and time as continuous predictors, the objective of the present meta-analyses was to assess the general responsiveness of transcriptional biomarker candidates in bivalves. We demonstrated an overall relative increase of the tested transcriptional responses on exposure to metal stressors (Figure 2A), suggesting that the transcripts are in fact sensitive to general metal stress. Without separation of transcripts and tissues, the average lnRR from metal exposure was 0.50. For an arbitrary metal exposure and a random transcript × tissue combination, this would translate to an expected 65% increase relative to a negative control treatment. By comparison, recent meta-analyses on pesticide-exposed fish demonstrated similar (although inverted) overall effect sizes for cholinesterase activity (Santana et al., 2021) but smaller effect sizes for enzymes involved in antioxidant defense and biotransformation (Santana et al., 2022). Similar effect sizes were also demonstrated in a meta-analysis on cortisol in fish exposed to various contaminants (Rohonczy et al., 2021). On the one hand, this could suggest that the robustness of transcriptional responses is comparable to that of other molecular biomarkers. However, an expected lnRR of 0.5 appears small considering the large variability (95% prediction intervals ranging from approximately −1 to 2) and high heterogeneity (I^2=97%). The overall response would therefore suggest only a moderate robustness of the selected biomarker candidates.

In contrast, there was no implication of concentration dependence (Figure 2B,D), giving no support for an overall monotonic response relative to the estimated amount of stress. In a meta-analysis on cortisol levels in fish, Rohonczy et al. (2021) were similarly unable to demonstrate concentration dependence relative to the contaminant exposure, despite positive overall responses. While one explanation could simply be a lack of concentration–response relationships, it could also result from comparing different toxicants on a common scale. In our study, it is possible that the toxic unit approach does not provide high enough resolution and/or that the between-group heterogeneity ($I^2 = 97\%$) or other sources of unaccounted variability obscure concentration dependence that could perhaps be demonstrated in wide-range concentration–response setups, using single species and single toxicants (Ekelund Ugge et al., 2022). On the other hand, the present data set covers a wide range of both response effect sizes and estimated stress exposures. If it were universally true that the biomarker candidates are highly sensitive to the relative amount of stress exposure, the applied meta-analytical models would most likely have captured a rough estimate of the concentration dependence. Consequently, on larger scales and in heterogenous data sets, metal stress appears to be a stronger predictor of transcriptional responses when assessed as a binary variable (exposed vs. nonexposed) than when treated as a continuous one (e.g., toxic unit).

Furthermore, we observed an overall time dependence, with response magnitudes increasing with longer exposure periods (Figure 2C,D). Although the slope was shallow, the credible interval did not overlap 0. Previous studies on single bivalve species (see Bao et al., 2018; Fang et al., 2010; Liu et al., 2014) have demonstrated how the selected transcripts peak after 3–15 days of metal exposure. In line with these findings, our results therefore suggest that exposures for at

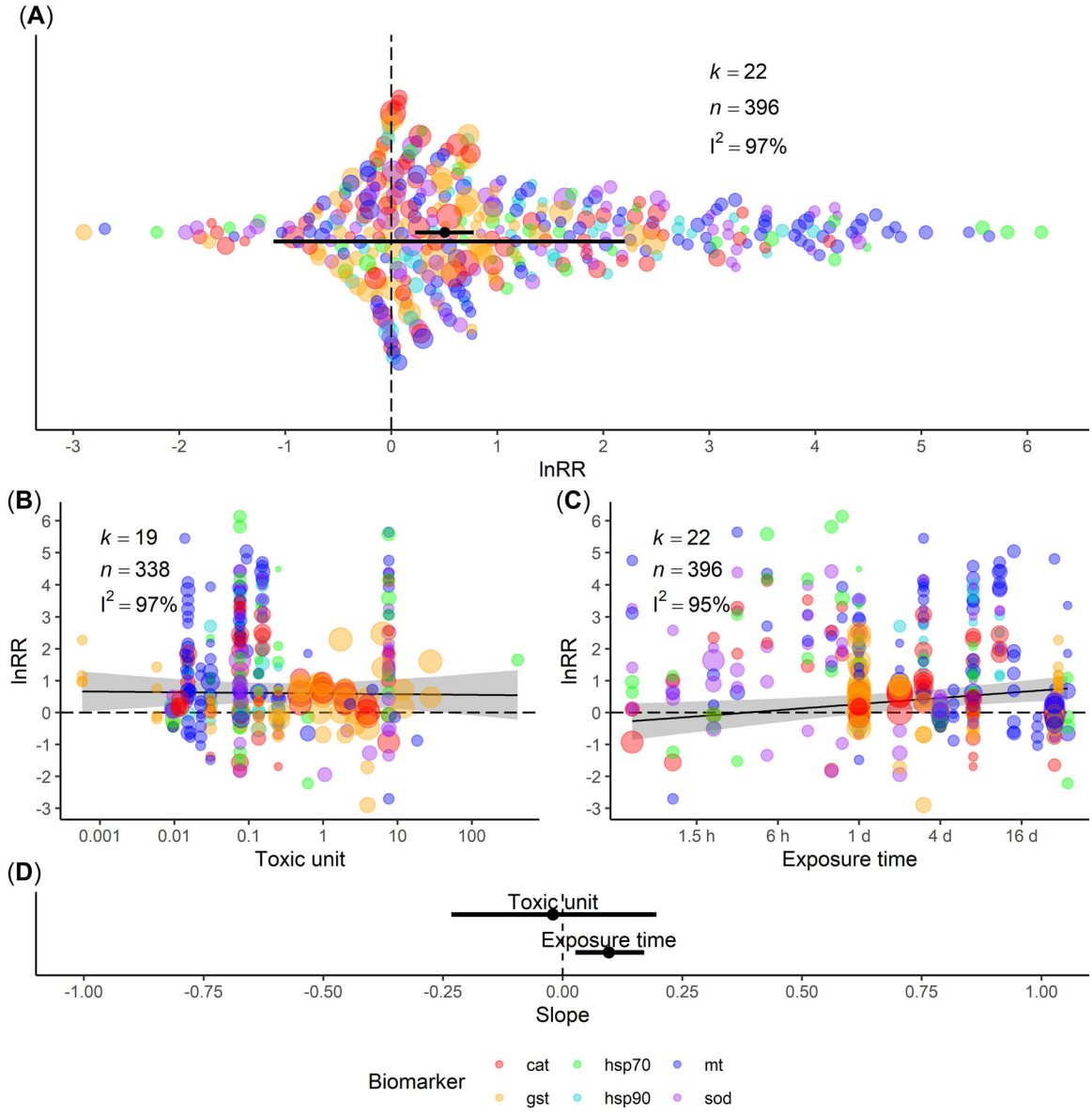

FIGURE 2: Effect of metal exposure on selected transcripts in bivalves. Effect size is expressed as log response ratio, determined according to Equation 2. The subplots demonstrate the overall effects of metal exposure (**A**), concentration dependence (**B**,**D**), and time dependence (**C**,**D**), determined by Bayesian hierarchical random effect models. The overall effect was determined by an intercept model without moderators, whereas concentration dependence and time dependence were determined by meta-regressions using toxic unit and exposure time as moderators. Each point represents an extracted effect size. Colors represent the different transcripts, and the point size represents the relative weight (inverted standard deviation). Shaded areas (**B**,**C**) and bars (**A**,**D**) represent 95% credible intervals, and a 95% prediction interval is represented by a horizontal line (**A**). Each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective analysis, as well as corresponding heterogeneity (I^2). *cat* = catalase; *gst* = glutathione-*S*-transferase; *hsp70*/*hsp90* = heat shock proteins 70 and 90; lnRR = log response ratio; *mt* = metallothionein; *sod* = superoxide dismutase.

least a few days are generally more likely to capture transcriptional responses than exposures of a few hours.

Transcript-specific effects

With regard to separate transcriptional responses, five out of six transcripts demonstrated average positive responses to exposure treatments (Figure 3). The implication would therefore be that the responsive transcripts *cat*, *gst*, *hsp70*, *mt*, and *sod* indeed have some potential as transcriptional biomarkers in bivalves. Despite a trend of positive responses, the credible interval of *hsp90* overlapped 0 (Figure 3D); and insufficient robustness is likely to limit the potential biomarker use of this transcript. In addition, there was a general lack of concentration dependence for separate transcripts (Figure 4). Despite positive responses to relative arbitrary metal exposure, there was a trend of decreasing response magnitudes with increasing toxic unit. Slopes were, however, shallow, with five out of six credible intervals overlapping 0. For *sod*, the upper confidence bound

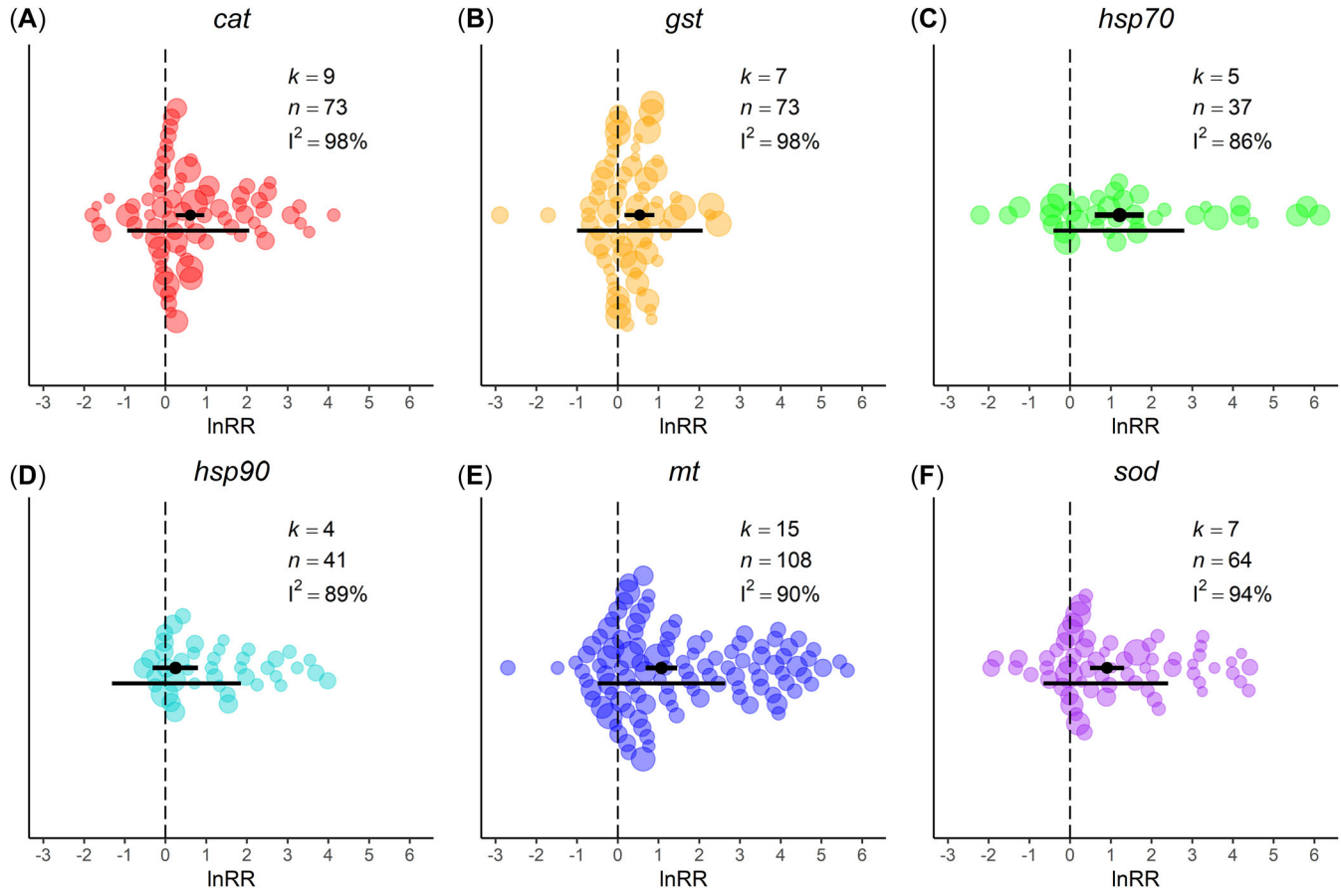

FIGURE 3: Effect of metal exposure on specific transcripts in bivalves. The subplots demonstrate the effects of arbitrary metal exposure on catalase (**A**), glutathione-*S*-transferase (**B**), heat shock protein 70 (**C**), heat shock protein 90 (**D**), metallothionein (**E**), and superoxide dismutase (**F**) expression. Effects (log response ratio) were determined by Bayesian hierarchical random effect models using transcript as moderator. Each point represents an extracted effect size, and the point size represents the relative weight (inverted standard deviation). Bars represent 95% credible intervals, and horizontal lines below represent 95% prediction intervals. Each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective subset, as well as corresponding heterogeneity (I^2). *cat* = catalase; *gst* = glutathione-*S*-transferase; *hsp70*/*hsp90* = heat shock proteins 70 and 90; lnRR = log response ratio; *mt* = metallothionein; *sod* = superoxide dismutase.

was just below 0, and the slope was also the steepest for this transcript (Figure 4F,G). In contrast, the general trend of time-dependent increases of response magnitudes was persistent in all biomarker candidates, although *gst*, *hsp70*, and *hsp90* credible intervals overlapped 0 (Figure 5). Heterogeneity was consistently high (I^2 = 83%–99%), with all transcript-specific models following the general pattern *gst* ≥ *cat* > *sod* > *mt* ≥ *hsp90* > *hsp70*. In summary, *cat*, *gst*, *hsp70*, and *mt* closely followed the trends of the overall effects, whereas *hsp90* credible intervals overlapped 0 for all moderators, and *sod* demonstrated a negative concentration–response relationship not observed in the other transcripts.

Tissue-specific effects

Two out of three tissues demonstrated positive average responses to exposure treatments (Figure 6). For the overall effect, digestive gland and gill credible intervals did not overlap 0 (Figure 6A,D), in contrast to gonads (Figure 6G). The potential for detecting responses therefore appears higher in gills and digestive glands, as could be expected from potential uptake and metabolism of metals in these tissues (Bonneris et al., 2005; Won et al., 2016). The general lack of concentration dependence was consistent in all tissues (Figure 6B,E,H,J), but interestingly, time dependence was weak for the separate tissues (Figure 6C,F,I,J). Specifically, gills and gonads showed a trend of responses increasing with time (Figure 6F,I), whereas digestive gland responses were largely unchanged (Figure 6C). Considering that all credible intervals overlapped 0, the general trend of time dependence, however, appears driven by factors other than tissue. Finally, heterogeneity was high across tissues in all models (I^2 = 91%–99%) and consistently highest in gonads.

Sensitivity analysis and limitations of the present meta-analysis

Generally, there was little influence from changing the correlation factor in the variance–covariance matrix to 0.1 or 0.9 (Supporting Information, Figure S2). In a few specific cases, credible intervals could change from just overlapping 0 to not doing so or vice versa, such as for overall effects in gonads, concentration dependence in *sod*, or time dependence in *gst*,

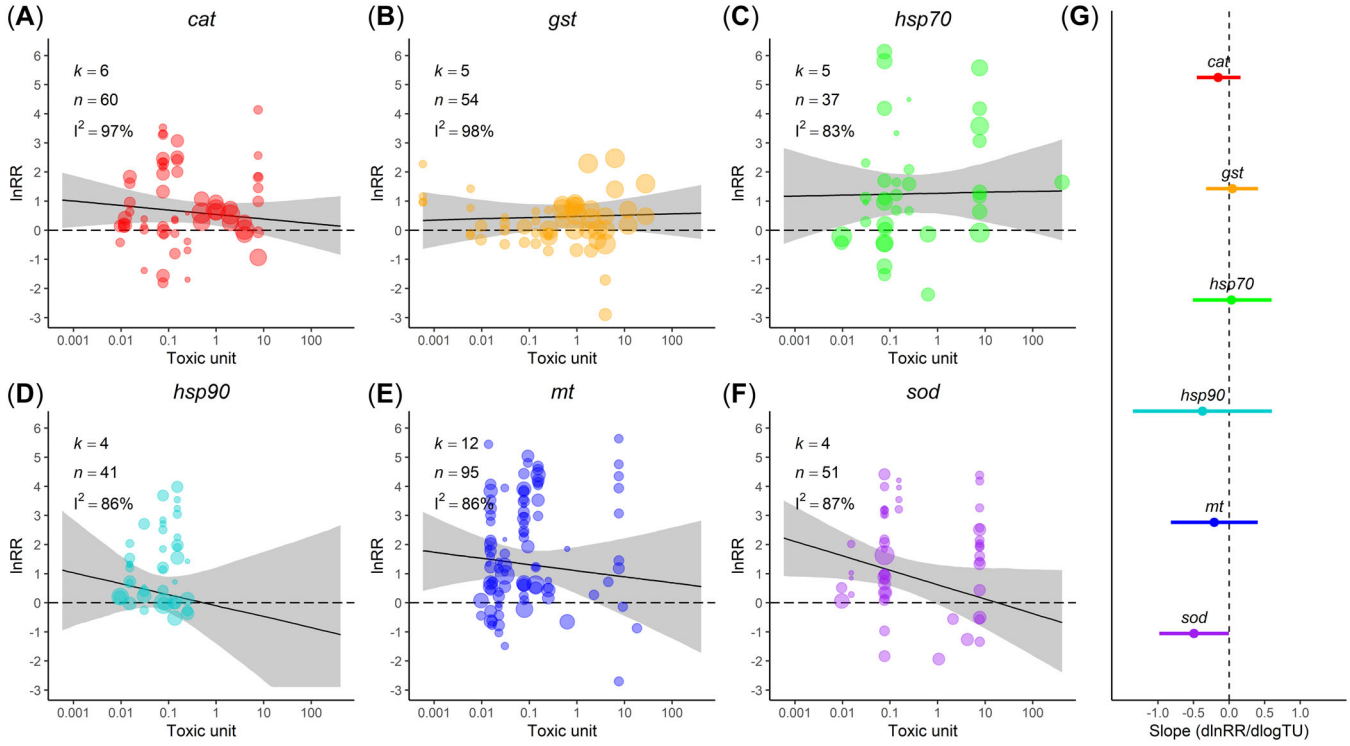

FIGURE 4: Concentration dependence of specific transcripts in metal-exposed bivalves. The sub-plots demonstrate the effects on catalase (**A**), glutathione-*S*-transferase (**B**), heat shock protein 70 (**C**), heat shock protein 90 (**D**), metallothionein (**E**), and superoxide dismutase (**F**) expression. Effects (log response ratio) were determined by Bayesian hierarchical random effect models using transcript and toxic unit as moderators. The model slopes are summarized in (**G**). Each point in a plot (**A–F**) represents an extracted effect size, and the point size represents the relative weight (inverted standard deviation). Shaded areas (**A–F**) and bars (**G**) represent 95% credible intervals, and each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective subset, as well as corresponding heterogeneity (I^2). *cat* = catalase; *gst* = glutathione-*S*-transferase; *hsp70*/*hsp90* = heat shock proteins 70 and 90; lnRR = log response ratio; *mt* = metallothionein; *sod* = superoxide dismutase; TU = toxic unit.

hsp70, and *mt* (Supporting Information, Figure S2). There is, however, no indication that a changed correlation factor would generally exaggerate or suppress effects in a way that would impact the general conclusions. Therefore, the results would support that our assumption of a 0.5 correlation factor represents a reasonable middle ground for addressing non-independence in our data set.

Transcriptional studies commonly present multiple effect sizes; for instance, there were only two studies in the data set used for the present meta-analysis from which we extracted a single effect size relative to control. Because publication bias can be driven by the reporting of positive results such as "statistically significant" differences (see Nakagawa et al., 2022), the likelihood of such detection, and hence publication, would increase with an increasing number of responses (transcripts, tissues) and/or exposure treatments (toxicants, concentrations, exposure durations). Indirectly, this could, however, also result in publication of negative results observed within the same study that would perhaps not be published on their own. It could therefore be possible that multibiomarker approaches in transcriptional studies might partially counteract the impacts of publication bias.

For the present data set, funnel plots revealed a slightly right-skewed distribution of effect sizes (Supporting Information, Figure S3A,B), which can be indicative of publication bias. Performing meta-regressions based on the inverted ESS, we also estimated new effect sizes that were adjusted for potential publication bias (Supporting Information, Figure S3C). Because there was no implication of dependence between ESS and toxic unit or exposure time (Supporting Information, Figure S1), we assumed that potential interactions with model slopes were negligible and that potential publication bias mainly affected estimates of model intercepts. That is, we would expect potential influence on the absolute effect size but not on the change relative to toxic unit or exposure time. Not surprisingly, adjusted effect size estimates were consistently smaller than nonadjusted ones for our intercept models (overall or separated by transcript or tissue), with credible intervals consistently overlapping 0 (Supporting Information, Figure S3C). Despite this apparent overestimation of the effect sizes by our original models, it is worth noting that even nonadjusted effect sizes were generally small. Also, high variation would generally appear to affect the model outcomes to a greater extent than the overestimation of effect sizes. Ultimately, the sensitivity analysis suggests that (1) our data set (presumably extending to the bulk of scientific literature) is biased, and (2) on a large scale, the expected transcriptional responses to arbitrary metal exposure are seemingly not distinguishable from 0, even when approaching infinite sample sizes.

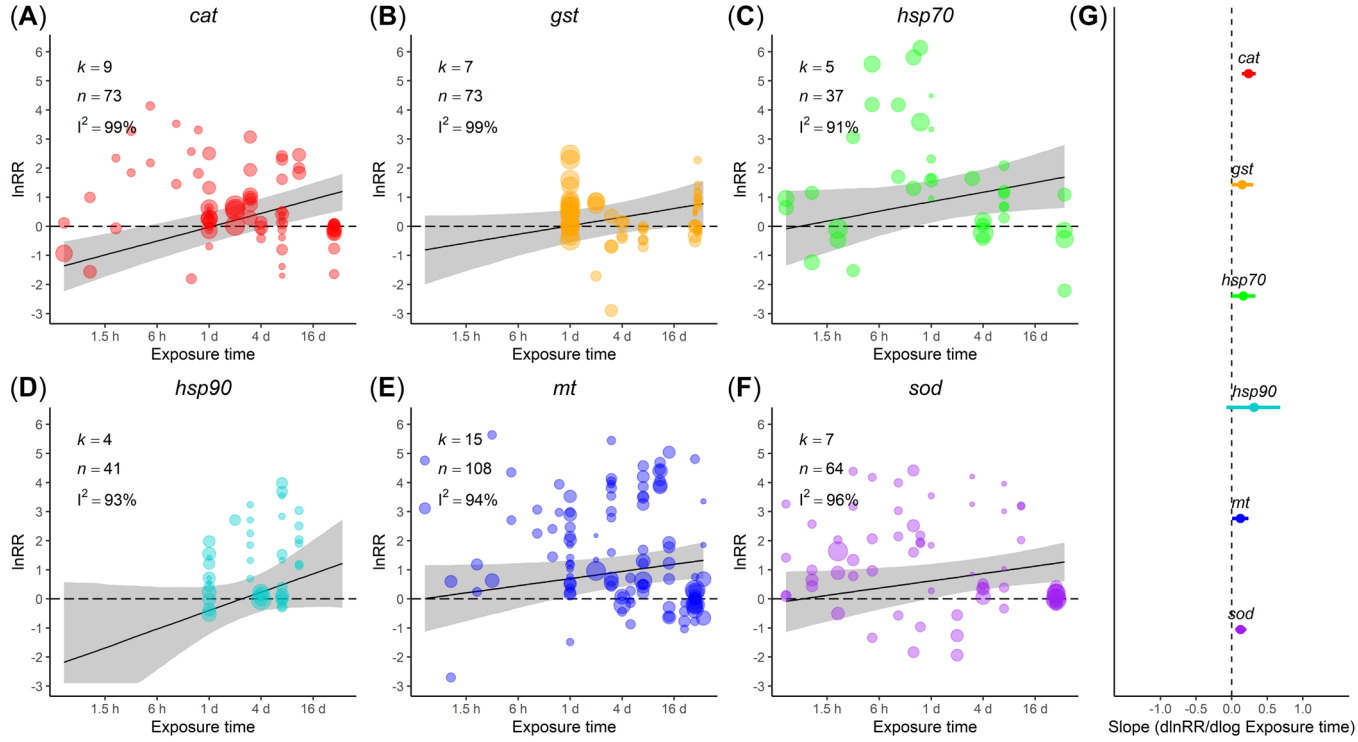

FIGURE 5: Time dependence of specific transcripts in metal-exposed bivalves. The subplots demonstrate the effects on catalase (**A**), glutathione-*S*-transferase (**B**), heat shock protein 70 (**C**), heat shock protein 90 (**D**), metallothionein (**E**), and superoxide dismutase (**F**) expression. Effects (log response ratio) were determined by Bayesian hierarchical random effect models using transcript and exposure time as moderators. The model slopes are summarized in (**G**). Each point in plot (**A–F**) represents an extracted effect size, and the point size represents the relative weight (inverted standard deviation). Shaded areas (**A–F**) and bars (**G**) represent 95% credible intervals, and each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective subset, as well as corresponding heterogeneity (I^2). *cat* = catalase; *gst* = glutathione-*S*-transferase; *hsp70*/*hsp90* = heat shock proteins 70 and 90; lnRR = log response ratio; *mt* = metallothionein; *sod* = superoxide dismutase.

In addition to the impact from publication bias, there are some other important limitations to the present data set. For instance, different isoforms were in some cases grouped together to represent a single transcript. This is in many ways analogous to using multiple species to represent "bivalves" or multiple compounds to represent "metals." Ultimately, it increases the generality of the results, while decreasing the specificity. Furthermore, the data points were not evenly distributed across either toxic units or exposure time points, in particular for certain subsets of the data. For *hsp90* and gonads, the coverage over both exposure concentrations and exposure time was rather narrow, which results in greater uncertainties of the respective meta-regressions. Similarly, there was not sufficient replication or representation of all combinations for us to consider transcript × tissue interactions. Provided sufficient data, such analyses would give a higher resolution and could help specify what transcript to analyze in which tissue for highest biomarker potential.

With regard to the meta-analyses themselves, one important limitation is the nonindependence of multiple data points from the same studies. This has been presented as a common phenomenon in meta-analyses on ecology and evolution (Nakagawa et al., 2022) and would in many cases likely extend to the adjacent research fields of ecotoxicology and environmental science. By taking measures to adjust the data and models (see section, *Nonindependence and effect size calculation*), we ultimately assume that nonindependence has been accounted for. Another important limitation is the use of toxic unit as a measure of relative concentration and/or chemical stress. The way we use it, toxic unit is a rough measure that assumes equal tolerance within the whole taxonomic group of bivalves. The transformation of a toxicant × concentration combination to toxic unit therefore adds uncertainty to each data point. Consequently, it might not be a suitable approach in, for instance, mechanistic response modeling. However, we argue that normalization of different toxicant exposures to a common scale makes it possible to better represent the general trends that we are currently addressing. Consequently, if a strong relationship between general metal stress and transcriptional responses were present, it should be detectable by meta-regression even when using a rough estimate such as toxic unit as moderator.

Finally, we once again acknowledge some underlying limitations that affect both the generality and the specificity of our results. Our objective was to identify the general trends of biomarker potentials rather than representing a fine-tuned mechanistic approach. Our results thus simply suggest what responses to expect from arbitrary exposure, as supported by available data. Still, there were important limitations to the scope of our study. We only used bivalves to represent

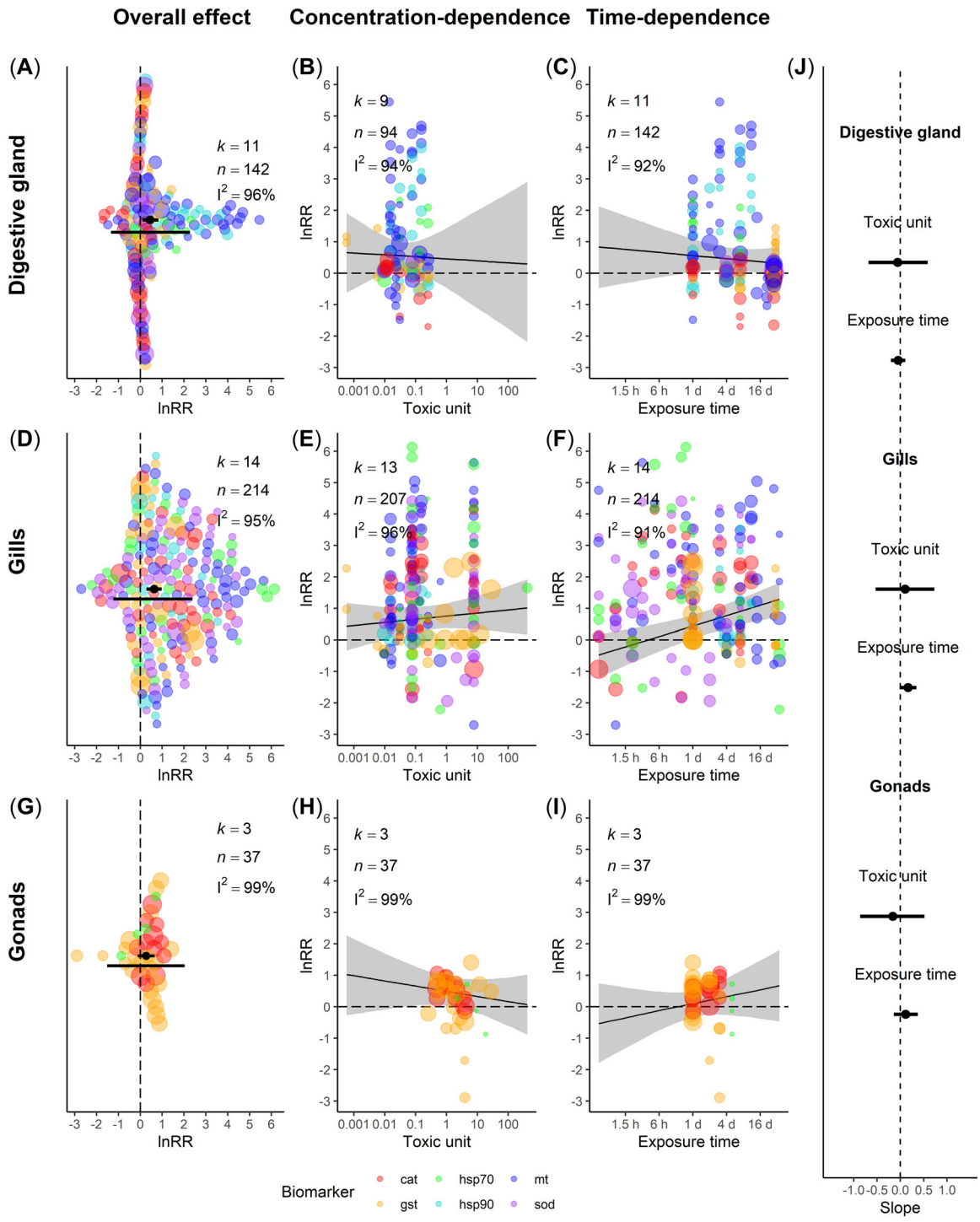

FIGURE 6: Effect of metal exposure on selected transcripts in three bivalve tissues. The subplots demonstrate the overall effects (log response ratio) of metal exposure in digestive glands (**A**), gills (**D**), and gonads (**G**); the concentration dependence of responses in digestive glands (**B**), gills (**E**), and gonads (**H**); and the time dependence of responses in digestive glands (**C**), gills (**F**), and gonads (**I**), determined by Bayesian hierarchical random effect models. The overall effects were determined using only tissue as moderator, whereas concentration and time dependence were determined by meta-regressions using toxic unit and exposure time as additional moderators. The model slopes are summarized in (**J**). Each point in plot (**A–I**) represents an extracted effect size. Colors represent the different transcripts, and the point size represents the relative weight (inverted standard deviation). Shaded areas (**B**,**C**,**E**,**F**,**H**,**I**) and bars (**A**,**D**,**G**,**J**) represent 95% credible intervals, and horizontal lines (**A**,**D**,**G**) represent 95% prediction intervals. Each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective subset, as well as corresponding heterogeneity (I^2). *cat* = catalase; *gst* = glutathione-*S*-transferase; *hsp70*/*hsp90* = heat shock proteins 70 and 90; lnRR = log response ratio; *mt* = metallothionein; *sod* = superoxide dismutase.

potential, environmentally relevant bioindicators and metal exposure as a proxy for general chemical stress. In addition, we limited the evaluation of biomarkers to six transcripts. Even so, between-group heterogeneity was consistently high ($I^2 \geq 83\%$), and it is plausible that other taxon × toxicant × transcript combinations would yield different results. Hence, for different setups and/or very specific exposure conditions, our results may be of limited use for prediction of specific responses. In that case, setups focusing on, for instance, single species/genera and/or single compounds can offer a higher resolution (Ekelund Ugge et al., 2022), which could potentially be necessary to address more specific questions. On the other hand, such setups would tend to be even less appropriate for extrapolations and for addressing more general questions. We have no apparent reason to believe that another selection of taxa, compounds, and/or genes would better fit our research questions. Therefore, we suggest that our results offer a fair representation of the general biomarker potentials of (assumed) stress genes for metal-exposed bivalves in particular, and to some extent pollutant-exposed organisms in general.

CONCLUSIONS

Based on the published scientific literature, there was support for slight positive responses of the assessed transcriptional biomarker candidates at arbitrary metal exposure, both overall and (with the exception of *hsp90*) when assessed separately. The same was also true for the overall responses in gills and digestive glands. However, there was also an implication of publication bias in favor of positive effect sizes, likely leading to a general overestimation of biomarker responsiveness. Predicted effect sizes from arbitrary metal exposure should therefore be interpreted with caution because it is not unlikely that the "true" effects in most cases would be close to 0. Taken together, this suggests low sensitivity and robustness of the biomarker candidates.

There was a slight increase in expected response with exposure time, although this effect was weaker for the transcript and tissue subsets than for the overall response. The general implication would be that sensitivity increases with time and that the probability of detecting differences is likely higher after days or weeks than after hours of exposure.

Finally, except for a slight decrease in *sod*, there was little support of concentration dependence of the responses with regard to toxic unit, either for overall responses or for transcript- or tissue-specific effects. As discussed, this could partially be due to low resolution resulting both from the various species × transcript × toxicant combinations and from the uncertainties around toxic units. Nonetheless, it gives a clear implication that, on a large scale, there is no universal concentration–response relationship for stress-related transcripts in metal-exposed mussels. Consequently, in the absence of species-, toxicant-, and/or tissue-specific data, robust responses should not necessarily be expected even at high exposure concentrations.

The present study illustrates a number of limitations of the selected transcriptional responses in bivalves, which would likely be true for a range of other taxa, transcript, and toxicant exposures. Prior to potential application of transcriptional biomarkers in ERA, it will therefore be crucial to further address, for example, concentration dependence, time dependence, and individual variation. Provided there is sufficient mechanistic understanding and/or empirical support, transcripts may have great potential for various approaches in ERA, such as adverse outcome pathways, multibiomarker models, or transcriptional points of departure. Whether or not there are transcripts that on their own can function as biomarkers of general toxicity and chemical stress, however, remains a question for future research.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://10.1002/etc.5494.

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Author Contributions Statement—**Gustaf M. O. Ekelund Ugge**: Conceptualization; Investigation; Formal analysis; Methodology; Visualization; Writing—original draft; Writing—review & editing. Data curation **Ullrika Sahlin**: Methodology; Formal analysis; Writing—review & editing. **Annie Jonsson**: Writing—review & editing. **Olof Berglund**: Supervision; Conceptualization; Writing—review & editing.

This article has earned both an Open Data and Open Materials badge for making publicly available the digitally shareable data necessary to reproduce the reported results. The data are available at https://doi.org/10.17632/83jc4yv35h, and the materials are available at https://github.com/gmoeu/transcriptional-biomarkers-metaanalysis. Learn more about the Open Practices badges from the Center for Open Science: https://osf.io/tvyxz/wiki.

Data Availability Statement—Data, associated metadata, and calculation tools are also available from the corresponding author (gustaf.ekelund_ugge@biol.lu.se).

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