Synergistic interactions of biotic and abiotic environmental stressors on gene expression

Ianina Altshuler, Anne M. McLeod, John K. Colbourne, Norman D. Yan, and Melania E. Cristescu

Abstract: Understanding the response of organisms to multiple stressors is critical for predicting if populations can adapt to rapid environmental change. Natural and anthropogenic stressors often interact, complicating general predictions. In this study, we examined the interactive and cumulative effects of two common environmental stressors, lowered calcium concentration, an anthropogenic stressor, and predator presence, a natural stressor, on the water flea Daphnia pulex. We analyzed expression changes of five genes involved in calcium homeostasis—cuticle proteins (Cutie, Icp2), calbindin (Calb), and calcium pump and channel (Serca and Ip3R)—using real-time quantitative PCR (RT-qPCR) in a full factorial experiment. We observed strong synergistic interactions between low calcium concentration and predator presence. While the Ip3R gene was not affected by the stressors, the other four genes were affected in their transcriptional levels by the combination of the stressors. Transcriptional patterns of genes that code for cuticle proteins (Cutie and Icp2) and a sarcoplasmic calcium pump (Serca) only responded to the combination of stressors, changing their relative expression levels in a synergistic response, while a calcium-binding protein (Calb) responded to low calcium stress and the combination of both stressors. The expression pattern of these genes (Cutie, Icp2, and Serca) were nonlinear, yet they were dose dependent across the calcium gradient. Multiple stressors can have complex, often unexpected effects on ecosystems. This study demonstrates that the dominant interaction for the set of tested genes appears to be synergism. We argue that gene expression patterns can be used to understand and predict the type of interaction expected when organisms are exposed simultaneously to natural and anthropogenic stressors.

Key words: gene expression, Daphnia pulex, calcium level, predator kairomones, anthropogenic stressors, biotic stressors.

Résumé : Il est critique de comprendre la réponse des organismes à de multiples stress afin de prédir si des populations peuvent s’adapter à des changements environnementaux rapides. Les stress naturels et anthropogéniques interagissent souvent, ce qui peut compliquer les prédicitions générales. Dans ce travail, les auteurs ont examiné les effets interactifs et cumulatifs de deux stress environnementaux communs, soit une faible concentration en calcium (un stress anthropogénique) et la présence d’un prédateur (stress naturel), sur la puce d’eau Daphnia pulex. Les auteurs ont analysé l’expression de cinq gènes impliqués dans l’homéostasie du calcium, soit les gènes codant pour des protéines de la cuticule (Cutie, Icp2), la calbindine (Calb) et une pompe et un canal calciques (Serca et Ip3R), au moyen de la PCR quantitative en temps réel (RT-qPCR) lors d’une expérience factorielle complète. Les auteurs ont observé de fortes interactions synergiques entre la faible concentration en calcium et la présence du prédateur. Tandis que le gène Ip3R n’était pas affecté par les stress, la transcription des quatre autres gènes a été affectée par la combinaison des stress. La transcription des gènes codant pour les protéines de la cuticule (Cutie et Icp2) ainsi que la pompe calcique sarcoplasmique (Serca) n’a changé qu’en réponse à une combinaison des stress, et ceci de manière synergique, tandis que celle du gène codant pour la protéine de liaison du calcium (Calb) a été affectée tant par le stress calcique que par la combinaison des deux stress. L’expression de ces gènes (Cutie, Icp2 et Serca) était non-linéaire, mais dose-dépendante tout au long du gradient calcique. De multiples stress peuvent entrainer des effets complexes et parfois inattendus sur des écosystèmes. Cette étude démontre que l’interaction dominante au sein de ces gènes semble être la synergie. Les auteurs avancent que l’expression génique permet de comprendre et de prédir le type d’interaction attendue lorsque des organismes sont exposés simultanément à des stress naturels et anthropogéniques. [Traduit par la Rédaction]

Mots-clés : expression génique, Daphnia pulex, niveau de calcium, kairomones de prédateurs, stress anthropogéniques, stress biotiques.

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Introduction

In nature, organisms are exposed to a plethora of natural and anthropogenic stressors, the combination of which can alter individual survival, growth, and reproduction. A huge challenge in studying the impacts of environmental stressors on biota is to understand their interactive effects. These combined effects are difficult to predict as the individual stressors may interact in an additive, synergistic, or antagonistic manner (Christensen et al. 2006; Coors and De Meester 2008; Folt et al. 1999). Often these interactions result in a negative synergistic impact on the organisms, and their cascading effects at the population and community levels can lead to local extinctions (Schiedek et al. 2007; Brook et al. 2008; Noyes et al. 2009; Schweiger et al. 2010). Aquatic ecosystems, in particular, are routinely, simultaneously subjected to a large variety of stressors, both biotic and abiotic, the latter often being anthropogenic (Christensen et al. 2006; Sala et al. 2000; Altshuler et al. 2011).

The need to understand the interactive and cumulative effects of multiple stressors is often considered as one of the most pressing questions in ecology and conservation (Crain et al. 2008). Many ecological studies have investigated the interactions among multiple stressors and provided a general level of understanding on when certain interaction types are expected (Crain et al. 2008). However, the impacts of multiple environmental stressors on the regulation of gene expression, especially in response to a mix of biotic and abiotic stressors, are not well understood. Yet, such mixes are common, e.g., changes in nutrient and mineral limitation, predation, and competition. Moreover, physiological responses to multiple stressors are preceded by transcriptional changes that can act as biomarkers of early detection of environmental stress (Viarengo et al. 1999; Quirós et al. 2007). Our understanding of the effects of mixtures on gene expressions is limited mainly to mixtures of toxic compounds, and these studies indicate that multiple compound exposures have complex, nonadditive effects on expression patterns compared to single compound exposures (Yang et al. 2007; García-Reyero et al. 2012; Maes et al. 2013). Counter-intuitively, in organisms from severely polluted habitats, the expression of detoxifying genes is down-regulated, in the face of contaminant mixtures, potentially reducing capacity for an appropriate detoxifying response. This contrasts with single contaminant exposures, in which the expression of detoxifying genes is commonly up-regulated (Quirós et al. 2007; Maes et al. 2013). Perhaps unsurprisingly, exposure to mixtures increases the number and interactions of transcription responses. For example, more genes were affected in zebrafish embryos in toxicant mixtures than in single toxicant exposures, and the interactions were largely synergistic (Yang et al. 2007). Similarly, the exposure of Daphnia to a combination of nickel with either lead or cadmium resulted in clear interactions of the mixtures on gene expression, increasing the number of differentially expressed genes in comparison with exposure to the individual metals (Vandenbrouck et al. 2009). Moreover, genes encoding cuticle proteins were substantially down-regulated in mixture treatments (Vandenbrouck et al. 2009).

Given the overall limited understanding of the interactive and cumulative effects of multiple stressors in highly impacted freshwater ecosystems, it is important to consider how chronic exposure to common stressors such as calcium decline and predator presence affect the transcriptional patterns of several selected target genes in sentinel species. Calcium decline is a recently recognized but widespread environmental stressor in soft-water lakes across the eastern Canadian and Fennoscandian shields (Jeziorski et al. 2008). Several species with high calcium requirements have been negatively impacted by this recent, substantial decline of ambient calcium levels (Cairns and Yan 2009; Edwards et al. 2009). Within the crustacean zooplankton community, daphniids are the first to be affected because they have a 10–100 fold higher requirement for calcium than copepods or nondaphnid cladocerans (Yan et al. 1989; Waervågen et al. 2002; Jeziorski and Yan 2006; Cairns and Yan 2009). Since calcium is a key element of the structural minerals of the daphniid carapace, the animal’s sensitivity to low calcium is due, in large part, to the frequent and substantial loss of calcium through molting and excretion, requiring the almost complete replenishment of calcium stores every few days (He and Wang 2009). Because of their great calcium need, declining calcium concentrations reduce daphniid growth, reproduction, and survival. For example, calcium concentrations under 1.5 mg/L lower the growth of Daphnia (Riessen et al. 2012), thus delaying reproduction (Ashforth and Yan 2008), while the animals die when calcium falls below 0.5 mg/L. Since daphniids are keystone organisms in a wide variety of freshwater habitats, their loss as a major grazer of algae and food source in freshwater ecosystems is expected to have strong repercussions up the food chain (Lampert 2006). Calcium uptake is energetically costly in soft-water lakes, given their low ambient concentrations, and as soft-water lakes are also commonly oligotrophic, low food availability may further reduce calcium uptake (He and Wang 2009). Therefore, the increased energy requirements of daphniids under low food and low calcium may lower their ability to effectively respond to other stressors that are energetically costly. One such natural stressor is predation.

The larvae of the phantom midge Chaoborus americanus (Diptera) often control Daphnia populations in nature, especially in small, ephemeral ponds, through heavy predation (Kurek et al. 2010). As the majority of the world’s lakes are small, Chaoborus larvae are the most common pelagic predators that regulate zooplankton communities (McQueen et al. 1986; Ramcharan et al. 2001). Chaoborus releases kairomones that induce species-specific changes in daphniids. Thus, under high predation level, Daphnia...
produces offspring that are larger at parturition, grow faster, have harder carapaces (Lafrance et al. 2004), and form species-specific projections such as neck spines that reduce daphniid vulnerability to Chaoborus (Riessen and Trevett-Smith 2009). Daphnia respond to Chaoborus presence by shifting their reproductive strategy to produce a smaller number of eggs of large size. Such responses are rapid and occur even if maternal exposure to kairomones is just before egg deposition (Feder and Walser 2005; Miyakawa et al. 2010). These phenotypic changes are preceded by molecular changes, including alterations in gene expression. For example, daphniids up-regulate their expression of genes encoding morphogenetic factors and endocrine genes involved in the juvenile hormone pathway to facilitate these physiological changes (Miyakawa et al. 2010). Since these responses have an energetic cost, daphniids have evolved to only manifest them in the presence of the predators, such that the same genotype can produce distinct phenotypes depending on environmental cues (Riessen and Trevett-Smith 2009; Tolrian and Leese 2010).

These two commonly concurrent stressors, low calcium and presence of Chaoborus predation, are intrinsically associated. Calcium is a critical component of the carapace and its availability may determine the size and growth rate of daphniids (Hessen and Rukke 2000). The phenoplastic changes induced by the predator greatly alter the structure, rigidity, ornaments, and size of the carapace in daphniids (Riessen and Trevett-Smith 2009), thus, likely increasing the animals already great calcium demand. Low environmental calcium interferes with the production of these defensive traits (carapace size, neck spines, and rigidity), rendering the animals more vulnerable to predation (Riessen et al. 2012). A significant percentage of Canadian Shield lakes currently have calcium levels of <1.5 mg/L, levels low enough to impede the production of these defensive traits (Riessen et al. 2012), but we do not know if the transcriptional patterns of genes linked to carapace growth and metabolism are influenced by the interactions of low calcium stress in the presence of Chaoborus kairomones.

Here, we used a targeted, a priori candidate gene approach to test the individual and interactive effects of a gradient of low calcium concentrations and the presence and absence of Chaoborus kairomones on the gene expression patterns in Daphnia pulex. By using clonally propagated Daphnia, we ensured that changes in transcription were due to the stressors and not genetic differences among individuals. Since calcium is integral to the formation of the carapace, candidate genes included two genes encoding carapace cuticle proteins (Cutie and Icp2) and three genes encoding proteins involved in calcium homeostasis (Serca, Ip3r, and Calb). As kairomones also influence carapace morphology, shape, and rigidity, we used the presence and absence of predator kairomones as our second, natural stressor to examine the interactions of an anthropogenic and a natural stressor. Overall, we hypothesize that the combination of the two stressors would have an interactive effect on daphniid transcription patterns, with the selected genes showing up-regulation under low calcium and predator presence. We predict that a joint exposure of kairomones and low calcium would increase the number of genes differentially transcribed in comparison with either low calcium stressor or kairomone exposure on their own. As kairomones increase Daphnia’s calcium requirements (Riessen et al. 2012), we predict that Calb, a gene involved in calcium absorption, and Serca, a gene involved in calcium storage, will be up-regulated at lower calcium concentration in the presence of predator kairomones.

Materials and methods

Exposure conditions

The experiment was conducted using the pond dwelling D. pulex, a close relative of Daphnia arenata, whose genome has been fully sequenced (Colbourne et al. 2011). We employed a clonal isolate whose lower calcium thresholds for survival (0.1–0.5 mg/L Ca+2) and reduced reproduction (1.5 mg/L Ca+2) are known (Ashforth and Yan 2008). This obligate parthenogenetic isolate undergoes inducible phenoplastic changes in the presence of Chaoborus, but low calcium reduces its ability to form defensive structures that otherwise are normally induced by Chaoborus (Riessen et al. 2012). The particular Daphnia lineage used in our study was isolated from Sherborne Road Pond, a shallow (less than 1 m maximum depth), ephemeral, forest pond situated near Dorset, Ontario, Canada. This pond contains Chaoborus larvae of two species (C. americanus and C. trivittatus) at high densities (often reaching 2 to 8 individuals per liter) and has a relatively high calcium level (Riessen et al. 2012). The isolated clone was maintained in the laboratory at 2.5 mg/L of Ca+2 for several years prior to our experiment (Riessen and Trevett-Smith 2009).

The experiment was performed in parallel to Riessen et al. (2012), on the same Daphnia isolate and under the same experimental conditions, to ensure that the exposures to kairomones trigger defensive structures and that lower calcium decreases the carapace rigidity of the test daphniids. Twenty parthenogenetic progeny of one progenitor mother were exposed in each of eight treatments: four calcium concentrations (0.5, 1.5, 2.5, and 5.0 mg/L of Ca+2) in the presence (+) or absence (−) of Chaoborus kairomones. The four calcium concentrations were chosen for ecologically relevant reasons. The animals die below 0.5 mg/L Ca+2 and their reproductive ability decreases below 1.5 mg/L Ca+2 (Ashforth and Yan 2008). The 2.5 mg/L Ca+2 concentration was used to replicate the soft-water FLAMES (Field Laboratory for the Assessment of Multiple Ecological Stressors) medium (Celis-Salgado et al. 2008), a medium designed to reflect the chemistry of two Dorset-area, Canadian Shield lakes,
Red Chalk and Blue Chalk, which support stable, multi-species assemblages of *Daphnia*. The highest concentration of 5.0 mg/L Ca^{2+} was chosen as a saturation point for native daphniids (Cairns and Yan 2009). The concentrations of other chemicals in our exposure media were identical to those in the FLAMES medium.

The *Chaoborus* kairomones were introduced into the experimental media by culturing fourth-instar *C. americanus* (with neonate daphniids supplemented as food) at a density of 20 *Chaoborus* individuals per liter of media for 24 h prior to use. All treatment media were filtered through a 1.2 μm Whatman filter to ensure that most bacterial and all algal cells were removed. The media were renewed daily to ensure fairly constant calcium and kairomone exposure.

The animals were kept in a temperature-controlled chamber, at 20 °C, under a 16 h light : 8 h dark cycle. Daphniids were fed daily ad libitum a 4:1 ratio of *Pseudokirchneriella subcapitata : Scenedesmus obliquus*. The algae were cultured in Bold’s Basal medium, a commonly used media for growing green algae, and harvested in log phase (Bischoff and Bold 1963). *Daphnia* neonates (20 individuals per treatment, <24 h old) were separated into the eight experimental treatments. The animals were reared to maturity (15 days) in the treatment media and, to ensure stable expression levels, were sampled during the inter molt stage, which was determined by the presence of eggs in the brood pouch. Four replicates of 3–4 individuals each were collected from each treatment. After collection, animals were stored in 1 mL of RNAlater at −80 °C for subsequent RNA extraction and qPCR.

**Target and reference gene selection**

Five genes were selected in our target gene approach (Table 1): insect cuticle proteins (*Cutie* and *Icp2*), calbindin (*Calb*), sarcoplasmic calcium ATPase (*Serca*), and inositol triphosphate receptor (*Ip3R*). These genes code for proteins involved in calcium homeostasis and building of the carapace. Specifically, cuticle proteins, which are structural components of the animal’s carapace, tend to be differentially expressed depending on the tissue type, development, and molt stage (Charles 2010). Calbindin is thought to be involved in calcium absorption (Bouillon et al. 2003; Reifegerste et al. 1993) and is expected to show differential expression under different calcium treatments, potentially increasing in expression with decreasing calcium concentrations and increased calcium demand due to predator presence. SERCA and IP3R control calcium homeostasis in the cells (Berridge et al. 2003), and they are likely to be affected by lower calcium concentrations and increased demand as the animal responds to the stressors. In few cases our targeted genes appear to have paralogs in the *D. arenata* genome. In such situations we selected the genes that had annotated gene ontology (GO) and predicted functional eukaryotic orthologous groups (KOG) classification. *Daphnia arenata* is a member of the *D. pulex* species complex (sensu lato) and
a close relative of the *D. pulex* (sensu stricto) used in our study. Three common housekeeping genes were used to control for quality and quantity of RNA: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Syntaxin 16 (*Stx16*), and C-terminal pdz ligand of neuronal nitric oxide synthase (*CAPON*) (Schwarzenberger et al. 2009; Spanier et al. 2010; Table 1). We opted to use these three housekeeping genes in favour of others since *GAPDH* is commonly used for this purpose and the expression of *CAPON* and *Stx16* remains stable under kairomone treatment (Spanier et al. 2010). Moreover, we tested the three housekeeping genes for stability using NormFind, a model-based approach that evaluates the stability of housekeeping gene candidates and has an advantage of being able to group samples into treatments while using inter- and intra-variation between treatments to assess the stability (Andersen et al. 2004). The three possible housekeeping genes had comparable stability values (*GAPDH* (0.196); *Stx16* (0.236); *Capon* (0.154)). Moreover, since the geometric average of multiple control genes is shown to produce more accurate results (Andersen et al. 2004; Vandesompele et al. 2002), we opted to use the geometric mean of all three housekeeping genes to normalize the target genes.

**RNA extraction and RT-qPCR**

RNA was extracted and purified from the frozen *Daphnia* samples preserved in RNAlater using the RNeasy Mini Kit (QIAGEN) and treated with RNase-free DNase I (QIAGEN) for 15 min to remove DNA contamination from the samples. The concentration and purity of the RNA samples was checked with the NanoVue spectrophotometer (GE Healthcare Technologies) using the 260/280 nm absorbance ratio, and all samples were found to be of high RNA purity (2.01–2.02 ratio). Prior to performing qPCR on the experimental RNA samples, we ran aliquots of them on a polyacrylamide gel to ensure that the RNA was not degraded. Forty nanograms of total RNA was reverse-transcribed into cDNA with the Sensiscript Reverse Transcription Kit (QIAGEN) using the Oligo (dt) Primer (50 μmol/L) from Ambion. The qPCR reactions were performed in triplicate for each biological replicate, using the Power SYBR Green PCR Master Mix (Applied Biosystems), and containing 2 μL of cDNA, 0.4 μL (10 μmol/L) of each primer, 10 μL of the 2X Power SYBR Green PCR Master Mix, and water to bring the final volume to 20 μL. Two types of negative control reactions were performed, one without cDNA and a second without reverse transcriptase (RT) to confirm the absence of DNA contamination. The qPCRs were run on the Applied Biosystems 7500 SDS Real-Time PCR System according to the manufacturer’s instructions. Each target gene for each treatment and biological replicate was run on the same plate with all three housekeeping genes to ensure that any differences between plates would be accounted for. Biological replicates of the treatments were run on separate plates. The thermal cycling program included an initial 10 min denaturation at 95 °C and 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

**Data analysis**

The normalization factor (n) was calculated from the geometric mean (Vandesompele et al. 2002) of the three housekeeping genes (*GAPDH*, *CAPON*, and *Stx16*; see Table S1) and used to normalize the target genes and acquire the ΔCT (cycle threshold) values (Livak and Schmittgen 2001; Spanier et al. 2010). The CT used was an average of the three technical replicates. The treatment with high calcium (5.0 mg/L) in the absence of *Chaoborus* (−) was used to calibrate the changes in gene expression of the other treatments via the standard 2−ΔΔCT method (Livak and Schmittgen 2001) and the values log2-transformed (see Tables S2 and S3 for calculations and CT values). A two-way ANOVA with log2-transformed fold change values was used to test whether calcium concentration, predator kairomones, or the interaction of the two variables affected the expression of each target gene (Table 2). Two separate 1-way ANOVA tests using the log2-transformed fold change values were used to determine if changes in calcium concentration had a significant effect on each gene’s expression in the kairomone and nonkairomone-treated animals separately (Table S4). This was performed to assess the effect of calcium alone. The treatment means deemed significant by the 1-way ANOVA were then analyzed by a Tukey’s test, to determine which specific calcium concentrations had a significant effect on gene expression (Table S5). A t-test was used to assess differences in gene expression between the kairomone and nonkairomone treatments at each calcium concentration (Table S6). Significance for every test was assessed as p < 0.05. All statistical tests were performed in R-Project version 2.7.

**Results and discussion**

The combination of calcium limitation and predator presence synergistically affected the expression of the cuticle protein genes *Cutie* and *Icp2*, and the calcium homeostasis gene *Serca* (Table 2). However, the individual stressors alone did not affect expression of these genes. On the other hand, expression of the calcium homeostasis *Calb* gene was significantly affected by changes in calcium concentration and the interaction of the two stressors (Table 2). Finally, the expression of *Ip3R* did not appear to change with either of the stressors or a combination of the two (Table 2).

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Table 2. Results of 2-way ANOVA testing if calcium, kairomones, or their interaction had a significant effect on gene expression of *Daphnia pulex*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source of variation</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutie</td>
<td>Calcium</td>
<td>3</td>
<td>0.226</td>
</tr>
<tr>
<td></td>
<td>Kairomones</td>
<td>1</td>
<td>0.273</td>
</tr>
<tr>
<td></td>
<td>Calcium × kairomones</td>
<td>3</td>
<td><strong>0.033</strong></td>
</tr>
<tr>
<td>Icp2</td>
<td>Calcium</td>
<td>3</td>
<td>0.126</td>
</tr>
<tr>
<td></td>
<td>Kairomones</td>
<td>1</td>
<td>0.929</td>
</tr>
<tr>
<td></td>
<td>Calcium × kairomones</td>
<td>3</td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td>Ip3R</td>
<td>Calcium</td>
<td>3</td>
<td>0.153</td>
</tr>
<tr>
<td></td>
<td>Kairomones</td>
<td>1</td>
<td>0.230</td>
</tr>
<tr>
<td></td>
<td>Calcium × kairomones</td>
<td>3</td>
<td>0.438</td>
</tr>
<tr>
<td>Serca</td>
<td>Calcium</td>
<td>3</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>Kairomones</td>
<td>1</td>
<td>0.189</td>
</tr>
<tr>
<td></td>
<td>Calcium × kairomones</td>
<td>3</td>
<td><strong>0.040</strong></td>
</tr>
<tr>
<td>Calb</td>
<td>Calcium</td>
<td>3</td>
<td><strong>0.028</strong></td>
</tr>
<tr>
<td></td>
<td>Kairomones</td>
<td>1</td>
<td>0.144</td>
</tr>
<tr>
<td></td>
<td>Calcium × kairomones</td>
<td>3</td>
<td><strong>0.010</strong></td>
</tr>
</tbody>
</table>

Note: This test was performed to examine if the two stressors or their interaction have an effect on gene expression. Significant values are in bold (p < 0.05).

Cuticle proteins: Cutie and Icp2

The carapace (cuticle) of insects and crustaceans is made up of interlinked chitin filaments and cuticle proteins (Andersen et al. 1995), with calcium minerals as an integral stiffening element (Willis 1999). The expression of both cuticle protein genes (*Cutie* and *Icp2*) was significantly affected by the interaction of calcium and predator kairomones (Table 2), but not affected by the individual stressors. The decrease in calcium only had an effect on *Cutie* expression if the animals were simultaneously exposed to kairomones (Fig. 1; Table S4). This decrease in calcium (between 5.0 and 0.5 mg/L) in the presence of kairomones increased *Cutie* expression by 6.2 fold ± 2.4 SEM (Fig. 1; Tables 2, S5). Furthermore, kairomone exposure decreased the expression of the second cuticle protein gene, *Icp2*, by 47.9 fold ± 26.7 SEM, but only at 2.5 mg/L Ca²⁺ (Fig. 1; Table S6).

Both of these genes encode structural constituents of the cuticle and belong to large multigene families with sequences ranging from very similar to having no homologous features (Andersen et al. 1995). Organisms have many cuticle proteins, which may be differentially expressed depending on the tissue type, development, and molt stage (Charles 2010). *Daphnia arenata* in particular has a multitude of cuticle protein genes (~295 genes; GO:0042302, *Daphnia pulex* version 1.0-Joint Genome Institute). The expression of the two cuticle proteins genes we examined was governed by ecological cues, i.e., calcium, kairomones, and their interactions (Table 2), though their expression patterns differed. The expression of *Icp2* was dose dependent and nonlinear across the calcium gradient. This nonlinear expression pattern is not unique; other stressors have similar effects on the expression of cuticular protein genes (Soetaert et al. 2007). For example, an intermediate cadmium exposure of 50 μg/L increased the expression of several cuticle genes compared to a control, but a level of 100 μg/L decreased their expression. Since many *Daphnia* genes are transcriptionally activated only during specific environmental challenges (Colbourne et al. 2011), this differential expression of cuticular protein genes is likely the organism’s response to a particular combination of kairomone exposure and calcium levels. This was true for *Cutie*, as well. Decreasing calcium concentration increased the expression of *Cutie*, but only in kairomone-exposed animals. Thus, *Cutie* is likely to be highly expressed in animals that have a higher demand but lower availability of Ca²⁺. Our result are consistent with previous studies that demonstrated differential expression of genes involved in molting, such as cuticle proteins and proteolytic enzymes, under other environmental stressors such as fungicides and metals (Soetaert et al. 2007; Vandenbrouck et al. 2009; Kim et al. 2011).

Sarcoplasmic proteins: Serca and Ip3R

Both *Serca* and *Ip3R* code for proteins found in the sarcoplasmic membrane of cells and play important roles in cell calcium signalling pathways and calcium homeostasis (Berridge et al. 2003). The expression of *Ip3R* was not affected by either of the stressors or their combined effects. In contrast, the interaction of calcium and kairomones (p < 0.05; Table 2) affected the transcription of *Serca*. However, the individual stressors did not have an effect on expression levels on their own. In the absence of kairomones, calcium concentration did not influence the expression of *Serca*, meaning expression was stable across the calcium gradient. However, in the presence of kairomones, the decrease in calcium between 5.0 and 1.5 mg/L Ca²⁺ increased the expression of *Serca* (p < 0.05; Fig. 1; Tables 2, S4, S5). Moreover, kairomone exposure decreased the expression of *Serca* (3.3 fold ± 0.30 SEM; p < 0.005) at the highest calcium concentration relative to nonkairomone-exposed animals (Fig. 1; Table S6).

Cells tightly regulate their cytoplasmic calcium concentrations, as Ca²⁺ ions are used in a multitude of concentration-dependent processes. For example, during signal transduction in a resting cell, various pumps and exchangers keep calcium concentrations low — one of the main pumps in this process is SERCA. It uses active transport to pump Ca²⁺ into the sarcoplasmic reticulum for storage (Wuytack et al. 2002). On the other hand, IP3R is a calcium channel protein that releases Ca²⁺ from the sarcoplasmic reticulum to propagate calcium signalling (Berridge et al. 2003). The role of IP3R is to generate local and global calcium signals that regulate a multitude of cell processes such as transcription and motility (Foskett et al. 2007). IP3R has several paralogs in the *Daphnia* genome; this may be one of the reasons we did not observe a response to the stressors, as some of the paralogs may have been differentially regulated.
This further demonstrates the need to carefully select candidate genes and resolve phylogenetic relationships between paralogs (Schwarzenberger et al. 2009; Altshuler et al. 2012). While neither stressor had an effect on Ip3R expression, the expression of Serca was up-regulated at lower calcium concentrations, but only in animals that were simultaneously exposed to kairomones.

Calcium-binding protein: Calb
Calbindin (Calb) encodes a calcium-binding protein and is part of the EF-hand homolog family containing six calcium-binding domains (Reifegerste et al. 1993). Calcium concentration alone and the interaction between the two stressors affected the expression of Calb ($p < 0.05$; Table 2). The decrease of calcium did not affect the
expression of Calb in the absence of kairomones (Table S4). However, in the presence of kairomones, the decline of calcium between 5.0 and 1.5 mg/L increased the expression of Calb 2.5 fold ± 1.2 SEM (Fig. 1; Tables S4, S5). When considering kairomone exposure solely, Calb expression increased 2.3 fold ± 0.8 SEM (Fig. 1; Table S5) in the presence of kairomone compared to its absence, however, this effect was only observed at 2.5 mg/L Ca+2 (Table S6).

Calbindin acts as a weak calcium buffer (Schwaller et al. 2002) and is expressed in vertebrates in renal and intestinal epithelia as well as in the nervous system (Reifegerste et al. 1993). One of its major functions in birds and mammals is vitamin-D dependant absorption of calcium (Reifegerste et al. 1993; Bouillon et al. 2003). Its function is less understood in arthropods, though a Drosophila calbindin gene has been characterized and dubbed as Calbindin-32 (Reifegerste et al. 1993). Chaoborus kairomones increase Daphnia’s demand for calcium by triggering the formation of defensive structures that require more calcium (Riessen et al. 2012; Riessen and Trevett-Smith 2009). If calbindin plays a similar role in crustaceans as it does in mammals and birds, then the increase in Calb expression observed under low calcium conditions and high calcium demand is expected. Our results provide further support for the suggestion that the function of calbindin in crustaceans may be related to calcium absorption. Thus, daphniids likely produce more calcium-binding proteins involved in calcium absorption in response to low ambient calcium concentrations and higher calcium demand.

Overall, low calcium alone was not enough to trigger changes in gene expression. However, when the animal’s need for calcium was augmented (increased) by kairomones, changes in gene expression were observed. The addition of the second stressor triggered changes in transcriptional patterns that the abiotic stressor alone did not induce. This finding is consistent with the results of Riessen et al. (2012) who showed that low calcium alone did not affect the strength of the daphniids carapace or formation of neck spines. Animals exposed to lower calcium in combination with kairomones exhibited decreased strength of the carapace and reduced formation of neck spines (Riessen et al. 2012). However, the size of the animals was significantly affected by both calcium alone and in combination with kairomones (Riessen et al. 2012). A similar effect was seen in Daphnia exposed to single and mixed metal compounds. Vandenbrouck et al. (2009) reported that at the transcriptional level more genes and pathways were effected under multiple stressors compared to single metal compounds and that transcriptional patterns of mixtures was not the simple sum of their individual compounds, which suggested an interaction among the compounds. Furthermore, Vandenbrouck et al. (2009) showed that a large portion of the genes differentially effected in the mixtures were coding for cuticle constituents.

Organisms alter their gene expression patterns in response to environmental perturbations. Hence, gene expression patterns are useful in understanding the initial response of individuals to multiple stressors (Viarengo et al. 1999; Shaw et al. 2007; Poynton et al. 2008; Maes et al. 2013). Consequently, gene expression levels can serve as useful bioindicators with an important diagnostic role for environmental and toxicological monitoring (Oleksiak 2008; Quiróz et al. 2007). However, as is often the case in controlled laboratory experiments, gene expression changes observed in experimental settings do not always reflect the changes recorded in the field (Maes et al. 2013). There are two likely explanations for this disagreement. First, wild populations vary naturally in their genetics. Hence, wild populations may have different or more varied gene expression patterns than laboratory populations of the same species (Normandeau et al. 2009; Leder et al. 2015). Second, natural systems are far more complicated, with organisms being inevitably simultaneously exposed to multiple stressors, complicating the profiles of gene expression (Maes et al. 2013). Thus, predicting the complex effects of interacting stressors remains an important challenge. Studies on gene expression offer the opportunity of early detection of environmental stress and the possibility of intervention before keystone species experience noticeable decline.

Concluding remarks

Four of the five candidate genes responded to the combination of the two stressors. Overall, they increased in expression at lower calcium levels, but only when simultaneously exposed to kairomones, while exposure to single stressors failed to elicit significant changes in gene expression. This reinforces our conclusion that multiple stressors can interactively effect gene expression patterns, producing complex physiological responses and influencing the survival of exposed organisms (Christensen et al. 2006; Riessen et al. 2012). The combination of low calcium and predator presence has previously been shown to interfere with Daphnia’s ability to produce physiological defensive structures, including neck spines, rendering them more vulnerable to Chaoborus predation (Riessen et al. 2012). Here, we demonstrated that this combined effect is also mirrored in gene expression patterns, with a strong interaction on the expression when both stressors are present. For now, our results are limited to a laboratory setting and one lineage and would need to be validated in a natural environment and a larger number of lineages. Background environmental quality, food and nutritional availability, and water chemistry all have distinct effects on an organism’s gene expression. Thus, we first need to understand how these factors, alone and in combination, affect gene expression, since we would be in a better position to discern the effects of anthropogenic stressors from abiotic and biotic environmental stressors (van Straalen and Feder 2012). The long-term
trend of calcium decline is likely to continue to negatively impact freshwater habitats. Understanding how this stressor will interact with other natural and anthropogenic stressors is important in predicting its effects on freshwater organisms and the ecosystem at large. Gene expression changes are likely to become effective methods for detecting the effects of multiple stressors in threatened ecosystems.

**Author contributions**

I.A. conceptualized the study and performed the experiments. I.A. and A.M.M. analyzed the data. I.A., M.E.C., and N.D.Y. prepared the manuscript. All authors read and approved the final manuscript.

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