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Effect of β-adrenergic receptor agents on cardiac structure and function and whole-body gene expression in *Daphnia magna*☆

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ABSTRACT

Propranolol (PRO), a human β-AR (β-adrenergic receptor) antagonist, is considered to result in specific effects in a non-target species, *D. magna*, based on our previous studies. The present study investigated the effects of β-AR agents, including an antagonist and agonist using pharmacologically relevant endpoints as well as a more holistic gene expression approach to reveal the impacts and potential mode of actions (MOAs) in the model non-target species. Results show that the responses in cardiac endpoints and gene expression in *D. magna* are partially similar but distinguishable from the observations in different organisms. No effect was observed on heart size growth in PRO and isoprenaline (ISO) exposure. The contraction capacity of the heart was decreased in ISO exposure, and the heart rate was decreased in PRO exposure. Time-series exposures showed different magnitudes of effect on heart rate and gene expression dependent on the type of chemical exposure. Significant enrichment of gene families involved in protein metabolism and biotransformation was observed within the differentially expressed genes, and we also observed differential expression in juvenile hormone-inducible proteins in ISO and PRO exposure, which is suspected of having endocrine disruption potential. Taken together, deviation between the effects of PRO and ISO in *D. magna* and other organisms suggests dissimilarity in MOAs or attributes of target bio-molecules between species. Additionally, PRO and ISO may act as endocrine disruptors based on the gene expression observation. Results in the present study confirm that it is challenging to predict ecological impact of active pharmaceutical ingredients (APIs) based on the available data acquired through human-focused studies. Furthermore, the present study provided unique data and a case study on the impact of APIs in a non-target organism.

1. Introduction

APIs that are introduced into the aquatic environment can affect non-target organisms and, consequently, result in unintended harmful effects on ecosystems. Unexpected detrimental effects of APIs in natural ecosystems have been reported and are a pressing concern in environmental science (Brodin et al., 2013; Cuthbert et al., 2006; Pérez et al., 2012). Studies have proven that vertebrate biochemical messengers react with receptors in wildlife, which are potential targets of several APIs and responsible for crucial physiological functions in non-target organisms (Buonomo et al., 1984; Kashian and Dodson, 2004). Although the homology of pharmaceutical target receptors is highly species-dependent, studies have shown therapeutic actions of APIs in non-targeted organisms (Brooks and Huggett, 2012; Campos et al., 2012; Gunnarsson et al., 2008). Considering persistent concentrations of APIs up to several μg/L in the aquatic environment (Tijani et al., 2016), deeper understanding of the ecological risk of APIs is necessary to investigate how the effects of APIs occur and whether those are pharmacologically relevant.

PRO, a cardiovascular drug, is one of the potentially harmful APIs to aquatic organisms. Results of our previous study on *D. magna*

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confirmed a heart-specific action of PRO in a non-target organism (Jeong et al., 2018), but details on the cardiac effects were limited due to the lack of observation on additional pharmacologically relevant endpoints. Furthermore, the complexity of the proposed MOAs of PRO in non-targeted organisms (Huggett et al., 2002; Massarsky et al., 2011) requires a study employing multiple biomarkers to further understand the effects and potential MOAs of the β-AR agent. Therefore, the current study focused on multiple pharmacologically relevant endpoints to investigate how β-AR binding agents affect *D. magna*. In addition, whole-body gene expression profiling in a time-course experiment was performed to provide deeper insight into the effects and potential MOAs. β-AR antagonist and agonist, PRO and ISO (Day and Roach, 1974), were used to activate and deactivate the target receptor. A mixture of the agents was also utilized to confirm a mixture effect as the effect of ISO is abolished by PRO (Hainsworth et al., 1973).

2. Materials and methods

2.1. Preparation of chemical solutions and model organisms

(±)-Propranolol hydrochloride and isoprenaline hydrochloride were purchased from Sigma-Aldrich. Reagents were handled as recommended by the manufacturer. Chemical solutions for each exposure test were generated using media identical to the culture media. For the investigation of cardiac functional and structural change, an in-house *D. magna* culture was used. Culturing methods and media composition were in compliance with the US Environmental Protection Agency (EPA) guideline (Weber, 1991). For the gene expression profiling, the Xinb3 genotype of *D. magna* was used, which had been raised in the laboratory of K. D. Schamphelaere (Asselman et al., 2016). COMBO media and Organization for Economic Co-operation and Development (OECD) guidelines were used for the test organism culture (Kilham et al., 1998; OECD, 2012). The Xinb3 isolate was specifically selected for the gene expression profiling because it has been used to develop the recently published transcriptome of *D. magna* and allowed us to easily identify potential targets using the recently published corresponding gene set of *D. magna* (Orsini et al., 2016).

2.2. Chemical exposure experiments and body sampling

The overall design of the exposure experiments is described in Fig. 1. The first and second experiments investigated the heart-related endpoints monitoring and whole body gene expression profiling, respectively. In the first experiment, animals were exposed to solutions of PRO and ISO during 6 days to study chronic effects in heart size, contraction capacity, and heart rate. The mixture of PRO and ISO was additionally used to investigate mixture effect of the target compounds on heart rate. The concentrations of solutions were 96 μg/L and 1755 μg/L for PRO and ISO solution, which are sub-lethal concentrations (Dzialowski et al., 2006). Seven-day-old animals were individually exposed to each chemical solution in 30-mL plastic beakers. After 6 days of exposure, cardiac size, body size, and heart volumes in relaxation and contraction states were measured under the microscope. Every exposure was replicated 6 times.

In the second experiment, animals were exposed for 24 h to 2 different concentrations of PRO, a single concentration of ISO, and a mixture of PRO and ISO to study changes in gene expression and heart rate. Five-day-old daphnids, which have no egg on their clutches, were used to avoid detection of gene expression in eggs. The solution concentrations were 0.9 mg/L and 3.6 mg/L for PRO solutions, 84.4 mg/L for an ISO solution, and 0.9 mg/L of PRO and 84.4 mg/L of ISO for a mixture. The concentrations in the exposure were chosen to be sufficiently high to observe clear time-series changes of heart rate and corresponding gene expression regulation within 24 h, which is the period of initial response of *D. magna* to the exposed chemicals. Twenty individuals were exposed to different chemical solutions separately in a volume of 35 ml and were harvested at 1, 3, 6, and 24 h of exposure. When the whole body sample was gathered, heart rates were recorded from separate exposure sets, an individual *D. magna* in 20 mL chemical solutions, under a microscope. The body sampling was triplicated, and heart rate measurement was replicated 6 times. Exposure conditions, including room temperature and food concentration, were the same across all exposures and identical to the culturing conditions. Solutions in all exposures were daily generated and renewed daily to prevent degradation of the chemicals.

2.3. Confirmation of chemical concentrations of solutions

Chemical concentrations in the exposure solutions were quantified separately using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in triplicate, and the averages of the estimated concentrations were used in this manuscript. The studies

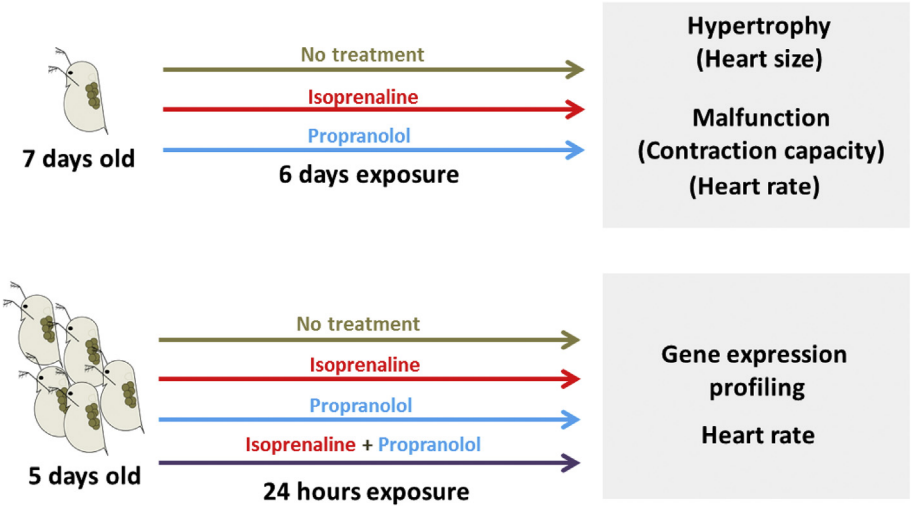

Fig. 1. Overall experiment design.

of Jeong et al. (2016) and Gu et al. (2008) were used as references for analysis method development, and metoprolol was used as an internal standard compound. In brief, quantification was performed using a Waters Quattro micro high-performance LC-MS/MS system. Chromatographic separations were performed on an ACQUITY UPLC BEH C18 column (2.1 × 150 mm, 3.5 μM, water). The mobile phases were Milli-Q water (0.1% formic acid) and acetonitrile (0.1% formic acid). The column temperature was 40 °C. The mass condition was as follows: ESI positive ion mode; source temperature of 150 °C; desolvation temperature of 350 °C; desolvation gas flow of 500 L/h; nitrogen gas for desolvation, and argon gas for collision. The instrument was operated in multiple reaction monitoring mode and ion masses of 260 > 183, 208 > 166, and 268 > 74 were used for PRO, ISO, and metoprolol, respectively.

2.4. Heart rate, heart size, and body size measurement from the 6-day exposure set

Animals of similar body size were chosen to minimize biological variation. After 6 days of exposure, individuals were placed under a microscope (CKX41SF, Olympus) equipped with a digital camera, and heart rate, heart size, and body size were measured. The heart rate for 30 s was counted for 30s in slow-motion mode using a GOM player (Gretech Corporation), and relative heart rate to control was calculated for comparison between different exposures. The heart size and body size were captured and estimated using ImageJ software, and raw units (pixel) were not transformed to actual-size units (Schindelin et al., 2015). Heart size and body size were measured at the start and at the end of the exposure to calculate the relative change. To compare heart sizes between individuals, the measured heart size was normalized to the measured body size.

The contraction capacity was defined as the area deduction between the heart sizes in relaxation and contraction states, and ImageJ was used for the measurements and calculations of the heart area. The details of the contraction capacity calculation are listed in Fig. S1.

2.5. Gene expression profiling and heart rate measurement from the 24-h exposure set

During the 24-h exposure period, the animals were harvested after 1, 3, 6, and 24 h of exposure, at which heart rate was also simultaneously recorded. The measurement method of heart rate was the same as that in the 6-day exposure experiment. Body samples of *D. magna* frozen using liquid nitrogen were stored in 1.5 ml microtube at −80 °C until RNA extraction. Total RNA was extracted from the body sample using the RNeasy kit and Qiashredder (Qiagen, Venlo, The Netherlands) following the manufacturer's protocol.

Concentration and quality of the extracted total RNA was measured using the Quant-it RiboGreen RNA assay (Life Technologies, Grand Island, NY, US) and using an RNA 6000 Pico Chip Kit on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US). An Illumina mRNA sequencing library was made from 500 ng of total RNA using the Truseq stranded messenger RNA Library Prep Kit (Illumina, San Diego, CA, US). Libraries were quantified by quantitative polymerase chain reaction, according to Illumina's protocol Sequencing Library qPCR Quantification protocol guide. A DNA 1000 chip (Agilent Technologies, Santa Clara, CA, US) was used to control the library's size distribution and quality. In total, 21 RNA-sequence (RNA-seq) libraries were equimolarly pooled and sequenced on an Illumina NextSeq 500 high throughput run, generating 1 × 75 base pair reads. All sequencing data was deposited in GEO and is available under accession number GSE104487.

2.6. Data analysis

For the data from cardiac endpoints monitoring, an analysis of variance (ANOVA) test was performed with a *post hoc* Tukey's test to compare the significant changes between exposure sets and control using SPSS 18.0.0 software. For the RNA-seq data, quality of the raw reads was assessed using FastQC (Babraham Institute, Cambridge, UK, version 0.11.5). Potential adapter contamination in the raw reads was removed using Trim Galore (Babraham Institute, Cambridge, UK, version 0.3.2.). Additionally, reads were dynamically trimmed to the longest stretch of bases to obtain at least 99.9% base-call accuracy. Reads were aligned to the Xinb3 transcriptome (Orsini et al., 2016) using Bowtie2 (version 2.1.0) (Langmead and Salzberg, 2012). Aligned reads were processed with HTseq to count the number of reads per gene (Anders et al., 2015). These counts files were then statistically analyzed in R and Bioconductor (Gentleman et al., 2004; Ihaka and Gentleman, 1996) for differential gene expression. Trimmed means of M-values were applied for normalization after data filtration. Quasi-likelihood dispersion was estimated (Lun et al., 2016). Gene expression at different time points and chemical exposures were compared with controls to identify significant differences using factorial designs to determine the effects of exposure time, PRO, ISO, and any potential interactions, e.g., interactions between PRO and time. This was done by fitting a quasi-likelihood negative binomial generalized log-linear model to the data and conducting gene-wise statistical tests for each statistical contrast or coefficient of the log-linear model, which includes both main effects and interaction effects (Lun et al., 2016). The Benjamini-Hochberg method was applied to adjust p values (Benjamini and Hochberg, 1995). Genes with a significant p-value (<0.05) and a positive log2 fold change were identified as significantly upregulated, genes with a significant p-value and a negative log2 fold change significantly downregulated. No additional cut-off value was used. Fisher's exact test was performed to identify enrichment or overrepresentation of gene families and pathways within the differentially expressed gene set (Asselman et al., 2012).

3. Results

3.1. Effect on heart rate, size, and contraction capacity after 6-day exposure

The effects of PRO and ISO on cardiac structure and function were determined by measuring heart size, heart contraction capacity, and heart rate (Fig. 2). Heart size did not significantly change after exposure to PRO and ISO (Fig. 2a). However, heart contraction capacity decreased significantly in ISO exposure (Fig. 2b). The heart rate measurement results are shown in Fig. 2c. Heart rate was significantly reduced after 6 days of PRO exposure, whereas it was not affected by ISO. Interestingly, when ISO was mixed with PRO, the heart rate was significantly higher than when *D. magna* was exposed to PRO alone. The lowered heart rate in the mixture was still significantly decreased from that of the control.

3.2. Heart rate and gene expression profile after 24 h exposure

Time series effects of PRO and ISO on gene expression were monitored with timely synchronized heart rate measurement. The decrease in heart rate in *D. magna* was time-dependent as well as dependent on types of exposures (Fig. 3). PRO at 3.6 mg/L had the biggest effect on heart rate among all exposure conditions and the lowest heart rate was observed at 24 h. ISO did not have a significant effect, and the mixture of ISO and PRO resulted in a decrease in heart rate. However, this effect was smaller than that of PRO alone.

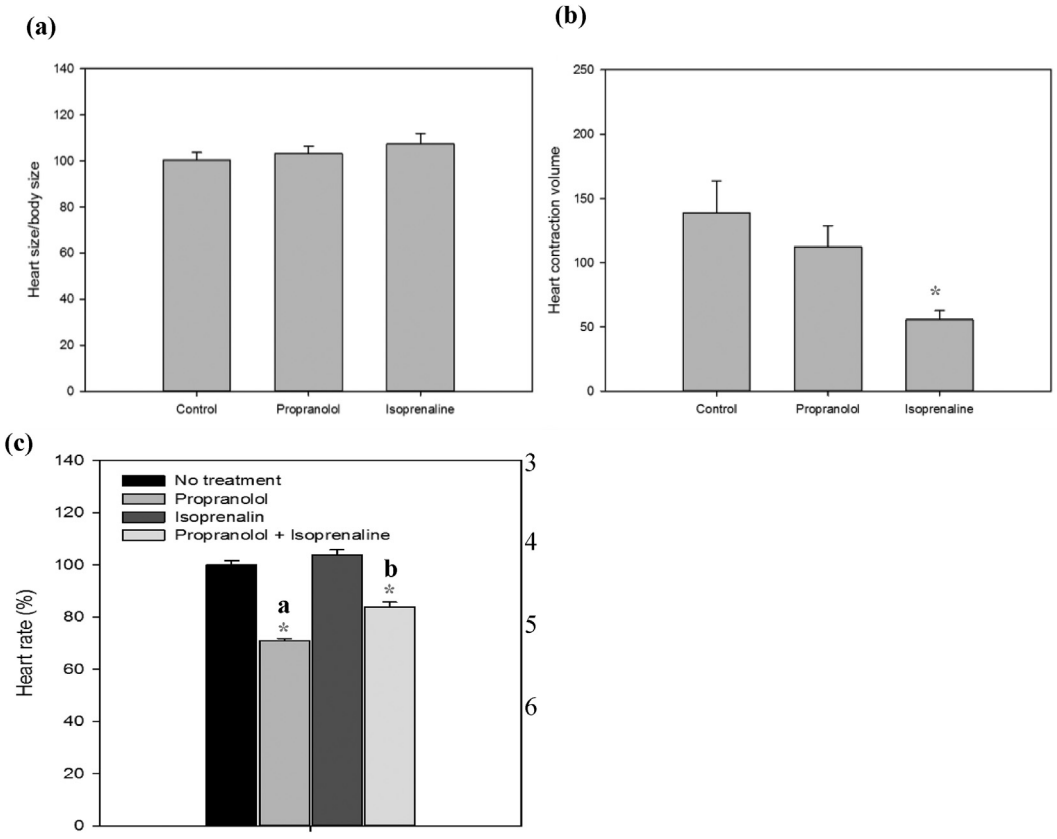

Fig. 2. (a) Heart size, (b) contraction capacity, and (c) heart rate after 6-day exposure. Each bar and line represents the average and ±standard error. *P ≤ 0.05. Propranolol and Isoprenaline were 96 μg/L and 1775 μg/L, respectively.

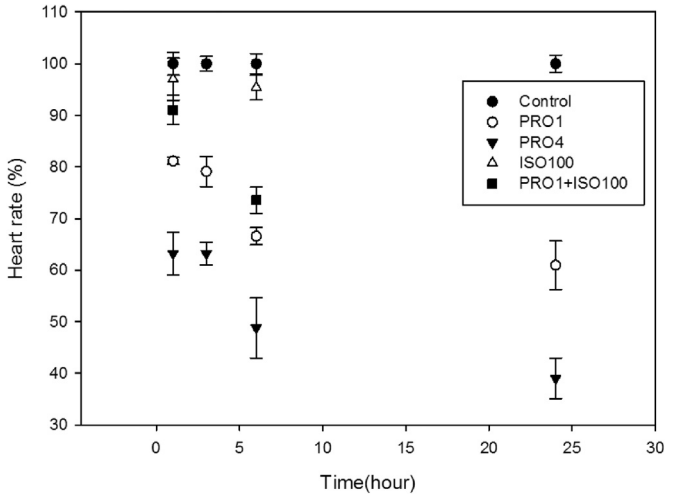

Fig. 3. Heart rate measurements for the different exposures at the different time points. Each scatter and line represent the average and ±standard error. Propranolol was 0.9 and 3.6 mg/L for PRO 1 and 4, and Isoprenaline was 84.4 mg/L for ISO 100, respectively.

At the gene expression level, we studied more than 19,000 genes and observed significantly different regulation of genes across exposures and time points. When comparing differentially expressed genes across the different statistical contrasts (Fig. 4), we observed a similarity between the different contrasts. Eleven and 261 genes were shared across the different main contrasts and different interaction contrasts with time, respectively. These overlapping genes between the contrasts imply that gene expressions and consequent physiological changes might be partly overlapped as results of those gene expressions. In Fig. 4, the effects of PRO on gene expression seem to be more stable across different time points as we observed more than 1300 genes significantly expressed at all time points (PRO) whereas we observed roughly 400 genes which were significantly expressed depending on the time point (PRO X TIME). We observed the opposite for ISO and ISO + PRO. For those treatments, gene expression depends on the time point as more genes were significantly expressed in ISO X TIME and ISO + PRO X TIME contrast than the main effects. Based on these observations, it is assumable that ISO and ISO + PRO showed different level of influence dependent on time than PRO.

The known main target of PRO and ISO is the β-AR, meaning that the changes in gene expression are not directly regulated by agents; however, it could provide hints on potential MOAs by comparing with results of previous studies focusing on effects of β-AR activity-related agents. Table 1 compares MOA-related or frequently reported gene expressions from the present and previous studies. If there were too many genes annotated in a gene function to be summarized, only differentially expressed genes were listed in the table. As shown in Table 1, gene expression results are partially matched to the previous studies; the kinds of differentially expressed genes are quite similar but the patterns of gene expressions are far different. The details of all genes differentially expressed are provided in Table S2.

Apart from the expression analysis on the single genes, we observed the enrichment of several gene families with differentially expressed genes in ISO, PRO, and mixture exposures (Table S1, Figs. 5–7). Given the small number of genes for the main ISO and

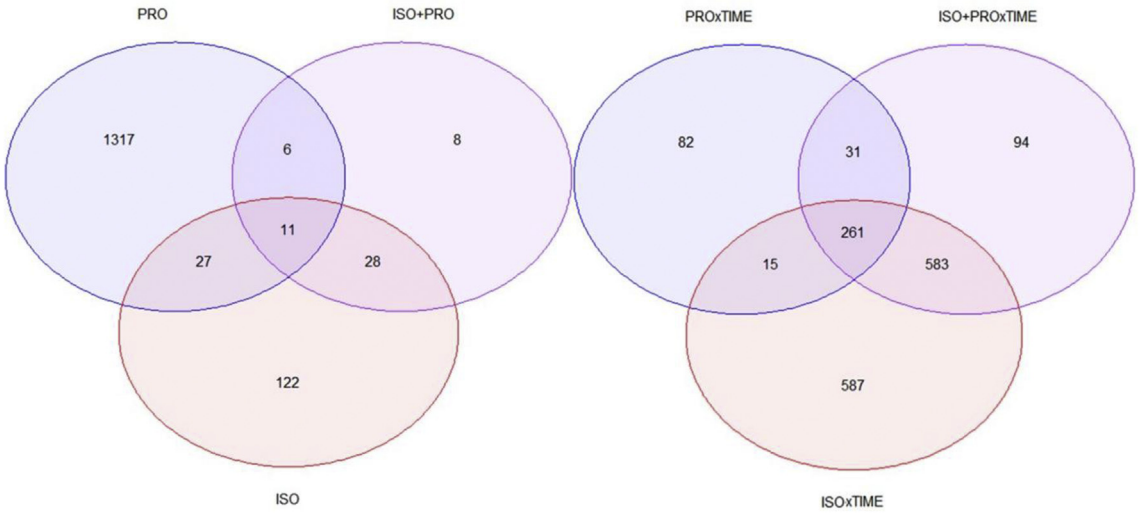

Fig. 4. Venn diagram of differentially expressed genes shared across different contrasts regardless of time (left): genes that differ significantly between control and PRO exposures regardless of time (PRO), between control and ISO exposures regardless of time (ISO), genes that show a significant interaction in a combined exposure of propranolol and isoprenaline regardless of time (ISO + PRO). Venn diagram of differentially expressed genes shared across different time contrasts (right): genes that showed a significant interaction between ISO and time (ISO X TIME), genes that showed a significant interaction between PRO and time (PRO X TIME), genes that showed a significant interaction between ISO and PRO exposures across time (ISO + PRO X TIME).

Table 1
Differential gene expressions in this study and previous studies. Genes from previous studies are all regulated by β-AR activity-related agents. Up and Down arrows indicate up and downregulation of gene expression. ND: Differential expression not detected.

Related function	Observation			References		
	Gene	Expression-Agent (across time)	Expression-Agent (regardless of time points)	Gene	Expression - Agent	Tissue
β-Adrenergic receptor	β-AR kinase	ND	ND	β_1-AR	↑↓ - ISO	Medaka heart (Kawasaki et al., 2008)
	β_2-AR	ND	ND			
Protein Kinase A	Camp-dependent protein kinase catalytic subunit	↑ - ISO ↓ - ISO + PRO	↓ - PRO			
Myosin	Myosin light chain kinase, smooth muscle	↑ - ISO, PRO ↓ - ISO + PRO	↓ - PRO	Myosin XVIIIA	↓ - PRO	Minnow brain (Lorenzi et al., 2012)
	Myosin-RhoGAP protein	↑ - ISO ↓ - ISO + PRO	↓ - PRO			
	Unconventional Myosin 16	↑ - ISO ↓ - ISO + PRO	↓ - PRO	Myosin light chain	↓ - PRO	Burned patients muscle (Herndon et al., 2003)
	Myosin 3	↓ - ISO				
Actin	α -Actinin-1	↑ - ISO ↓ - ISO + PRO	↓ - PRO	skeletal α-actin	↑ - ISO	Rat ventricular myocytes (Bishopric et al., 1992)
Apoptosis	B-cell lymphoma/ leukemia 11A	↓ - ISO + PRO	ND	Caspase 8	↓ - PRO	Minnow brain (Lorenzi et al., 2012)
	Tumor necrosis factor ligand superfamily member	↑ - ISO	↓ - PRO	Caspase 3	↓ - PRO	
	Calcium/calmodulin dependent protein kinase	↑ - ISO ↓ - ISO + PRO	↓ - PRO	TGF-β3	↓- ISO	Rat cardiac fibroblasts(Colombo et al., 2001)
Immediate early gene	c-Fos	ND	ND	c-Fos	↑- ISO + CHT	Rat myocardial cell, Rat ventricular tissue (Brand et al., 1993; Iwaki et al., 1990)
				c-Jun	↑- ISO + CHT	Rat myocardial cell (Iwaki et al., 1990)
				Jun-B	↑ - ISO	Rat ventricular tissue (Brand et al., 1993)
	Early growth response	ND	ND	Jun-D	↑ - ISO	Rodent parotid gland (Ten Hagen et al., 2002)
				Early growth response 1	↑-ISO + CHT	Rat myocardial cell (Iwaki et al., 1990)

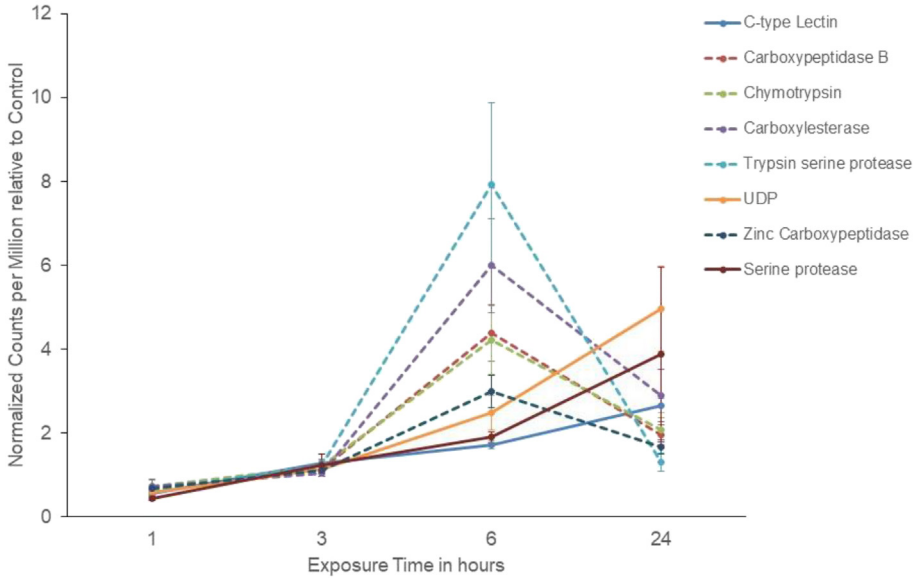

Fig. 5. Average gene expression patterns for different gene families in PRO 0.9 mg/L relative to control exposure across four time points. (e.g., Value 1 means that normalized counts per million are equal in propranolol and control exposures). Error bars represent standard errors.

ISO × PRO contrasts, few significant enrichments could be detected (Table S1, Figs. 5–7), and, as such, gene family enrichment for the contrasts without time interactions will not be discussed further. Eight gene families were significantly enriched with differentially expressed genes across the different exposures in time × exposure interactions (Table 2, Figs. 5–7). For all gene families, the largest upregulation was observed after 6 or 24 h for the PRO exposure as shown in the case of PRO exposure (Fig. 5). In addition, we observed dose-dependent gene expression after 6 h for all these gene families for the different PRO exposures (Fig. 6). For all gene families, the gene expression patterns can be described as similar for all exposures. Only C-type lectin and carboxylesterase gene families showed different regulation patterns by the mixture of PRO and ISO at 6 h, suggesting a potential mixture interaction between the effects of ISO and PRO at the gene level.

Among the gene families significantly enriched, only 2 gene families were enriched by ISO and PRO individually, but not by ISO + PRO. Surprisingly, these 2 gene families showed a time-dependent pattern for ISO, while they showed a consistent pattern over time for PRO (Fig. 7, Table S1). Indeed, ISO exposure across time regulated genes, which have endocrine disrupting (ED) potential, the juvenile hormone inducible proteins while this was regulated significantly but consistently regulated over time in PRO (Fig. 7, Table S1). Similarly, the mRNA capping enzymes are significantly enriched in the ISO X TIME and in the PRO contrast, but not in any others. These genes are involved in gene expression and splicing. While it seems likely that exposure to toxicants affects gene expression and splicing, it is unclear as to why these effects are observed for the single stressors but not for the combined treatment.

4. Discussion

4.1. Effect on cardiac structure and function after 6-day exposure

Heart size is known to be affected by β-AR activity (Osadchii, 2007; Stanton et al., 1969). Abnormal enlargement of the heart, or cardiomegaly, has been reported to be induced by β-AR activation via ISO administration in rats (Osadchii, 2007; Stanton et al., 1969). Both PRO and ISO have demonstrated negative and positive effects in the heart size of fish (Kawasaki et al., 2008). Here, the size of the heart of *D. manga* was not affected (Fig. 2a). In addition, the results of contraction capacity were not comparable to previous studies (Fig. 2b). In general, cardiac output, which is a function of the heart rate and stroke volume, is known to be increased by ISO (McQueen et al., 2005) with increases in both heart rate and stroke volume (Fenyvesi and Hadházy, 1973; Kouchoukos et al., 1970). Because the stroke volume is the amount of blood per heartbeat, the contraction capacity must be positively related to the stroke volume. As the reduction of the contraction capacity in this study implies a potential reduction in stroke volume, the effect caused by ISO contrasts with the results of previous studies, in which an increase in heart rate and stroke volume were reported.

ISO and PRO are known to have opposite regulatory effects on the heart rate in humans, and PRO is able to negate the effect of ISO (Brick et al., 1968). The decreased heart rate in the PRO exposure group, and the decreased but higher level than that of PRO alone in the mixture, supports a similar action of the chosen drugs in *D. magna* (Figs. 2c and 3). On the other hand, no change in ISO exposure suggests a weak binding affinity or a difference in pharmacodynamics of ISO in the heart of *D. magna*. Berghmans et al. reported a non-significant mild increase of zebrafish heart rate at 1 mM of ISO exposure; otherwise, gut contraction was severely affected in the same exposure condition (Berghmans et al., 2008). Their results are comparable to ours in terms of the influence of ISO on certain biological functions, but not heart rate, in non-targeted organisms.

Overall, the observed responses in *D. magna* hearts highlight clear differences from the known actions of PRO and ISO. We observed effects partially identical to the pharmacological effects on heart rate, but the observations on heart size and contraction capacity were totally unpredictable from the known MOA. It is assumed that the discrepancy in actions of the APIs results from structural differences of target receptors or variations in the distribution and function of target receptors between species. Such species deviations have been reported; for example, β_2-AR activation induces a positive inotropic response in myocytes of cats and dogs, but not in guinea pigs, due to variations in receptor

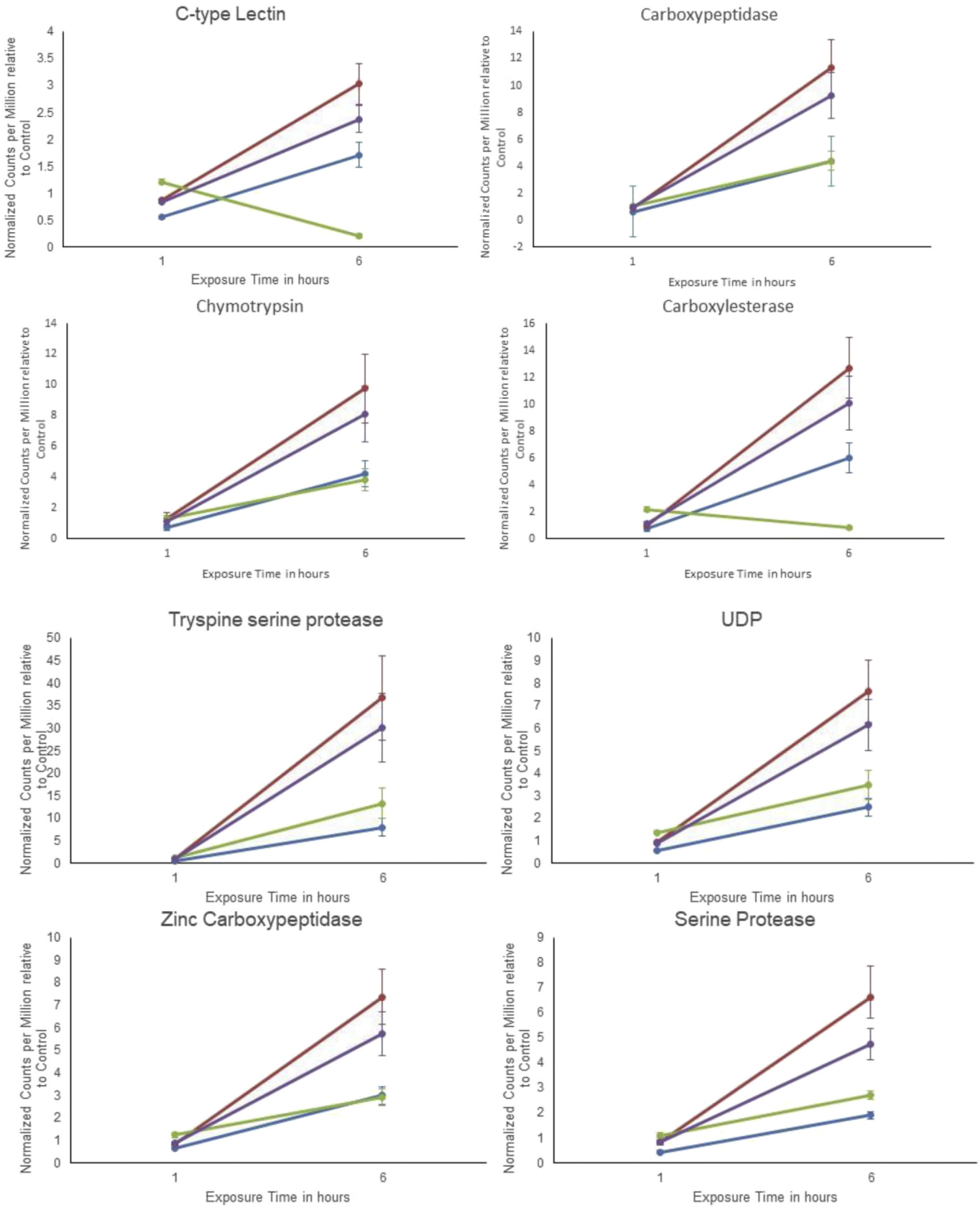

Fig. 6. Average gene expression patterns for different gene families in PRO 1 mg/L (blue), PRO 4 mg/L (purple), ISO 100 mg/L (red), PRO 1 mg/L + ISO 100 mg/L (green) across two time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

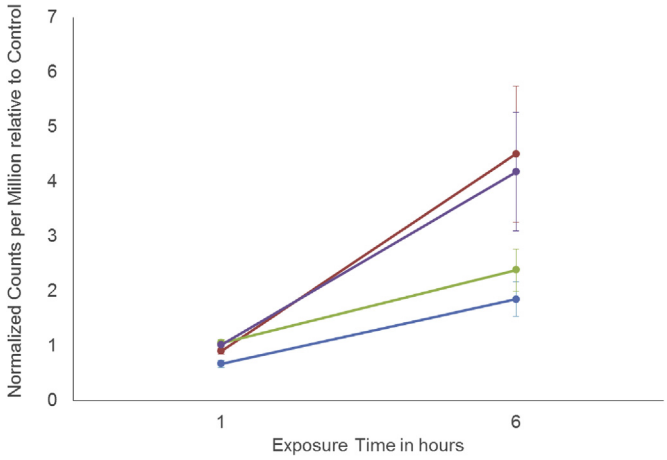

Fig. 7. Average gene expression patterns for the juvenile hormone inducible proteins in PRO 1 mg/L (blue), PRO 4 mg/L (purple), ISO 100 mg/L (red), PRO 1 mg/L + ISO 100 mg/L (green) across two time points. Error bars represent standard errors. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

distribution and physiological function (Booze et al., 1989; Steinberg, 1999). Different amino acid sequences of target receptor subtypes could also contribute to functional differences (Finch et al., 2006; Michel and Insel, 2006). Because these differences occur between relatively closely related mammals, it is not surprising that differences exist between mammals and *D. magna,* and this supports a predicted low similarity of β_2-AR between *Daphnia* and humans based on genome sequence data (Gunnarsson et al., 2008).

4.2. Effect on whole-body gene expression during 24 h exposure

In Fig. 4, more stable effects on gene expression were observed across time in PRO than ISO and ISO + PRO. This may suggest that the pathways triggered by PRO require continuous expression whereas the pathways triggered by ISO and ISO + PRO require subsequent expression of different genes over different time points. Temporal patterns for gene expression have already been observed for metallothionein genes in response to metal exposure most likely due to the long half-life of metallothionein proteins (Asselman et al., 2013). As such, this could also suggest that genes regulated by PRO encode for proteins with a shorter half-life, thus leading to more continuous RNA expression, while genes regulated by ISO and ISO + PRO encode for proteins with a long half-life, thus requiring only RNA expression at specific time points.

Table 1 shows similar genes were affected in expression but the patterns of gene expression were different compared to the previous studies. This tendency is in the same context of the observed impacts on the cardiac endpoints in this study. β-AR activity has been known to be involved to muscle contraction, cell growth, apoptosis and a variety of other functions in different organs, particularly in the heart (Communal et al., 1998; Devic et al., 2001; Simpson et al., 1991). In the major signaling pathway, stimulation of β-AR results in a signaling cascade sequentially consisting of G protein-mediated adenylyl cyclase activation, c-AMP generation, PKA activation, and phosphorylation of diverse proteins, which leads to physiological changes (Perez, 2006). β_1-AR gene, a gene of the target receptor of PRO and ISO, did not influence the expression, unlike the up- and downregulation by ISO in the fish model (Kawasaki et al., 2008). Cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA), a major downstream enzyme, phosphorylates various substrates in the AR signaling pathway; for example, the L-type Ca_2^+ channel is phosphorylated by PKA and the increased inner cell Ca2+ concentration of myocytes (Perez, 2006). Expression of the PKA catalytic subunit gene was up- and downregulated by agents and its mixture in the present study. Myosin-related gene expressions were reported to be downregulated by PRO in fish and burned patients (Herndon et al., 2003; Lorenzi et al., 2012). In the present study, several myosin-related gene expressions were significantly regulated by drug exposures; a mixture and solutions of PRO and ISO up and downregulated most of the gene expressions, except Myosin-3, which is downregulated by ISO only. Actin is supposed to be related to muscle cell morphological and developmental regulation along with myosin. A study found that gene expression of the skeletal α-actin is upregulated by ISO in rat ventricular myocytes (Bishopric et al., 1992). Expression of the α-actinin-1 was found to be up and downregulated by the mixture and single solutions of PRO and ISO. Apoptosis mediated by β_1-AR activation has been reported (Communal et al., 1999) and in-vitro studies have revealed that myocyte apoptosis results from PKA-independent activation of calcium/calmoduline-dependent kinaseII (CaMKII) (Zhu et al., 2003). Differential expressions of the gene related to apoptosis and CaMKII are detected in the present study, but the impacts on gene expression were not comparable to those of previous studies. Additionally, early response gene expressions were compared to those of the previous studies; however, there was also lack of similarity.

Obviously, there are limitations in the comparison of gene expression between studies. The studies used different organisms, tissues, effect concentrations, and exposure times. Complexity of the MOA of agents is another factor making the translation of gene expression results challenging; for example, ISO and PRO also targets mitogen-activated protein kinase 1 (MAPK1), phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R, phosphodiesterase 4 (PDE4), superoxide dismutase 1 (SOD1), and 5-HTR1, which share so many molecules in their cascade signaling pathways with β-AR

Table 2
Overview of the gene families that were enriched for differentially expressed genes in statistic contrasts: (1) genes that differed significantly between PRO and control exposures across time: PRO X TIME; (2) genes that showed a significant interaction between ISO and control exposures across time: ISO X TIME; (3) genes that differed significantly between ISO and PRO exposures across time: ISO + PRO X TIME. P values are Benjamini-Hochberg adjusted P values and the result of Fisher exact test for enrichment analysis. Visualization of the gene expression patterns can be found in Figs. 5–7.

	PRO X TIME	ISO X TIME	ISO + PRO X TIME
C-type lectins	9.52 e−04	5.4 e−15	9.53 e−16
Carboxypeptidase B	1.51 e−05	4.04 e−04	2.55 e−05
Chymotrypsin BI precursor	2.78 e−05	8.72 e−04	1.53 e−04
Carboxylesterase	3.00 e−09	6.22 e−08	6.66 e−10
Putative serine protease	7.68 e−06	1.24 e−04	7.63 e−06
Trypsin serine protease	1.15 e−04	8.72 e−04	9.30 e−05
UDP-glucorosonyltransferase 2A1	8.67 e−04	5.69 e−05	2.4 e−06
Zinc carboxypeptidase	8.57 e−03	2.25 e−06	6.66 e−10

pathway (Masson et al., 2012; Wishart et al., 2017). Although there are uncertainties caused by the limitations, it seems clear that PRO and ISO affect genes involved in the AR pathway, and the patterns of gene expressions are quite different from the previous studies, as shown in Table 1, which is in the same context of physiological monitoring results because the results are only partially comparable to those of other organisms.

Table 2 shows that most of the gene families that were significantly enriched are related to protein metabolism. Carboxypeptidase (Carboxypeptidase B and Zinc Carboxypeptidase) is responsible for the hydrolytic cleavage of a peptide bond at the C-terminal of protein or polypeptide (Christianson and Lipscomb, 1989). Serine protease (serine protease, trypsin serine protease, and chymotrypsin) also cleaves peptide bonds; trypsin-like and chymotrypsin-like protease respectively target basic and non-polar amino acids (Ovaere et al., 2009). Those protein-degrading enzymes are involved in a wide range of biological functions, including digestion, immune response, reproduction, and protein post-translation modification (Hedstrom, 2002). According to a recent observation, PRO exposure caused a depletion of free amino acids in the *D. magna* metabolome (Jeong et al., 2018). The present results are not sufficient to suggest which specific mechanisms result in the gene family enrichments; however, as the peptide-cleaving enzymes are related to the protein metabolism, the enrichment of the 5 gene families in this study may be associated with the downregulation of the free amino acid contents. In addition to the carboxypeptidase and protease, the other 2 gene families are related to biotransformation. Carboxylesterase and UDP are individually phaseland II enzymes (Parkinson and Ogilvie, 2001). They catalyze hydrolysis and glucuronidation of xenobiotics. It was confirmed from our previous study that a major metabolite of PRO in humans is also generated by *D. magna* (Jeong et al., 2016); thus, the gene family enrichment related to the drug metabolism seems rational.

The ED ability of APIs has been discussed in previous studies, and AR drugs were also mentioned as a potential ED chemical (Massarsky et al., 2011). Despite the structural similarity between PRO and ISO, only ISO showed time-dependent impacts on ED-related gene expressions (Fig. 7). It seems needed to be further studied about the effect of ISO on *D. magna* requires further study, as ISO showed distinguishable and significant impacts on cardiac endpoints along with the ED-related gene expression.

5. Conclusion

Our results highlight that effects of PRO and ISO on a non-target species, *D. magna*, is unpredictable, as indicated in the available pharmacological database. Cardiac endpoints and gene expression in *D. magna* were affected by PRO and ISO in a manner similar to that of other organisms, but the results do not seem to be extrapolatable based on the results of other species. Furthermore, enrichment analysis indicated that AR drugs affect to expression of genes involved in protein metabolism, drug metabolism and, more importantly, endocrine system disruption which suggests needs for future studies for ED potential of ISO and PRO. Although this study still has limitations in study design to reveal MOAs precisely, at the same time, it proves the novelty of co-employment of physiological and transcriptional measurements in the investigation of impacts on APIs in non-target species study.

Author contributions

TJ and SDK designed the study. TJ executed all the experiments and collected all the samples. TJ analyzed the physiological data. TJ, JA, KDS, and FVN designed the gene expression experiment. JA analyzed the sequencing data with input from TJ, KDS and FVN. TJ wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.06.026.

References

Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169.
Asselman, J., De Coninck, D.I., Beert, E., Janssen, C.R., Orsini, L., Pfrender, M.E., Decaestecker, E., De Schamphelaere, K.A., 2016. Bisulfite sequencing with Daphnia highlights a role for epigenetics in regulating stress response to Microcystis through preferential differential methylation of serine and threonine amino acids. Environ. Sci. Technol. 51, 924–931.
Asselman, J., De Coninck, D.I., Glaholt, S., Colbourne, J.K., Janssen, C.R., Shaw, J.R., De Schamphelaere, K.A., 2012. Identification of pathways, gene networks, and paralogous gene families in Daphnia pulex responding to exposure to the toxic cyanobacterium Microcystis aeruginosa. Environ. Sci. Technol. 46, 8448–8457.
Asselman, J., Shaw, J.R., Glaholt, S.P., Colbourne, J.K., De Schamphelaere, K.A., 2013. Transcription patterns of genes encoding four metallothionein homologs in Daphnia pulex exposed to copper and cadmium are time-and homolog-dependent. Aquat. Toxicol. 142, 422–430.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Roy. Stat. Soc. B 289–300.
Berghmans, S., Butler, P., Goldsmith, P., Waldron, G., Gardner, I., Golder, Z., Richards, F.M., Kimber, G., Roach, A., Alderton, W., 2008. Zebrafish based assays for the assessment of cardiac, visual and gut function—potential safety screens for early drug discovery. J. Pharmacol. Toxicol. Meth. 58, 59–68.
Bishopric, N.H., Sato, B., Webster, K.A., 1992. Beta-adrenergic regulation of a myocardial actin gene via a cyclic AMP-independent pathway. J. Biol. Chem. 267, 20932–20936.
Booze, R.M., Crisostomo, E.A., Davis, J.N., 1989. Species differences in the localization and number of CNS beta adrenergic receptors: rat versus Guinea pig. J. Pharmacol. Exp. Therapeut. 249, 911–920.
Brand, T., Sharma, H.S., Schaper, W., 1993. Expression of nuclear proto-oncogenes in isoproterenol-induced cardiac hypertrophy. J. Mol. Cell. Cardiol. 25, 1325–1337.
Brick, I., Hutchison, K., McDevitt, D., Roddie, I., Shanks, R., 1968. Comparison of the effects of ICI 50172 and propranolol on the cardiovascular responses to adrenaline, isoprenaline and exercise. Br. J. Pharmacol. 34, 127–140.
Brodin, T., Fick, J., Jonsson, M., Klaminder, J., 2013. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 339, 814–815.
Brooks, B.W., Huggett, D.B., 2012. Human Pharmaceuticals in the Environment: Current and Future Perspectives. Springer Science & Business Media.
Buonomo, F.C., Zimmermann, N.G., Lauterio, T.J., Scanes, C.G., 1984. Catecholamine involvement in the control of growth hormone secretion in the domestic fowl. Gen. Comp. Endocrinol. 54, 360–371.
Campos, B., Piña, B., Barata, C.C., 2012. Mechanisms of action of selective serotonin reuptake inhibitors in Daphnia magna. Environ. Sci. Technol. 46, 2943–2950.
Christianson, D.W., Lipscomb, W.N., 1989. Carboxypeptidase a. Accounts Chem. Res. 22, 62–69.
Colombo, F., Noël, J., Mayers, P., Mercier, I., Calderone, A., 2001. β-Adrenergic stimulation of rat cardiac fibroblasts promotes protein synthesis via the activation of phosphatidylinositol 3-kinase. J. Mol. Cell. Cardiol. 33, 1091–1106.
Communal, C., Singh, K., Pimentel, D.R., Colucci, W.S., 1998. Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the β-adrenergic pathway. Circulation 98, 1329–1334.
Communal, C., Singh, K., Sawyer, D.B., Colucci, W.S., 1999. Opposing effects of β 1- and β 2-adrenergic receptors on cardiac myocyte apoptosis. Circulation 100, 2210–2212.
Cuthbert, R., Green, R., Ranade, S., Saravanan, S., Pain, D., Prakash, V., Cunningham, A., 2006. Rapid population declines of Egyptian vulture (Neophron percnopterus) and red-headed vulture (Sarcogyps calvus) in India. Anim. Conserv. 9, 349–354.
Day, M., Roach, A., 1974. Central α-and β-adrenoceptors modifying arterial blood pressure and heart rate in conscious cats. Br. J. Pharmacol. 51, 325–333.

Devic, E., Xiang, Y., Gould, D., Kobilka, B., 2001. β-adrenergic receptor subtype-specific signaling in cardiac myocytes from β1 and β2 adrenoceptor knockout mice. Mol. Pharmacol. 60, 577–583.
Dzialowski, E.M., Turner, P.K., Brooks, B.W., 2006. Physiological and reproductive effects of beta adrenergic receptor antagonists in Daphnia magna. Arch. Environ. Contam. Toxicol. 50, 503–510.
Fenyvesi, T., Hadházy, P., 1973. Action of isoprenaline and β-blocking agents on stroke volume regulation. Eur. J. Pharmacol. 22, 105–108.
Finch, A.M., Sarramegna, V., Graham, R.M., 2006. Ligand Binding, Activation, and Agonist Trafficking, the Adrenergic Receptors. Springer, pp. 25–85.
Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.
Gu, Q., Shi, X., Yin, P., Gao, P., Lu, X., Xu, G., 2008. Analysis of catecholamines and their metabolites in adrenal gland by liquid chromatography tandem mass spectrometry. Anal. Chim. Acta 609, 192–200.
Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42, 5807–5813.
Hainsworth, R., Karim, F., Stoker, J., Harry, J., 1973. The blocking effects of propranolol, practolol and ICI-66082 on the peripheral vascular responses to isoprenaline. Br. J. Pharmacol. 48, 342P.
Hedstrom, L., 2002. Serine protease mechanism and specificity. Chem. Rev. 102, 4501–4524.
Herndon, D.N., Dasu, M.R., Wolfe, R.R., Barrow, R.E., 2003. Gene expression profiles and protein balance in skeletal muscle of burned children after β-adrenergic blockade. Am. J. Physiol. Endocrinol. Metab. 285, E783–E789.
Huggett, D., Brooks, B., Peterson, B., Foran, C., Schlenk, D., 2002. Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. Arch. Environ. Contam. Toxicol. 43, 229–235.
Ihaka, R., Gentleman, R., 1996. R: a language for data analysis and graphics. J. Comput. Graph Stat. 5, 299–314.
Iwaki, K., Sukhatme, V.P., Shubeita, H.E., Chien, K.R., 1990. Alpha-and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. fos/jun expression is associated with sarcomere assembly; Egr-1 induction is primarily an alpha 1-mediated response. J. Biol. Chem. 265, 13809–13817.
Jeong, T.-Y., Kim, T.-H., Kim, S.D., 2016. Bioaccumulation and biotransformation of the beta-blocker propranolol in multigenerational exposure to Daphnia magna. Environ. Pollut. 216, 811–818.
Jeong, T.-Y., Yoon, D., Kim, S., Kim, H.Y., Kim, S.D., 2018. Mode of action characterization for adverse effect of propranolol in Daphnia magna based on behavior and physiology monitoring and metabolite profiling. Environ. Pollut. 233, 99–108.
Kashian, D.R., Dodson, S.I., 2004. Effects of vertebrate hormones on development and sex determination in Daphnia magna. Environ. Toxicol. Chem. 23, 1282–1288.
Kawasaki, T., Saito, K., Deguchi, T., Fujimori, K., Tadokoro, M., Yuba, S., Ohgushi, H., Kawarabayasi, Y., 2008. Pharmacological characterization of isoproterenol-treated medaka fish. Pharmacol. Res. 58, 348–355.
Kilham, S.S., Kreeger, D.A., Lynn, S.G., Goulden, C.E., Herrera, L., 1998. COMBO: a defined freshwater culture medium for algae and zooplankton. Hydrobiologia 377, 147–159.
Kouchoukos, N.T., Sheppard, L.C., Mcdonald, D.A., 1970. Estimation of stroke volume in the dog by a pulse contour method. Circ. Res. 26, 611–623.
Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.
Lorenzi, V., Mehinto, A.C., Denslow, N.D., Schlenk, D., 2012. Effects of exposure to the β-blocker propranolol on the reproductive behavior and gene expression of the fathead minnow, Pimephales promelas. Aquat. Toxicol. 116, 8–15.
Lun, A.T., Chen, Y., Smyth, G.K., 2016. It's DE-licious: a recipe for differential expression analyses of RNA-seq experiments using quasi-likelihood methods in edgeR. Stat. Genom. Met. Protoc. 391–416.
Massarsky, A., Trudeau, V.L., Moon, T.W., 2011. β-blockers as endocrine disruptors: the potential effects of human β-blockers on aquatic organisms. J. Exp. Zool. Part A: Ecological Genetics and Physiology 315, 251–265.
Masson, J., Emerit, M.B., Hamon, M., Darmon, M., 2012. Serotonergic signaling: multiple effectors and pleiotropic effects. Wiley Interdiscipl. Rev. Membr. Transport Signal. 1, 685–713.
McQueen, A.P., Zhang, D., Hu, P., Swenson, L., Yang, Y., Zaha, V.G., Hoffman, J.L., Yun, U.J., Chakrabarti, G., Wang, Z., 2005. Contractile dysfunction in hypertrophied hearts with deficient insulin receptor signaling: possible role of reduced capillary density. J. Mol. Cell. Cardiol. 39, 882–892.
Michel, M.C., Insel, P.A., 2006. Adrenergic Receptors in Clinical Medicine, the Adrenergic Receptors. Springer, pp. 129–147.
OECD, 2012. Test No. 211: Daphnia Magna Reproduction Test. OECD Publishing.
Orsini, L., Gilbert, D., Podicheti, R., Jansen, M., Brown, J.B., Solari, O.S., Spanier, K.I., Colbourne, J.K., Rush, D., Decaestecker, E., 2016. Daphnia magna transcriptome by RNA-Seq across 12 environmental stressors. Sci. Data Manag. 3.
Osadchii, O.E., 2007. Cardiac hypertrophy induced by sustained β-adrenoreceptor activation: pathophysiological aspects. Heart Fail. Rev. 12, 66–86.
Ovaere, P., Lippens, S., Vandenabeele, P., Declercq, W., 2009. The emerging roles of serine protease cascades in the epidermis. Trends Biochem. Sci. 34, 453–463.
Parkinson, A., Ogilvie, B.W., 2001. Biotransformation of Xenobiotics. McGraw-Hill, New York.
Perez, D.M., 2006. The Adrenergic Receptors: in the 21st Century. Springer Science & Business Media.
Pérez, M.R., Fernandino, J.I., Carriquiriborde, P., Somoza, G.M., 2012. Feminization and altered gonadal gene expression profile by ethinylestradiol exposure to pejerrey, Odontesthes bonariensis, a South American teleost fish. Environ. Toxicol. Chem. 31, 941–946.
Schindelin, J., Rueden, C.T., Hiner, M.C., Eliceiri, K.W., 2015. The ImageJ ecosystem: an open platform for biomedical image analysis. Mol. Reprod. Dev. 82, 518–529.
Simpson, P.C., Kariya, K.-I., Karns, L.R., Long, C.S., Karliner, J.S., 1991. Adrenergic Hormones and Control of Cardiac Myocyte Growth, Molecular Mechanisms of Cellular Growth. Springer, pp. 35–43.
Stanton, H.C., Brenner, G., Mayfield, E.D., 1969. Studies on isoproterenol-induced cardiomegaly in rats. Am. Heart J. 77, 72–80.
Steinberg, S.F., 1999. The molecular basis for distinct β-adrenergic receptor subtype actions in cardiomyocytes. Circ. Res. 85, 1101–1111.
Ten Hagen, K.G., Balys, M.M., Tabak, L.A., Melvin, J.E., 2002. Analysis of isoproterenol-induced changes in parotid gland gene expression. Physiol. Genom. 8, 107–114.
Tijani, J.O., Fatoba, O.O., Babajide, O.O., Petrik, L.F., 2016. Pharmaceuticals, endocrine disruptors, personal care products, nanomaterials and perfluorinated pollutants: a review. Environ. Chem. Lett. 14, 27–49.
Weber, C.I., 1991. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Environmental Monitoring Systems Laboratory, Office of Research and Development. US Environmental Protection Agency.
Wishart, D.S., Feunang, Y.D., Guo, A.C., Lo, E.J., Marcu, A., Grant, J.R., Sajed, T., Johnson, D., Li, C., Sayeeda, Z., 2017. DrugBank 5.0: a major update to the DrugBank database for 2018. Nucleic Acids Res. 46, D1074–D1082.
Zhu, W.-Z., Wang, S.-Q., Chakir, K., Yang, D., Zhang, T., Brown, J.H., Devic, E., Kobilka, B.K., Cheng, H., Xiao, R.-P., 2003. Linkage of β1-adrenergic stimulation to apoptotic heart cell death through protein kinase A–independent activation of Ca2+/calmodulin kinase II. J. Clin. Invest. 111, 617.