Uranium accumulation and toxicokinetics in the crustacean *Daphnia magna* provide perspective to toxicodynamic responses

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**ABSTRACT**

The importance of incorporating kinetic approaches in order to gain information on underlying physiological processes explaining species sensitivity to environmental stressors has been highlighted in recent years. Uranium is present in the aquatic environment worldwide due to naturally occurring and anthropogenic sources, posing a potential risk to freshwater taxa in contaminated areas. Although literature shows that organisms vary widely with respect to susceptibility to U, information on toxicokinetics that may explain the variation in toxicodynamic responses is scarce. In the present work, *Daphnia magna* were exposed to a range of environmentally relevant U concentrations (0 – 200 µg L\(^{-1}\)) followed by a 48 h depuration phase to obtain information on toxicokinetic parameters and toxic responses. Results showed time-dependent and concentration-dependent uptake of U in daphnia (\(k_u = 1.2 - 3.8\) L g\(^{-1}\) day\(^{-1}\)) with bioconcentration factors (BCFs) ranging from 1,641 – 5,204 (L kg\(^{-1}\)), a high depuration rate constant (\(k_d = 0.75\) day\(^{-1}\)), the majority of U tightly bound to the exoskeleton (~ 50 – 60%) and maternal transfer of U (1 – 7%). Effects on growth, survivorship and major ion homeostasis strongly correlated with exposure (external or internal) and toxicokinetic parameters (uptake rates, \(k_u\), BCF), indicating that uptake and internalization drives U toxicity responses in *D. magna*. Interference from U with ion uptake pathways and homeostasis was highlighted by the alteration in whole-body ion concentrations, their ionic ratios (e.g., Ca:Mg and Na:K) and the increased expression in some ion regulating genes. Together, this work adds to the limited data examining U kinetics in freshwater taxa and, in addition, provides perspective on factors influencing stress, toxicity and adaptive response to environmental contaminants such as uranium.

1. Introduction

Uranium (U) is a naturally occurring radioactive element with three isotopes (\(^{234}\)U, \(^{235}\)U and \(^{238}\)U) and enters aquatic environments due to the release from naturally occurring (U containing minerals) or anthropogenic sources (UNSCEAR, 2016). Due to the long half-life of \(^{238}\)U, the most abounded U isotope, the radioactivity is relatively low, and it is assumed that adverse effects to aquatic organisms in U contaminated environments result chiefly from chemotoxicity (Sheppard et al., 2005; Zeman et al., 2008). As a concern for the environment and human health, regulatory agencies developed U water quality criteria for the protection of aquatic life and drinking water guidelines with concentrations ranging from 15 – 33 µg L\(^{-1}\) (CCME, 2011; USEPA, 2000; WHO, 2017). However, other evaluations of U toxicological thresholds (benchmarks) concluded that water concentrations should not exceed 3.2 – 5 µg L\(^{-1}\) (Mathews et al., 2009; Sheppard et al., 2005). Large exceedance of benchmark safety thresholds in various water-bodies (range: < 1 µg L\(^{-1}\) to > 7 mg L\(^{-1}\)) suggests needs for more accurate assessment of environmental and physiological factors modifying toxicity to evaluate risks and whether stricter regulation of U should be required (Waseem et al., 2015).

It is recognized that physiological processes such as uptake, accumulation and turnover of most trace metals will influence toxic responses in freshwater organisms, but the same breadth of knowledge for U in most species still lacks. Physical and chemical parameters can alter the mobility and bioavailability of U which can drive toxicokinetic...
processes. In oxic surface waters, U is primarily present as U(VI) ions, which is more bioavailable and toxic than anodically derived U(IV) ions. The bioavailable fractions of U (UO$_2^+$ and UO$_2$OH $^-$) depend on the pH conditions (Lofts et al., 2015) whereas, complexation (e.g., natural organic compounds, carbonates and phosphates) or competition with other elements (e.g., major ions) can also play a critical role in U uptake and toxicity (Fortin et al., 2004; Goulet et al., 2015; Sheppard et al., 2005). Given similar exposure conditions, taxa can also vary widely in U susceptibility (Goulet et al., 2015; Poston et al., 1984; Sheppard et al., 2005), indicating that differences in underlying physiological processes related to metal ion transport and detoxification may contribute to some of the variability. However, substantial data gaps exist for understanding the toxicokinetic processes for many freshwater organisms, and in particular planktonic microcrustaceans inhabiting relatively static waters being key to ecological functions (Miner et al., 2012).

Daphnia are widely used as a model organism in the field of ecotoxicology due to simple culture conditions, short reproductive cycles, susceptibility to many toxicants and a natural abundance in aquatic environments worldwide. A growing body of evidence shows daphnia to exhibit water chemistry-dependent responses with negative effects on the overall degree of maternal transfer and (4) investigate early stress responses in $D$. magna. The overall objective was to assess whether U toxicokinetics may provide insight into toxicodynamic response, when appropriate.

2. Materials and Methods

2.1. Animal maintenance

The water flea $D$. magna (DHI strain) was obtained from culture at the Norwegian Institute for Water Research (Oslo, Norway) and continuously maintained (22 ± 1°C, 16 h light: 8 h dark) at the Norwegian University of Life Sciences (NMBU; Ås, Norway) using M7 media and fed green algae ($Raphidiocelis subcapitata$) in accordance with Organization for Economic Cooperation and Development (OECD) test guidelines (OECD, 2012).

2.2. Water characterisation

Water used for experiments was ASTM moderately hard water (MHW) (96 mg L$^{-1}$ NaHCO$_3$, 60 mg L$^{-1}$ CaSO$_4$·2H$_2$O, 60 mg L$^{-1}$ MgSO$_4$ and 4 mg L$^{-1}$ KCl) and adjusted to pH = 6.7 ± 0.1 using 0.1 M HCl. Premade MHW (1 L batches) with environmentally realistic U concentrations of 0, 10, 50, 100 and 200 µg L$^{-1}$ (Nominal U stock: 1 g U $L^{-1}$ U as uranyl nitrate hexahydrate, Sigma Aldrich, St. Louis, USA) were aerated 24 h prior to start of exposure. Water samples ($n = 3 − 5$, 10 mL) from each treatment were collected after filtration (0.45 µm polyethersulfone syringe filters, VWR International) at all timepoints to characterize dissolved U and major cations (Ca, Mg, Na and K). Temperature, light and pH were constantly measured using a HOBO temperature/light logger (Onset Computer Corp., Bourne, USA) and WTW Multi 3420 probe (Xylem Analytics, Weilheim, Germany).

2.3. Determination of U uptake and depuration in $D$. magna

Daphnia were synchronized to < 6 h after release from the brood chamber, transferred to clean MHW, supplied with adequate food levels (500 cells daphnia$^{-1}$ day$^{-1}$) until 4 days old and starved 4 h prior to the start of experiment. The age of daphnia (4-day) was selected to minimize the need for feeding during the uptake phase, while limiting offspring production, as both of which can influence kinetics. For each treatment, daphnia ($n = 20$) were introduced into 5 × 100 mL beakers (100 total daphnia/treatment) with a parafilm cover to reduce evaporative loss, but
**LOQ**

Figure 1. U uptake in *D. magna* (µg g⁻¹ d.w.) over the course of 2 days from exposure to a range of dissolved U (6.7–194 µg L⁻¹). Symbols represent mean (n = 5/timepoint) ± S.E.

Table 2

| Nominal U (µg L⁻¹) | Measured U (Pre-exposure) (µg L⁻¹)* | Measured U (Post-exposure) (µg L⁻¹)* | U loss from solution (%) | Na (mg L⁻¹) | Mg (mg L⁻¹) | K (mg L⁻¹) | Ca (mg L⁻¹) | pH | Temperature (°C) |
|---------------------|-------------------------------------|--------------------------------------|--------------------------|-------------|-------------|-------------|-------------|----|-----------------|
| 0                   | <LOQ**                              | <LOQ**                               |                          | 19.8 ± 0.1  | 4.9 ± 0.1   | 2.9 ± 0.1   | 14.1 ± 0.1  | 6.72 ± 0.2 | 21.3 ± 0.02 (n = 333) |
| 10                  | 6.7 ± 0.02                           | 4.7 ± 0.1                            | 28.7                     | 0.10 ± 0.0  | 0.01 ± 0.0  | 0.1 ± 0.0   | 0.04 ± 0.0   | 0.01 ± 0.0 | 6.71 ± 0.1 |
| 50                  | 45.4 ± 0.2                           | 32.5 ± 0.7                           | 28.5                     | 0.04 ± 0.0  | 0.01 ± 0.0  | 0.03 ± 0.0  | 0.03 ± 0.0   | 0.03 ± 0.0 | 6.72 ± 0.1 |
| 100                 | 94.3 ± 0.2                           | 78.3 ± 0.6                           | 17.3                     | 0.05 ± 0.0  | 0.01 ± 0.0  | 0.04 ± 0.0  | 0.03 ± 0.0   | 0.03 ± 0.0 | 6.72 ± 0.1 |
| 200                 | 193.5 ± 0.6                          | 168.4 ± 2.0                          | 12.9                     | 0.05 ± 0.0  | 0.01 ± 0.0  | 0.05 ± 0.0  | 0.04 ± 0.0   | 0.03 ± 0.0 | 6.72 ± 0.1 |

* n = 3 for pre-exposure, n = 5 for post-exposure
* *LOQ = 0.0015 µg L⁻¹; LOD = 0.0004 µg L⁻¹
N = 33 for major ions and pH

with sufficient air space to allow O₂ exchange. Daphnia were removed (n = 1/beaker, 5/treatment) from exposure at 1, 2, 3, 6, 24 and 48 h, rinsed quickly in clean MHW (194 µg L⁻¹), and placed into a clean beaker containing 450 mL premade MHW with 100 µg L⁻¹ U in the water samples and digested daphnia (NIST-1640a, National Institute of Standards and Technology; Spinach, NCS ZC73013, National Analysis Center for Iron and Steel; Mixed fish, IAEA-414, International Atomic Energy Agency) were included in the digestion and measurements to confirm that the accuracy of concentrations were within 5% of the certified values for all measurements. The limit of quantification (LOQ) for U was 0.0015 µg L⁻¹.

GR = \frac{ln(w_i) - ln(w_f)}{t} \times 100

(1)

where the GR is growth rate, w₁ is average final weight (g), wᵢ is average initial weight (g) and t is time in days.

2.4. Determination of external U binding to daphnia and maternal transfer

A parallel experiment examined tightly bound U associated with the external daphnid surface (molted exoskeleton). Synchronized daphnia (< 2 h, n = 50) from hatching were raised until 7 days old, transferred to clean MHW without food for 4 h and placed in a single polyethylene beaker containing 450 mL premade MHW with 100 µg L⁻¹ U (pH = 6.7 ± 0.1). After 4 h, daphnia were removed from exposure and rinsed twice with clean MHW. A subsample of daphnia (n = 6) were taken directly after exposure to quantify total U in daphnia without any molting. The remaining daphnia were placed into individual well plates (polyethylene 6-well, VWR) containing clean MHW (~ 10 mL) to observe molting every 10 mins over the course of 4 h. Upon a molting event, the daphnid, molt and any offspring (n = 1 – 10 neonates/adult daphnid) were quickly removed from solution and similarly processed for U determination as described in section 2.3. Control daphnia (n = 5) were obtained in case of background U subtraction, but U concentrations were negligible (0.033 ± 0.01 µg g⁻¹ d.w.). U concentration in molt and molted daphnia (µg g⁻¹ d.w.) was normalized to total mass (molt weight + daphnid weight). The proportion of U associated with the molt was calculated by dividing total U in molt (µg) by the combination of total U in molt and daphnia (µg); the remaining fraction was considered to be internalized. Maternal transfer factors (from adult to offspring) were calculated by dividing U concentration in offspring (µg g⁻¹ d.w.) by internalized U concentration in adult (µg g⁻¹ d.w.).

2.5. Determination of U by inductively coupled plasma mass spectrometry (ICP-MS)

Acidified (5% ultrapure HNO₃) water samples and digested daphnia samples were measured using a triple quadrupole ICP-MS (Agilent 8900, Hachii, Japan) to quantify U and major ion (Ca, Mg; Na and K). Certified reference materials (Trace Elements in Natural Water, NIST-1640a, National Institute of Standards and Technology; Spinach, NCS ZC73013, National Analysis Center for Iron and Steel; Mixed fish, IAEA-414, International Atomic Energy Agency) were included in the digestion and measurements to confirm that the accuracy of concentrations were within 5% of the certified values for all measurements. The limit of quantification (LOQ) for U was 0.0015 µg L⁻¹.
2.6. U kinetic modelling

Depuration rates (k_d’s) were determined as the slope of the natural log of the proportion of metal retained in the daphnia across time of depuration (in days). The first few time points (1 and 2 h) were removed from calculations as rapid desorption can cause misinterpretation (R. Q. Yu and Wang, 2002). Assuming steady-state conditions were reached, depuration rate constants were calculated using equation 2:

\[ k_d = \frac{\ln\left(\frac{C_{\text{ss}}}{C_t}\right)}{t} \]  

where \( C_{\text{ss}} \) = organism concentration at steady-state, \( C_t \) = organism concentration at time \( t \), and \( k_d \) = depuration rate constant (day^{-1}). Kinetic BCF’s were compared to steady-state BCF’s, as calculated by dividing the steady-state U concentration in daphnia (\( C_{\text{ss}} \)) by the U concentration in water (\( C_w \)). Uptake rates (\( k_u \) g^{-1} day^{-1}) and depuration rate (day^{-1}) were determined by multiplying the uptake rate constant (\( k_u \)) with the dissolved U concentration in the water.

2.7. Early stress responses

2.7.1. Reactive oxygen species

Mitochondrial reactive oxygen species (ROS) were measured using a fluorescent probe, dihydrorhodamine 123 (DHR123, Thermo Fisher Scientific, Waltham, USA), as previously described (Gomes et al., 2018). In brief, one \( D. magna \) per replicate (\( n = 5 \)) was incubated with 5 \( \mu \)M probe (prepared in 200 \( \mu \)L M7 medium) in a black 96-well microplate (Corning Costar, Cambridge, MA, USA) for 1 h (room temperature, dark). After incubation, the daphnids were gently washed with clean medium to remove unbound probe. The plate was immediately read by a VICTOR 3 microplate reader (PerkinElmer, Waltham, USA) at excitation/emission wavelengths of 485/538 nm. Images for length measurements were taken using a digital camera (FinePix S2500HD, Fujifilm, Tokyo, Japan). The weight of \( D. magna \) was calculated using a length-weight regression model (Cauchie et al., 2000). The ROS data was further normalized to the estimated weight of the corresponding daphnid.

2.7.2. Mitochondrial membrane potential

Tetramethylrhodamine methyl ester perchlorate (TMRM, Thermo Fisher Scientific) was used as a fluorescent probe to determine changes in mitochondrial membrane potential (MMP) in \( D. magna \), as previously described (Song et al., 2020). In brief, one \( D. magna \) per replicate (\( n = 5 \)) was incubated with 2 \( \mu \)M probe (prepared in 200 \( \mu \)L M7 medium) in a black 96-well microplate (Corning Costar) for 1 h (room temperature, dark). After incubation, the daphnids were gently washed with clean medium to remove unbound probe. The plate was immediately read by a VICTOR 3 microplate reader (PerkinElmer, Waltham, USA) at excitation/emission wavelengths of 530/590 nm. Images for length measurements were taken using a digital camera (FinePix S2500HD). The weight of \( D. magna \) was calculated using a length-weight regression model (Cauchie et al., 2000). The MMP data was further normalized to the estimated weight of the corresponding daphnid.

2.7.3. Gene expression

Total RNA was isolated from \( D. magna \) after 48 h exposure to U (3 pooled daphnids/replicate, \( n = 5 \)) using the ZR Tissue & Insect RNA MicroPrep™ kit (Zymo Research, Irvine, USA), as previously described (Song et al., 2016). The quality of the purified RNA was assessed using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) and a Bioanalyzer (Agilent Technologies, Santa Clara, USA). RNA samples with yield > 200 ng, 280/260 > 1.8 and high integrity (clear RNA bands with no overlapping or background signal on gel) were stored at −80°C until gene expression analysis.

A high-throughput quantitative real-time reverse transcription
polymerase chain reaction (HT-qPCR) assay was used to determine gene expression changes in *D. magna* after U exposure. Primers for a selection of biomarker genes (Table A1) were designed in Primer3 v4.0.0 (http://primer3.ut.ee/) and synthesized by Thermo Fisher Scientific (Wal-tham, USA). The qPCR assay was conducted using a Bio-Rad CFX384 platform (Bio-Rad Laboratories, Hercules, CA), according to a previously established protocol (Song et al., 2016). In brief, RNA (200 ng) was reversely transcribed into cDNA using qScript™ cDNA SuperMix (Quanta BioSciences, Gaithersburg, USA). The cDNA template (1 ng/2.5 μL) was amplified in a 10 μL reaction containing 5 μL PerfeCTa® SYBR® Green FastMix® (Quanta BioSciences) and 2.5 μL of primers (400 nM). A dilution series of pooled templates from all samples (0.5, 1, 2, 4, 8 ng) was included to generate a standard curve for calculating amplification efficiency (90 – 105%) and correlation coefficient ($R^2 > 0.98$). Relative gene expression was calculated according to the Pfaffl Method (Pfaffl, 2001) and normalized to the expression of a reference gene, ubiquitin-conjugating enzyme E2 (*Ube2*) which displayed stable transcription across treatment groups (data not shown) compared to two other reference genes tested. A list of genes as it relates to functional category is described in Table 1.

### 2.8. Statistical Analyses

Statistical analyses were performed using SigmaPlot version 14.0 (Systat Software, San Jose, Ca). Comparisons between treatments were analyzed using one-way ANOVA (Holm-Sidak method for multiple comparisons). Linear correlations were performed to determine the relationship between dissolved U and kinetic parameters and, in addition, when examining maternal transfer. Pearson correlation matrix was conducted for all endpoints and principle component analysis (PCA) was performed to examine relationships between toxicokinetics and observed responses. Data are mean with their respective standard error (S.E.) unless otherwise stated.
Figure 4. Maternal transfer of U to offspring across range of internalized U in mother (µg g⁻¹ d.w.). Mothers were exposed for 4 h to a single U concentration (100 µg U L⁻¹). Maternal transfer factors were calculated by dividing the U concentration in offspring by U concentration in mother (post-molt). Each point represents an individual offspring paired with its mother. The regression line indicates the linear reduction in maternal transfer across the range of internalized U in mother (r = 0.65, p ≤ 0.01).

3. Results

3.1. Exposure water characterization

In the main exposure experiment, the dissolved (≤ 0.45 µm) U concentrations (Table 2) were close to the nominal concentrations (< 0.0015, 6.7, 45, 94 and 194 µg L⁻¹) and thus used to express the results herein. After 48 h exposures, the dissolved U loss from the exposure solution ranged from 13 − 29%, with higher loss occurring at lower ambient U concentrations (i.e., 6.7 µg L⁻¹). The U concentrations in the control exposures were below the LOQ (0.0015 µg L⁻¹) for both the uptake and depuration phases in the studies. Major ion concentrations (Ca, Mg, Na and K) and pH in the water did not differ between treatments (Table 2). In the experiment examining U bound to exoskeleton/molt, the initial dissolved U concentration in solution averaged 104 ± 0.1 µg L⁻¹ and were reduced to 91 ± 0.1 µg L⁻¹ after 4 h exposure.

3.2. Dissolved U uptake and depuration in daphnia

The uptake of U in D. magna followed first-order kinetic expression (Fig. 1) with pseudo-steady-state conditions reached at ~ 24 h across all U concentrations (global R² = 0.79, p < 0.0001). Uptake rates increased with U concentration (r = 0.85) and were in the range of 27 − 240 µg g⁻¹ day⁻¹ across all dissolved U concentrations (data not illustrated). Uptake rate constants (k_u) were not significantly different at the two lowest U concentrations tested (k_u = 3.8 ± 0.5 L g⁻¹ day⁻¹ at 6.7 µg U L⁻¹ and k_u = 3.1 ± 0.2 L g⁻¹ day⁻¹ at 45 µg U L⁻¹), but a significant reduction (p < 0.001) in k_u was observed at higher U concentrations (k_u = 2.5 ± 0.3 L g⁻¹ day⁻¹ at 94 µg U L⁻¹ and k_u = 1.2 ± 0.2 L g⁻¹ day⁻¹ at 194 µg U L⁻¹) (Fig. 2A). The reduction in k_u with increasing U concentrations followed a linear fit (slope: −0.01, r = −0.99, p ≤ 0.001) and due to the concentration dependency, k_u could not be considered constant. No significant difference in depuration rate constants (k_d) was observed across the U concentrations tested (Fig. 2B) with a global average across all treatment levels equaling 0.74 ± 0.02 day⁻¹. The method for describing bio-concentration utilized kinetic parameters (k_u/k_d) as opposed to steady-state values (C_m/C_a), but comparison of the methods (data not shown) indicated only marginal differences (≈ 5% across U concentrations). Therefore, either method was appropriate to use in this case. The bio-concentration factors (BCF’s) decreased (one-way ANOVA, p < 0.01) with U concentration (Fig. 2C) and ranged 1,641 − 5,204 (L kg⁻¹) across all concentrations. The linear decrease in BCF as a function of U concentration is described by a slope of −18.05 and r = −0.98 (p < 0.001) and correlated well with the linear decrease in uptake kinetics (r = 0.99).

3.3. U bound to daphnia exoskeleton and maternal transfer

After 4 h exposures, whole-body daphnia U concentration averaged 46 ± 5.5 (µg g⁻¹ d.w.). More than half of the daphnia (29 out of 50 total) molted during the following 4 h observational period. U concentrations in daphnid exoskeletons averaged 16 ± 0.8 (µg g⁻¹ d.w.), whereas U in post/molted daphnia averaged 12 ± 0.5 (µg g⁻¹ d.w.) (Fig. 3A). A significant linear reduction in U concentration in exoskeleton occurred over the course of the observational time period (r = −0.66, p < 0.001), whereas no reduction in U concentration in molted daphnia was observed. The proportion of total U was almost always higher in the exoskeleton (58 ± 1.7%) compared to the paired daphniid (Fig. 3B).

During the molting process, a proportion of adult daphnia (n = 12/29) released offspring into the clean water (n = 1-10) with an average U concentration of 0.40 ± 0.02 µg g⁻¹ d.w. There was no significant relationship between offspring U and adult daphnia U. Corresponding maternal transfer factors ranged from 0.007 − 0.069 (Fig. 4). A significant negative linear relationship (slope = −0.0028, r = −0.65, p ≤ 0.01) was observed between the maternal transfer factor and the U accumulated in adult daphnia.

3.6. Early stress responses

No significant induction of mitROS or alteration in mitochondrial membrane potential (TMRRM) could be identified (see appendix Fig. A1). A number of biomarker genes (Table 1) displayed either target- or concentration-dependent responses to U after 48 h (Fig. 5). Six out of 20 genes were significantly expressed at one or more U concentrations and all displayed non-monotonic responses. The metal exposure (detoxification) marker metallothionein (Mt) displayed up-regulation (2.2 − 2.7-fold increase) in all except one exposure concentration (94 µg U L⁻¹). All other significant responses exhibited up-regulation at intermediary U (45 µg U L⁻¹), followed by a reduced expression at 94 to 194 µg U L⁻¹ and were grouped below in terms of their biological function. Plasma ion regulators Cacnb1 and Atp1a1 were up-regulated (2 − 2.3-fold increase), whereas other ion transporters (GaM, Atp2b1, Magt, Slc41) showed no response. One gene associated with antioxidant defense (Nrf2) was also up-regulated (2.6-fold increase), whereas Gst, Sod and Cat did not significantly differ between treatments. One gene involved in the apoptotic signaling pathway (Dapk) was up-regulated (1.8-fold increase), whereas Aifm1 and Casp2 did not change significantly compared to control. The daphnia juvenile hormone receptor methoprene-tolerant (Met) was up-regulated (2.1-fold increase), whereas genes involved in the ecdysone signaling such as Shd and Ecrb did not significantly differ between groups. No significant change in oxidative phosphorylation (Atp5a1) or DNA damage (Rev1 and Rad50) related gene expression was identified.

3.5. Whole-body major ion concentrations (Ca, Mg, Na, K)

Initial whole-body Ca, Mg, Na and K averaged 55.1 ± 1.8, 2.7 ± 0.1, 11.0 ± 0.2 and 7.6 ± 0.1 mg g⁻¹ d.w. (n = 24 − 25), respectively (Fig. 6). A general increase (63 − 74%), p < 0.001 in Ca, Mg and Na concentrations as well for Ca:Mg and Na:K ratios occurred in all treatments over the course of the first 48 h (while starved). After switching to clean water with food, concentrations of the same ions significantly decreased (p < 0.001) until the end of experiment (96 h).

For the divalent elements Ca and Mg (Fig. 6A, C and E), no significant alteration in whole-body concentrations or in the Ca:Mg ratio (19.6 ± 0.3) occurred as a result of U exposure during the first 48 h, but only during the post exposure period. A significant reduction (p < 0.001) in whole-body concentration of these ions was first observed at 54 h after placed
into clean media) in daphnia exposed to the highest U treatment (194 µg L⁻¹). However, in daphnia exposed to 94 µg L⁻¹ U, a significant increase (p ≤ 0.05) in whole-body Mg was observed at 96 h. The Ca:Mg ratio in daphnia exposed to the highest U concentration (194 µg L⁻¹) was significantly reduced during the whole depuration phase, ranging 26% to 37% at 54 h (and 96 h, respectively (one-way ANOVA, p ≤ 0.05).

For the monovalent elements Na and K (Fig. 6 B, D and F), a significant reduction (p ≤ 0.05) in whole body concentrations was observed at the end of exposure (48 h after start) in the group exposed to highest U concentration (194 µg L⁻¹), while no difference in Na:K ratio (1.45 ± 0.02) was observed during the uptake phase. During depuration, the Na:K ratio significantly increased (p ≤ 0.05) compared to control post-exposure at 72 h (20 - 29 %) and 96 h (43 - 62 %) for both the two highest U exposures (94 and 194 µg L⁻¹). The only other significant difference occurred at 96 h, where whole-body Na increased in daphnia exposed to 94 µg L⁻¹ (p ≤ 0.05).

### 3.6. Molting, growth and survival

No significant reduction in molts, growth or survivorship occurred across U exposure (data not shown) during the uptake phase (first 48 h). The vast majority (97%) of molting occurred between 24 and 48 h time-points and no significant difference in total molts between exposures occurred (Fig. 7A). However, 48 h post exposure (96 h after start of exposure) significant reduction in growth (day⁻¹) was observed in daphnia exposed to 45, 94 and 194 µg L⁻¹ U (One-way ANOVA; *p ≤ 0.05, **p ≤ 0.001) (Fig. 7B). Significant (p ≤ 0.001) cumulative mortality after 96 h was also observed in the highest U treatment (194 µg L⁻¹) (Fig. 7C).

### 3.7. Correlations

Pearson’s correlations and principle component analysis (PCA) identified positive relationships between dissolved U (µg L⁻¹), uptake rate (µg g⁻¹ day⁻¹) and steady-state U in daphnia (µg g⁻¹ d.w.) (Table A2 and Fig. 8). Likewise, toxicokinetic parameters of uptake (k_u), BCF and to a lesser extent depuration rate (k_e) shared positive
correlations with each other. Relationships between exposure and sublethal effects (gene expression, mitochondrial response) and with toxicokinetic parameters were limited (Fig. 8). However, survivorship, total molts, growth rates and whole-body major ion concentration (Ca, Mg, K, Na) were all negatively correlated with both dissolved U, uptake rate and daphnia steady-state U (Fig. 8).

4. Discussion

4.1. Exposure, toxicokinetics and maternal transfer

4.1.1. Water exposure

In the present study, initial dissolved U concentrations were close to nominal and measurements of U post-exposure showed a ~13 – 29% loss of U from the exposure solution (Table 2). Based on mass-balance calculations, uptake into daphnia over 48 h accounted for 1 – 4% of U removed from solution. This was similarly observed in the parallel experiment examining exoskeleton U association/maternal transfer. U precipitation is not expected given the water characteristics (i.e., pH, ion concentrations, etc.) and relatively low U concentrations used in this experiment (Lofts et al., 2015). Therefore, the observed U loss from water may result from adsorption to external sources (e.g. beaker wall, daphnid exudates or molts), an occurrence highlighted in similar studies (Alves et al., 2009). Since a majority of U is strongly associated with molts (Fig. 3) and that nearly all of molting occurred between 24 – 48 h, it is possible that a large fraction of U lost from solution was a result of U being attached to molted exoskeletons.

4.1.2. U uptake into D. magna

Kinetic studies are most useful for understanding processes that occur when metals, including U, are released into freshwaters, and subsequently taken up by taxa with different tolerance for pollutants. They also are highly relevant for predicting models and can provide direct linkage between exposure and toxic effects. Present results showed rapid and concentration-dependent uptake of U in D. magna, whereby reaching pseudo-equilibrium with depuration (i.e., steady-state) after ~ 24 h across all concentrations tested (Fig. 1). For a given biological species, the uptake rate constant (k_u) is typically
considered concentration independent (Luoma and Rainbow, 2005). Deviations usually only occur when physical – chemical parameters such as major ion concentrations (e.g., Na or Ca), U speciation or factors influencing speciation (e.g., pH or dissolved organic C) differ between exposures (Lam and Wang, 2006; Yu and Wang, 2002). The k_u reports in the current study did not exhibit concentration independency, but were inversely related to U concentrations (Fig. 2A) even though there were no differences in water parameters. An evaluation of uranyl species (UO_2^{2+}) in solution using the speciation model Wham7 (Natural Environment Research Council; version 7.0.5) revealed no differences in the relative fraction across the U concentration range (data not shown) as observed in the present work. Uptake of metals can, however, reach a saturation level at excessive concentrations (e.g., Michaelis-Menten type kinetics), leading to k_u concentration-dependency. In another freshwater crustacean (Hyalella azteca), U uptake exhibited saturation-type kinetics when exposed to U concentrations similar to the present study (Alves et al., 2009), suggesting that saturation of binding sites can occur at more moderate U concentrations.

In D. magna, reported k_u values for other trace metals vary widely: Se = 0.2, Zn = 1.1, Cd = 1.5, Ag = 6.2, Hg = 8.4 and MeHg = 11.0 L g^{-1} day^{-1} (Lam and Wang, 2006; Tsui and Wang, 2004; Yu and Wang, 2002). Lower k_u values indicate less uptake potential from the aqueous phase and vice versa, and the data obtained here (k_u range = 1.2-3.8 L g^{-1} day^{-1}) suggests a relatively high U uptake potential compared to other metals. Studies investigating U kinetics in other freshwater taxa are scarce, but one study has reported k_u in the aquatic insect Chironomus tentans (0.02 L g^{-1} day^{-1}) (Muscatello and Liber, 2010). This implies a much lower U uptake capacity in chironomids (a group of insects considered to be tolerant to many pollutants, including metals) compared to D. magna; however, study conditions (e.g., pH and water chemistry) and thus U speciation were not identical.

4.1.3. U depuration in D. magna

Metal loss in invertebrates comes from different depuration routes (i.e., excretion, reproduction, egestion, molting) and can be an important defense mechanism against toxicity. In this study, depuration rates (k_u) did not differ across concentrations and equated to ~75% U lost in daphnia on a daily basis, which is comparatively higher than for other trace metals such as Ag, Cd, Cr, Se, Zn, Hg and MeHg (Lam and Wang, 2006; Tsui and Wang, 2004; Yu and Wang, 2002). Daphnia appear to have the highest depuration rate for U among invertebrates tested (Alves et al., 2009; Muscatello and Liber, 2010); however, the lack of thorough testing even within a taxon limits the ability to generalize to a larger assembly of biological species. Since D. magna have high depuration rates and a majority of U is lost per molt (Fig. 3), daphnia could be considered “poor accumulators” of U compared to other trace metals even though uptake rates are moderately high.

4.1.4. Factors contributing to U accumulation and bioconcentration

Bioconcentration factors obtained here (1,641 – 5,204 L kg^{-1}) corresponded inversely with exposure concentrations, an observation seen in other taxa exposed to U or other trace metals (Barillet et al., 2011; McGeer et al., 2003). Concentration-dependent uptake in daphnia appears to be the main factor influencing U bioconcentration since depuration rates did not change across concentration. Daphnia have relatively high BCF’s compared to other freshwater biota such as the amphipod Hyalella azteca (240 – 740 L kg^{-1}), zebrafish Danio rerio (92 – 1,030 L kg^{-1}), iridescent shark Pangasius sutchi (0.15 – 1.32 L kg^{-1}) and water lily Nymphaea tetragona (14 – 73 L kg^{-1}) (Alves et al., 2009; Annamalai and Arunachalam, 2017; Barillet et al., 2011; Li et al., 2019). The exception to this case exists for lower trophic level freshwater algae, where estimated BCFs range ~ 2,000 – 5,000 L kg^{-1} at similar pH (Horikoshi et al., 1979; Zhang et al., 1997).

In the present study, sublethal responses support that U was accumulated and internalized. This is indicated by the increased expression of some ion transport genes often associated with metal transport (e.g., Cacnb1 and Ap tightened; e.g., Cacnb1 and Ap1a1), an imbalance in whole-body ion concentrations and an increased expression of the biomarker gene metallothionein (Mt). In addition, since only a fraction of U was associated with the exoskeleton, molting events did not release all U from daphnia (Fig. 3) and a certain fraction of U was still retained in the organisms. The high relative fraction of U associated with the exoskeleton (50 – 60 %) is similarly observed in daphnia after exposure to other divalent elements such as Cd and Zn (Yu and Wang, 2002) and pairs with daphnid Ca distribution (Alstad et al., 1999). It stands to reason that a high fraction of U would be associated with the external surface since uranyl (UO_2^{2+}; the bioavailable U species) behaves similarly to other divalent elements with respect to sorption processes. Fractions associated with the exoskeleton should therefore be considered when examining...
toxicokinetics in aquatic invertebrates, especially since there is often a need to relate body burden to toxic effects. Thus, the current results highlight the importance of considering surface sorption in quantifying body burden, uptake and uptake kinetics for U and other trace metals.

The maternal transfer of essential elements is a fundamental mechanism for early life-stage development; however, imbalance (either excess or depleted supply) can have deleterious effects on survivorship and fecundity. As U is not an essential element, there is no biological imperative to transfer U to offspring, but the short-term transfer (1–7%) observed in this study (Fig. 4) most likely stemmed from U mimicking essential analogue elements (e.g., Ca). The transfer of U from mother to offspring is in agreement with other studies which show U transfer to daphnia eggs (Plaire et al., 2013) and the increased impact on successful generations (Massarin et al., 2011, 2010). Taken together, the maternal transfer of U to offspring could potentially result in adverse effects on sustainable populations in natural ecosystems or lead to adaptive responses, both of which require further investigation.

4.2. Linkages between U toxicokinetics and early stress responses in D. magna

4.2.1. Detoxification and other relevant toxicity pathways

Metallothionein (Mt) aids in metal detoxification, often indicates exposure (i.e., accumulation and internalization) and can protect against U toxicity (e.g., C-T Jiang et al., 2009). The present work showed elevated expression of Mt across U exposure (except 94 µg L\(^{-1}\)) but not in a linearly manner with exposure (Fig. 5). This suggested that induction of Mt helps protect against toxicity at lower U concentrations, but at higher concentrations that mechanism is saturated and not in proper function. The inability to detoxify and remove metals can effectively lead to cellular disruption and downstream adverse effects. For many metals, the production of ROS can form highly reactive radicals that may cause oxidative stress and biomolecule damage. Since U can initiate antioxidant responses in other biological species (Song et al., 2014, 2012), we hypothesized that increased U exposure would lead to increased production of mitochondria and increased expression of antioxidant regulating genes in daphnia. In the present work, however, ROS production and antioxidant gene expression (except nuclear erythroid 2-related factor 2 (Nrf2)) did not show any increase with U concentration (dissolved or whole-body U) during 48 h exposure. This could partly be attributed to the short exposure as strong evidence exists that time and concentration play a critical role in antioxidant defense from U (Barillet et al., 2011; Li et al., 2019) and, in addition, the increased expression of Nrf2 (an upstream regulator) would also be expected prior to other antioxidant genes (Gst, Cat and Sod).

As a result of U exposure, inability to cope with oxidative stress could directly or indirectly result in alterations in energy production (oxidative phosphorylation), mitochondrial disfunction, DNA damage, protein degradation and apoptosis leading to adverse effects on growth and reproduction (Barillet et al., 2011; Plaire et al., 2013; Song et al., 2012). In the current study, the general lack in gene expression associated with the aforementioned processes does not necessarily discount oxidative stress as a mode of action for U in daphnia since some genes were up-regulated in a bell-shaped manner. This type of response likely results from kinetic and detoxification processes limiting U accumulation.

4.2.2. Disruption in ion homeostasis and expression of transporter genes

Maintenance of major ions (Ca, Mg, Na and K) in freshwater invertebrates, including daphnia, is an important physiological process as they are hypertonic to the surrounding and require constant ionic or osmotic regulation to maintain homeostasis. Disruption of ion- and osmoregulation can have direct implications for cellular function (e.g., exoskeleton calcification, cell signaling, metabolism, hormone secretion) and may lead to organismal effects. In D. magna, exposure to trace metals (e.g., Ag, Ni, and Zn) can result in the disruption of major ion uptake (Na, Ca and Mg) and alter whole body ion concentrations (Bianchini and Wood, 2002; Muyssen et al., 2006; Pane et al., 2003). However, ion imbalance also results from periods of starvation, which can contribute to increased filtration rates, altered ion uptake from dietary sources and increased intermolt periods (Muyssen et al., 2006; Pane et al., 2003; Porcella et al., 1969). In this study, daphnia did not feed during uptake to maintain dissolved exposure conditions. Starvation increased the whole-body ion concentrations and reduced molt frequency since one true molting event occurred over 96 h and juvenile daphnia typically molt every 24–48 h (Porcella et al., 1969). The lack of feed, in addition to reduced molting, most likely caused the increased trend in whole-body ion concentrations over the first 48 h and is further highlighted by a return to pre-exposure whole-body concentrations once returned to food in the depuration phase. Nevertheless, it is clear that U uptake resulted in an overall ionic disruption (even with starvation effects observed), which could provoke deleterious effects on other cellular systems and the organism itself.

The general disruption of ion homeostasis in the current study was strongly associated with the concentrations of U in water and whole-body U concentrations, along with kinetic parameters \( k_u \) and BCF (Fig. 8). At the highest U exposure (194 µg L\(^{-1}\)) daphnia were no longer able to adequately regulate whole-body Ca or Mg (Fig. 6A and C) and this is further indicated by evaluating the whole-body Ca:Mg ratio (Fig. 6E). Calcium transport mechanisms believed to be a primary means by which dissolved U accumulates in aquatic biota; however, it is conceivable that U could interact with other divalent transporters (i.e., for Mg). The non-monotonic up-regulation of the Ca transport protein Cacnb1 (Fig. 6) indicated an interaction with U and implied elevated demand for restoring Ca homeostasis. It cannot be ruled out that U interacted with Mg transport since whole-body Mg was affected, but due to a lack of Mg transport gene expression as a function of U exposure, Ca transport pathways were more likely affected. Given that whole-body Ca and Cacnb1 ratio disruption occurred, the bell-shaped response in Ca-related gene expression (2.3-fold-change increase from 0 – 45 µg U L\(^{-1}\), and similar fold-change decrease from 45 – 194 µg U L\(^{-1}\)) indicated that the daphnia were no longer able to cope with higher U.

The inability to regulate whole-body Na, K and NaK ratio (Fig. 6) coupled with the increased expression of Na/K transport pump Atp1a1 (Fig. 5) suggested osmoregulatory disruption, yet causal explanations...
are currently scarce. Reduced blood Na and Cl levels in fish as a response to U exposure supports the idea that U exposure can cause an osmotic stress response (Teien et al., 2015). In addition, Na/K ion pumps can facilitate transport of molecules (including Ca) and cell signaling (Clausen et al., 2017). Whether these processes are inherently linked is currently unknown and require future investigation. Nevertheless, the similar bell shaped response in Na/K transport gene expression indicated that above 45 µg L\(^{-1}\), the daphnia are not able to efficiently regulate osmolarity across plasma membranes, leading to the disruption in the Na:K ratio at 94 – 194 µg L\(^{-1}\).

### 4.2.3. Adverse effects

The present study showed deleterious effects on daphnid growth and survivorship (Fig. 7) resulting from U concentrations between 45 – 194 µg L\(^{-1}\) or maximum U body-burdens ranging 175 – 320 µg g\(^{-1}\) (d.w.). It should be noted that these effects only occurred after uptake and during depuration and were likely caused by irreversible damage from the short time exposure. Based on external U concentrations, these results are in agreement with studies of similar length and exposure conditions (Zeman et al., 2008), but comparisons to other toxicity assays are difficult since exposure conditions differed and body-burdens were not obtained.

The work here shows relatively strong correlations (Table A2 and Fig. 8) between adversity (growth, survivorship and total molts), toxicokinetics and ion homeostasis; all of which support the notion that U uptake and accumulation/internalization drives toxicity. U accumulation is linked to toxicity in green algae (Lavoie et al., 2014) and amphipods (Alves et al., 2009), but authors highlighted that internal distribution is an important factor to consider due to the high sorption potential for U. Extensive literature now exists underscoring the importance of subcellular fractionation and determining internal distribution as it directly correlates with toxicity to certain metals (e.g., Wallace et al., 2003; Wallace and Luoma, 2003). Although we did not quantify the sub-cellular fractions of U in this study, we did show high accumulation in the exoskeleton, which is most likely not toxicologically relevant. Results also demonstrate a significant fraction of U not associated with the molted exoskeleton, which is most likely internalized. Significant maternal transfer of U also strongly supports internalization of U in daphnia. It is conceivable that evaluation of internalized soluble U fractions might be the best indicator of observed effects, and since U does act on the gut epithelium, identifying U bound directly with the gut could also provide further insight to toxicity.

### 5. Conclusions

The overall aim of this work was to investigate U toxicokinetics in *D. magna* under short term exposure and provide perspective to observed effects, where applicable. Daphnia have relatively high U uptake rate constants and depuration rates when comparing inter-specifically or intra-specifically to other trace metals. Yet, the major fraction of U was associated with the exoskeleton (> 50%), a finding in agreement with other divalent metals for the same biological species. Daphnia maternally transfer a significant proportion of U to offspring (1 – 7%), which may have implications for successful offspring development. Significant alteration in whole-body ion concentrations and their ionic ratios (e.g., Ca:Mg and Na:K) paired with increased expression in some ion regulating genes indicated that U interfered with ion uptake pathways and homeostasis. Furthermore, adverse effects (i.e., growth and survivorship) were associated both with external and internal exposure, perturbations in ion homeostasis and toxicokinetic parameters of uptake, and highlights the importance of incorporating toxicokinetic approaches to aid in explaining toxic response and species sensitivity.

**CRediT authorship contribution statement**

**Shane Scheibener:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft. **You Song:** Investigation, Methodology, Writing - review & editing. **Knut Erik Tollefsen:** Supervision, Conceptualization, Funding acquisition, Writing - review & editing. **Brit Salbu:** Supervision, Conceptualization, Funding acquisition, Writing - review & editing. **Hans-Christian Teien:** Supervision, Conceptualization, Funding acquisition, Writing - review & editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Figure A1.** Fold change in mitochondrial reactive oxygen species (mitROS) formation (A) and mitochondrial membrane potential (TMRM assay) (B) in *D. magna* after 48 h U exposures.
Table A1
Biomarker genes and primer sequence for transcriptional analysis.

| Functional category     | Gene name                                      | Gene symbol | Forward primer (5′-3′)          | Reverse primer (5′-3′)       | Amplicon size (bp) | Annealing temperature (°C) | Efficiency (%) | WFleaBase ID/GenBank Accession |
|-------------------------|------------------------------------------------|-------------|---------------------------------|------------------------------|--------------------|---------------------------|----------------|--------------------------------|
| Reference               | Ubiquitin-conjugating enzyme E2-17 kDa        | Ube2        | CTCTGCTATCGGACCCAAAC            | CATTGCCGTTCCAGTTATT          | 100                | 64.2                      | 97.5           | Dapma7bEvm015277t1             |
| Reference               | 18S ribosomal RNA/Uncharacterized protein     | 18S         | GATTCTGGTTCTAGGGGAG            | GGCGGACATGACGAATAA           | 180                | 62.5                      | 90             | Dapma7bEvm020523t1             |
| Reference               | Actin, alpha skeletal muscle                  | Actin       | TGGGTATTGAGATCTGCGG            | AAGGGGTCGTTCCCTTTGG          | 160                | 53.8                      | 91.9           | Dapma7bEvm018420t1             |
| Exposure marker         | Metallothionein protein crs5                  | Mt          | TACCACTTTGCTCCCTGCTG          | TTACCTGTACAGTTCCACCA          | 115                | 62.5                      | 102.4          | Dapma7bEvm0282491              |
| Calcium homeostasis     | Calmodulin                                     | CaM         | CAGACAACCTCGAGCAAGAAAT         | CAGGGATATTGGCCACATC          | 111                | 64.2                      | 92.1           | Dapma7bEvm067465t1             |
| Calcium homeostasis     | Voltage-dependent L-type calcium channel subunit beta-1 protein | Cacnb1 | CAAAAGCTGTTTGAATTGATT          | TGTGTAAGGCGTGGCGAAGG         | 138                | 60                        | 100.3          | Dapma7bEvm009498t1             |
| Calcium homeostasis     | Plasma membrane calcium-transporting ATPase   | Ap2b1       | CTTTTGATCCGTGTGCTTCA          | CAATGTTTACCATGGACAGG        | 184                | 60                        | 100            | Dapma7bEvm027353t1             |
| Magnesium homeostasis   | Magnesium transporter protein                  | Magt        | CCGTACAGGTTGACGACTG          | GAAGGAGATGACGAACGC           | 107                | 62.5                      | 90.2           | Dapma7bEvm002840t1             |
| Magnesium homeostasis   | Solute carrier family 41, member 1 (SLC41A1)  | Slc41       | ACAATGAGGCTGGAGCTGG          | ACGCTTAACGTGCGATGCA          | 198                | 62.5                      | 97.2           | Dapma7bEvm003211t1             |
| Osmoregulation          | Sodium/potassium-transporting ATPase subunit alpha | Ap1a1 | GAAGCGCCTTTGCTTAAATG          | TTTCTGGATGAGACATTTGA         | 127                | 60                        | 100            | Dapma7bEvm023379t1             |
| Oxidative stress response | Nuclear factor erythroid 2-related factor 2 | Nrf2        | ATCCACACACATCGCCCTA          | AAGGAAGACGTGCCACCA           | 159                | 64.2                      | 102.5          | Dapma7bEvm015075t1             |
| Oxidative stress response | Glutathione s-transferase | Gst         | GTCACTTGATGAGCCGAAGA          | TAGAGGCGAGAGCAGCAGT          | 134                | 64.2                      | 100            | KP736983.1                     |
| Oxidative stress response | Superoxide dismutase (Cu-Zn) | Sod         | GCTGTTGTTGGTCCTACGTC          | TCAAATGAGGACACCTCG           | 102                | 62.5                      | 100            | JX456347.1                     |
| Oxidative stress response | Catalase                                      | Cat         | CCCCCAAAGCATTATTTGCCG         | TGAGGAGAAAGACGCCCG           | 112                | 62.5                      | 100            | GQ389639.1                     |
| DNA damage response     | DNA repair protein REV1                        | Rev1        | ATGCTGTACCTGTTGACTG          | GTGCTCTGCCTTTGACTGC          | 156                | 62.5                      | 99.6           | Dapma7bEvm006311t1             |
| DNA damage response     | DNA repair protein RAD50                      | Rad50       | TGGACACAGGGTACATGGG          | CTTTGTTCTGCGACTTCT          | 184                | 53.8                      | 91.5           | Dapma7bEvm002105t1             |
| Apoptotic signaling     | Apoptosis-inducing factor 1, mitochondrial    | Aifm1       | CCTAACGAGGCCACCCACT          | ACGTAAAGAGCCACATTTCT         | 176                | 53.8                      | 93.3           | Dapma7bEvm003619t1             |
| Apoptotic signaling     | Death associated protein kinase                | Dapk        | GGTAAAGAGGCTGGACGAGG        | AAGGGGTCGTTCCAAAGA           | 124                | 64.2                      | 100            | Dapma7bEvm005012t1             |
| Apoptotic signaling     | Caspase-2                                      | Casp2       | GCGAGACATGACAGAAGTTGA        | GAAAGGACATGCTGACAGGA          | 186                | 62.5                      | 102            | Dapma7bEvm007428t1             |
| Oxidative phosphorylation | ATP synthase subunit alpha, mitochondrial    | AtpS1a       | GTGTTGTTGCCTTTGACTG          | AAGATACGTGCGACACCA           | 101                | 62.5                      | 100            | Dapma7bEvm009971t1             |
| Endocrine regulation    | Shade                                          | Shd         | GACCTGGATGAGGCGTACCA        | CGGCTGCACTAGTTGCA            | 62                 | 64.2                      | 104.1          | Dapma7bEvm010135t1             |
| Endocrine regulation    | Ecdysone receptor B                            | EcRb        | GCTGTTAGTGGGACGAGCAG         | GCGGCTTGCACTACACTCT          | 62                 | 64.2                      | 94.6           | AB274824.1                     |
| Endocrine regulation    | Methoprene tolerant                           | Met         | CGTGAACAGCTCATGCTTA        | GTGTTGTAAGGCGTTCCTG          | 157                | 53.8                      | 91.5           | Dapma7bEvm011426t1             |
assistance in sample analysis.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.105836.

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