



## SYMPOSIUM

# An Integrated Multi-Disciplinary Approach for Studying Multiple Stressors in Freshwater Ecosystems: *Daphnia* as a Model Organism

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**Synopsis** The increased overexploitation of freshwater ecosystems and their extended watersheds often generates a cascade of anthropogenic stressors (e.g., acidification, eutrophication, metal contamination, Ca decline, changes in the physical environment, introduction of invasive species, over-harvesting of resources). The combined effect of these stressors is particularly difficult to study, requiring a coordinated multi-disciplinary effort and insights from various sub-disciplines of biology, including ecology, evolution, toxicology, and genetics. It also would benefit from a well-developed and broadly accepted model systems. The freshwater crustacean *Daphnia* is an excellent model organism for studying multiple stressors because it has been a chosen focus of study in all four of these fields. *Daphnia* is a widespread keystone species in most freshwater ecosystems, where it is routinely exposed to a multitude of anthropogenic and natural stressors. It has a fully sequenced genome, a well-understood life history and ecology, and a huge library of responses to toxicity. To make the case for its value as a model species, we consider the joint and separate effects of natural and three anthropogenic stressors—climatic change, calcium decline, and metal contaminants on daphniids. We propose that integrative approaches marrying various subfields of biology can advance our understanding of the combined effects of stressors. Such approaches can involve the measuring of multiple responses at several levels of biological organization from molecules to natural populations. For example, novel interdisciplinary approaches such as transcriptome profiling and mutation accumulation experiments can offer insights into how multiple stressors influence gene transcription and mutation rates across genomes, and, thus, help determine the causal mechanism between environmental stressors and population/community effects as well as long-term evolutionary patterns.

## Introduction

In the age of global climatic change, increased industrialization and over-exploitation of natural resources, many ecosystems, and the biodiversity they support, are at a tipping point from anthropogenic stress (Sala et al. 2000). The Millennium Ecosystem Assessment leaves no doubt that global ecosystems are in peril, but we still have far to go with the urgent task of understanding how these rapid environmental changes are going to impact ecosystem functioning and ecological services, as well as the future of our own species. While many

studies have looked at the effects of individual stressors and their results have provided a solid foundation of our understanding of how specific stressors affect natural systems, few studies have considered their combined effects (Schindler 2001; Vinebrooke et al. 2004; Coors and De Meester 2008; Yan et al. 2008). There is an urgent need to understand the effects of multiple stressors, since individual stressors now commonly co-occur, with unexpected and complex interactions (Christensen et al. 2006). The combinations of stressors could have: (1) additive effects—whereby the effect of the mix of stressors equals the sum of the effects of individual stressors,

(2) synergistic effects—whereby the combined effect of two or more stressors is greater than the sum of the individual stressors, or (3) antagonistic effects—whereby the combined effect of the stressors is less than the summed effects of individual stressors (Folt et al. 1999). Therefore, we cannot necessarily predict with confidence what the combined effects of multiple stressors will be simply by looking at the effects of single stressors (Christensen et al. 2006); the effects of multiple stressors must be specifically assessed.

Freshwater ecosystems are among the most stressed of global environments, bombarded with all classes of stressors, including changes in water chemistry (acidification, eutrophication, metal contaminants, and decline in calcium), changes in the physical environment (changes in land use, climatic change, and increase in UV radiation), the introduction of invasive species (zebra mussels, sea lamprey, and spiny water flea), and over-harvesting of resources (over-fishing). We argue that the most effective way to understand the combined effect of these stressors is to use a multi-disciplinary approach that integrates knowledge derived from ecological, physiological, toxicological, evolutionary, and genetics data, and by following ecological and biological responses at all levels of biological organization from molecules to natural populations and communities. One of the most important challenges that an integrative approach has to overcome is the transferability of data between disciplines and multiple biological levels and the application of the developed models beyond singular keystone species and ecosystems and across large sets of multiple stressors. There is a pressing need for selecting model organisms that play a critical role in maintaining the stability of the aquatic ecosystems, while also being both ecologically sensitive (responsive) and geographically ubiquitous. We propose *Daphnia*, a freshwater microcrustacean, as one of the best model organisms for studying multiple stressors in limnetic ecosystems. *Daphnia* has been used as a model organism in studies of evolution, ecology, toxicology, and genomics (Colbourne et al. 2011). More recently, it has been suggested as a model species for ecological genomics, transcriptomics, and ecotoxicogenomics—fields that aim to understand how biotic and abiotic stressors, as well as evolutionary and life-history characteristics, affect gene expression and other biological and ecological changes (Watanabe et al. 2008; Orsini et al. 2011). Such studies can be used to understand how multiple stressors affect the organisms on a genetic and biochemical level. For example, microarray experiments are used to screen the patterns of

gene expression of thousands of genes in animals exposed to a particular stressor or to a combination of stressors. In addition, evolutionary and ecological studies have examined how *Daphnia* populations adapt to changing environmental conditions such as climatic warming (Mitchell and Lampert 2000; Van Doorslaer et al. 2010). This broad range of approaches can be used in integrative ways to shed light on the effects of multiple stressors on aquatic organisms.

Here, we review the ecology and evolutionary history of *Daphnia* that justifies its increased use in large-scale ecological-genomics studies and its potential use in understanding the effects of multiple stressors. We examine the effects of three widespread anthropogenic stressors: (1) climatic change, (2) calcium decline, and (3) metal contaminants in lakes. We consider these stressors in the context of their interacting effects with each other and with other anthropogenic and natural stressors. Finally, we propose combining transcriptome profiling and mutation accumulation experiments as a novel approach to studying responses to multiple environmental stresses.

### ***Daphnia* as an emerging model system for studying multiple stressors**

*Daphnia* is a keystone species in the pelagic zone of most freshwater habitats (e.g., arctic and temperate lakes, lakes at high elevations, ephemeral ponds, ponds in sand-dunes) and provides a key link between primary production and higher trophic levels (Hebert 1978; Lampert 2006). Because *Daphnia*'s ecology, phylogeny, toxicology, and physiology is relatively well understood, access to its genome sequence (wFleabase.org) and other genetic tools such as genetic linkage map (Cristescu et al. 2006), cDNA libraries, microarrays (Supplementary Table S1) allows for the study of environmental influences on gene functions in ways that are difficult in other model species (Eads et al. 2008; Colbourne et al. 2011). For example, the availability of genetic linkage maps and the transferability of crossing panels between laboratories can greatly advance the diagnosis of important ecological and environmental traits through quantitative trait locus (QTL) studies or the identification of heritable genotypes that affect gene expression using eQTL (expression QTL) approaches.

Compared to most other freshwater taxa, daphniids are highly sensitive to environmental perturbations (Schindler 1987) and they typically respond rapidly to stressors by altering their mode of

reproduction (Hebert and Crease 1983), changing their pattern of vertical migration within the water column (Stich and Lampert 1984; Dawidowicz 1992), and/or undergoing behavioral (Gerhardt et al. 2005) and phenoplastic changes (Tollrian 1993). These common natural stressors include environmental toxicants, bacterial infections, vertebrate and invertebrate predators and parasites, synthetic hormones, varying diets, UV radiation, hypoxia, acidity, salinity, and low ambient calcium levels (Colbourne et al. 2011). *Daphnia's* extremely “ecoresponsive genome” (Colbourne et al. 2011; Tautz 2011) may account for the multiple habitat transitions among closely related species. For example, members of *Daphnia* species complexes (e.g., *Daphnia pulex*, *Daphnia galeata mendotae*, and *Daphnia longispina* species complexes) evolved ecologically relevant traits, enabling these closely related species to colonize habitats with distinct environmental conditions (Fryer 1991; Hebert 1995; Wellborn et al. 1996; Colbourne et al. 1997). The multiple lineages independently colonized and adapted to these freshwater habitats and are characterized by various degrees of reproductive isolation and significant intraspecific (among populations) genetic subdivision (Crease et al. 1997; Pfrender et al. 2000).

*Daphnia* typically reproduces by cyclical parthenogenesis; this strategy entails both clonal reproduction during optimal environmental conditions and sexual reproduction otherwise. The sexual phase is often triggered by environmental stresses such as crowding, cooling, or change in photoperiod, as well as by predation (but see Paland et al. 2005). This unique reproductive system of *Daphnia* allows us to maintain both lines of genetically identical individuals in the laboratory, as well as lines of genetically variant clones; this allows us to understand if responses elicited from stressors do, or do not, have a genetic basis. *Daphnia* is an ideal system for studying multiple stressors because of its short generation time, well-studied ecology and evolutionary history, wide geographical distribution across many limnetic systems, high mutation and recombination rates, high sensitivity to changes in environmental conditions, unique cyclical parthenogenetic life history, and recent availability of many genomic tools.

## Climatic change

It is now widely accepted that climatic change is impacting the structure, function, and biodiversity of freshwater ecosystems (Sala et al. 2000; Carvalho and Kirika 2003), both directly and indirectly. Indirect interactions producing complex outcomes

in populations in freshwater communities have been noted with food quality and availability, toxic contaminants, and UV radiation. The effects may be also direct, i.e., changes in ambient temperatures are likely to have a strong impact on the life-history parameters of many aquatic species. Moreover, under climatic change, temperature fluctuations may exceed the thermal tolerance limits of keystone species, such as *Daphnia*, and lead to cascading effects up and down the trophic web, thereby impacting the functioning of the freshwater ecosystem (Jeppesen et al. 2010). Therefore, studying how climatic change independently and in concert with other stressors impacts the life history, ecology, physiology, and evolution of *Daphnia* should help us understand and predict major changes in freshwater ecosystems.

Temperature has been shown to have great effects on the life history of *Daphnia*, inducing earlier emergence (Carvalho and Kirika 2003) and shorter life-span (Bottrell 1974). In addition, as temperature increases, the filtering rate, metabolic rate, and demand for food also increase (Burn 1969). Even an increase in temperature of as little as 1.7°C during a short, but critical, seasonal period, can adversely affect *Daphnia* populations, and induce significant changes in entire food webs of lake systems (Wagner and Benndorf 2007). For example, for every rising degree of temperature, the predation on *Daphnia galeata* by the predatory cladoceran *Leptodora kindtii* and by planktivorous fish, started distinctly earlier in the season by 13.0 and 6.5 days, respectively, resulting in lower densities of *Daphnia* (Wagner and Benndorf 2007). Moreover, temperature changes can cause rapid microevolution of *Daphnia* populations and alter the community structure of freshwater habitats (Van Doorslaer et al. 2010). In a common garden experiment, *Daphnia* genetically adapted to increased temperature within one growing season (Van Doorslaer et al. 2010). Clones of *D. pulex* and *D. magna* were exposed for 6 months to different temperature treatments in large outdoor mesocosms that simulated small ponds. The clones showed evidence of micro-evolutionary adaptation to higher temperature by an increase in size at maturity (Van Doorslaer et al. 2010). Clearly, daphniids may adapt genetically to higher temperature quite rapidly. In addition, changes from more thermophobic to more thermophilic daphniid taxa are likely as the climate warms. For example, Lennon et al. (2001) suggested that the thermo-tolerant *Daphnia lumholtzi*, a species native to Africa, Asia, and Australia that has invaded North America, may replace many native taxa as the

climate warms. These findings suggest that it is important to incorporate micro-evolutionary responses of keystone species and colonization of nonnative clones in models that aim to predict the effects of climatic change on populations, communities, and the dynamics of food webs (Van Doorslaer et al. 2010).

Climatic change may directly affect water quality, by decreasing water availability, concentrating pollutants and contaminants, and increasing salinity (Schindler 2001). The exposure and sensitivity of organisms to chemical toxins and heavy metals in water appear to be closely linked to ambient temperature and tend to be toxin-specific. Climatic change affects the rate of uptake and detoxification of toxins and the sensitivity of organisms through changes in rates of metabolism and feeding (Cairns et al. 1975; Heugens et al. 2003). For example, in *Daphnia*, the rate of cadmium uptake, a major metal contaminant, significantly increases at higher temperatures, possibly because of elevated ventilation rates in response to increased metabolic rate and demand for oxygen (Cairns et al. 1975; Heugens et al. 2003); at the same time growth rate decreases (Heugens et al. 2006). These studies show that metals are more toxic at higher temperatures because of increased bioaccumulation. Nonmetallic toxins such as sodium dodecyl sulfate (SDS) also exhibit increased toxicity at high temperatures (Folt et al. 1999). However, once food is taken into consideration, the interaction of temperature and toxins dramatically changes. While higher food levels appear to protect *Daphnia* from the toxic effects of cadmium (Heugens et al. 2006), SDS is more toxic at higher food concentrations (Folt et al. 1999).

Global warming has been also shown to affect the exposure of freshwater organisms to UV radiation (Schindler et al. 1996; Yan et al. 1996) and the ability to repair the damage caused by UV (MacFadyen et al. 2004; Connelly et al. 2009). Elevated temperature in the environment leads to decreased influx of water to lakes from streams and ground-water tables. In consequence, the supply of dissolved organic carbon from these terrestrial and wetland catchments decreases, and penetration of UV-B into lakes increases exponentially as dissolved organic carbon declines (Schindler et al. 1996). Furthermore, an increase in the ambient temperature induces changes at the molecular level that help *Daphnia* deal with damage from UV radiation. Zooplankton have two main mechanisms for repairing UV-induced DNA damage: the light-dependent photoenzymatic repair (PER) and light-independent nucleotide-excision repair (NER) (Rautio and Tartarotti 2010).

MacFadyen et al. (2004) found that PER in *Daphnia pulicaria* is temperature dependent. The repair is less effective for UV-induced DNA damage at lower temperatures, owing to decreased efficiency of the enzyme. Although, UV-induced DNA damage was observed to be higher at elevated temperatures, the efficiency of the repair mechanism also increased; thus, the net UV-induced DNA damage at lower temperature is still more serious compared to that occurring at higher temperatures (MacFadyen et al. 2004). In contrast, Connelly et al. (2009) found that survival as well as the repair of DNA was higher at lower temperatures in four *Daphnia* species exposed to UV radiation. While many studies have reported the effects of UV radiation on *Daphnia*, very few studies focused on the interactive effects of UV and temperature on this important keystone species (Rautio and Tartarotti 2010). Furthermore, *Daphnia* inhabits lakes with varying temperatures and UV exposures, ranging from clear-water, oligotrophic arctic lakes with high permeability of UV to canopy-covered eutrophic ponds (Hessen 1999). This combination of a broad ecological setting provides the opportunity for investigating the interactive effects of UV and temperature.

## Decline in calcium

Soft-water lakes on the Boreal Shield and other parts of North America and Northern Europe are currently experiencing a trend of rapid decline of calcium (Keller et al. 2001; Jeziorski et al. 2008). In lakes of Ontario, Canada, for example, the levels of calcium have dropped by 13% since the mid-1980s (Jeziorski et al. 2008). Calcium decline has likely emerged from a combination of at least three unrelated anthropogenic stressors: multiple cycles of forest re-growth after logging in the watershed, historical and ongoing deposition of acid (Watmough 2003; Jeziorski et al. 2008), and reduced runoff as climate changes (Yao et al. 2011). Since crustaceans have high demands for calcium, they are expected to be most sensitive and to be the first impacted by this sharp decline in calcium levels (Cairns and Yan 2009). Indeed, daphniids have a significantly higher demand for calcium, averaging 2.38% Ca by dry weight, compared to 0.23% Ca in non-daphniid planktonic cladocerans and copepods (Waervagen 2002; Jeziorski and Yan 2006). In laboratory experiments where animals are well-fed, calcium concentrations of 1.5 mg/l and below have a negative impact on the animals' reproductive capabilities, and animals die below 0.5 mg/l  $\text{Ca}^{2+}$  (Ashforth and Yan 2008). However, under



**Table 1** Fold of increase (max EC50/min EC50) in toxicity of heavy metals attributable to high and low concentrations of ambient calcium

Metal tested	Range in concentration of Ca <sup>2+</sup> (mg/l)	Metal EC <sub>50</sub> concentrations range (free ion activity)	Fold increase in toxicity of the metal from high to low [Ca <sup>2+</sup> ]	Source
Cadmium	1.2–64.4	0.1–1.05 μM	10.5	Clifford and McGreer (2010)
Zinc	1.6–59.7	1.25–21 μM	18	Clifford and McGreer (2009)
Copper	10–160.3	8.6–48.7 nM	5.6	De Schamphelaere and Janssen (2002)
Nickel	0.8–58.8	3–62.5 μM	20.8	Kozlova et al. (2009)

natural conditions of ambient food, the animals do not survive at calcium concentrations below 1.4 mg/l (Cairns 2011, personal communication). This likely explains the observation that the probability of observing daphniids significantly drops in lakes with calcium concentrations below 1.5–2.0 mg/l Ca<sup>2+</sup> (Jeziorski et al 2008). Unfortunately, about 35% of the lakes in Ontario are already below this threshold (Jeziorski et al. 2008).

In the context of multiple stressors, several anthropogenic and natural stressors could have complex interactions with declining calcium levels. For example, animals experiencing limited calcium supplies have been proven to be more prone to UV-induced DNA damage, suffering reduced survival in low-Ca/high UV treatments (Hessen and Rukke 2000a). In addition, declining calcium levels may certainly interact synergistically with heavy metal contaminants in freshwater habitats. Water hardness is well known to protect *Daphnia* and other animals from toxic effects of metal contaminants. This protection has been mainly attributed to Ca<sup>2+</sup> concentrations and to a lesser extent Mg<sup>2+</sup> (Borgmann et al. 2005; Kozlova et al. 2009). For example, increased aqueous and body calcium in *Daphnia* directly decreases the efficiency of the uptake and assimilation efficiency of zinc and cadmium (Tan and Wang 2008). Calcium acts as a competitive inhibitor of toxic metals by binding more efficiently to sites on the biotic ligand than do other metal cations (Paquin et al. 2000; Santore et al. 2002). Increasing calcium concentrations in the medium drastically diminish the toxic effects of cadmium (Clifford and McGreer 2010), zinc (Clifford and McGreer 2009), copper (De Schamphelaere and Janssen 2002), and nickel (Kozlova et al. 2009). Consequently, calcium offers protection for *Daphnia* and other freshwater organisms from heavy metal contaminants (Table 1). The biota of soft-water lakes are already more susceptible to metal toxicity than are hard-water organisms, and with decreasing calcium levels, the

exposure of animals to toxic levels of metal contaminants may well increase.

In addition to these physical and chemical anthropogenic stressors, *Daphnia* may become more prone to biotic stressors with declining concentration of ambient calcium. The animals could suffer greater susceptibility to invertebrate predators such as the phantom midge, *Chaoborus*. The midge is a gape-limited predator that targets *Daphnia* neonates because of the smaller size of this life-history stage (Pastorok 1981). *Daphnia* have evolved ways that reduce predation by triggering phenotypically plastic changes in juveniles in response to kairomones released by the *Chaoborus*. These changes include formation of neck teeth (Tollrian 1993) and an increased stability of the carapace (Laforsch et al. 2004). Since calcium forms a major part of the carapace (Hessen and Rukke 2000b), declining calcium levels could lead to reduced formation of neck teeth and to loss of rigidity of the carapace, making the animals more vulnerable to predation by *Chaoborus*. Clearly, declining calcium concentrations have drastic effects on the survival and reproduction of *Daphnia* and other crustaceans. However, interacting effects with other anthropogenic and natural stressors seem to have a cascade of complex effects that are as yet not well understood.

## Metal stressors

Pollution by heavy metal contaminants in aquatic ecosystems, mainly caused by the mining industry, is an ongoing concern around the world. For several decades, ecotoxicologists have intensively studied the adverse biological impact of excessive amounts of metal elements such as copper, cadmium, lead, and arsenic on various aquatic organisms (Heugens et al. 2001). *Daphnia* have been an important model system for investigating the adverse impact that metal toxins exert on freshwater ecosystems. Metal contaminants usually have negative effects on individual development, population growth rate, longevity,

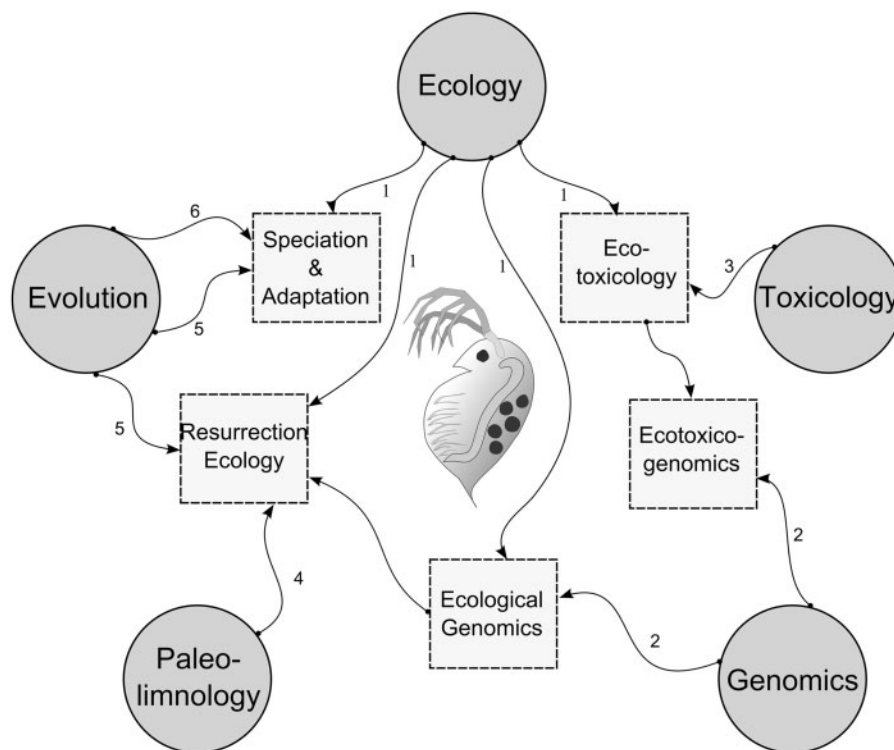
and reproduction. In the past decade, the rapid development of genomic technologies, such as microarrays, has revolutionized the field of ecotoxicology by revealing how metal contaminants can affect gene expression in many aquatic taxa (Neumann and Galvez 2002). In this genomic era, *Daphnia* is an important model system for crustaceans and freshwater organisms due to the availability of its whole genome sequence and microarrays (Colbourne et al. 2011). The numerous ecotoxicogenomic studies employing *Daphnia* as a model have made three types of contributions. First, characteristic profiles of gene expression have been established for various metal contaminants including copper, cadmium, zinc, and nickel (Poynton et al. 2007; Vandegheuchte et al. 2010; Poynton et al. 2011). Second, these studies have facilitated the development of novel biomarkers to monitor and identify the level and types of metal contaminants in aquatic ecosystems (Connon et al. 2003; Poynton et al. 2008; Garcia-Reyero et al. 2009). Third, because *Daphnia* have a large number of genes (i.e., 36% of the minimal set of ~30,907 genes) that have no detectable homologues in other sequenced animal lineages (Colbourne et al. 2011), a growing catalogue of novel genes involved in a response to acute metal stress have the potential to be discovered. For example, three new genes coding for the cadmium detoxification protein metallothionein have been identified in *D. pulex* (Shaw et al. 2007).

### Approaches to the study of multiple stressors

Despite these substantial advances, it remains a grand challenge for ecotoxicologists to link changes in genetic expression to adverse outcomes at the individual and population level (Snell et al. 2003; Snape et al. 2004; Fedorenkova et al. 2010). Because of the well-understood ecology of *Daphnia*, we have the potential of using the information of metal-induced molecular changes to predict ecological outcomes on a population scale. For example, Connon et al. (2008) performed a microarray study to investigate the changes in gene expression of *Daphnia magna* exposed to cadmium in relation to the pattern of population growth. In light of a lowered population growth rate observed in their experiment, these authors examined the detrimental effects of changes in gene expression on cellular processes related to growth, moulting, and metabolism, thereby establishing a logical link between measured responses of gene to population-level changes. However, this kind of work has been infrequent, and a great amount of gene-expression data and

endpoint toxicological tests are still needed to establish meaningful correlations between changes in gene expression and population levels (Fedorenkova et al. 2010), and eventually to responses by entire communities and ecosystems (Whitham et al. 2006). Compared to other model organisms, *Daphnia* has a few unique advantages. *Daphnia* is widely distributed in aquatic habitats with different ecological conditions (lake versus pond, contaminated versus uncontaminated habitats). The ecology of *Daphnia* in these habitats is also well studied. With the extraordinary ease of laboratory maintenance, it is possible to perform common garden experiments on populations of *Daphnia* with a particular genotype and directly examine the link between gene expression and population-level changes in these environmentally relevant scenarios.

However, to better understand the long-term impact of metal contaminants and multiple stressors on aquatic ecosystems, it is necessary to quantify induced heritable genetic changes, such as mutations and transcriptional changes, to better predict the acclimation, evolution, adaptation and the future of biota in stressed environments. Mutations provide the raw material upon which natural selection can work and are thus essential for the adaptation of populations to new environments. On the other hand, environmental stressors, such as heavy metals, may stimulate increased mutation rates which could be damaging to the integrity of the genome. To date, few efforts have been devoted to relating temperature changes, decreased level of calcium, and metal stress to changes in mutational and transcriptional processes. We suggest that combining the classic mutation accumulation (MA) experiments with controlled exposure to multiple stressors in *Daphnia* would provide insights into this largely unexplored field. The classic MA experiments consist of multiple isogenic lines derived from the same asexual ancestor or a common highly inbred base population (Halligan and Keightley 2009). Each of these MA lines is propagated in a benign, unchallenging environment and is bottlenecked at each generation (e.g., a single, randomly picked individual for clonal or hermaphroditic species). This results in an extremely low effective population size for each MA line, thus reducing the efficiency of natural selection to a minimum and allowing the majority of mutations, except those having extreme effects such as lethality, to accumulate over time in a neutral manner. Numerous MA studies have been performed and have provided insights into the rate and the spectra of spontaneous mutations for several model species in nonstressful environments (Denver et al.



**Fig. 1** Integrative approaches to studying multiple stressors using *Daphnia* as a model organism. The arrows connect primary fields of study (in circles) to form interdisciplinary fields (in squares). The numbers on the arrows indicate the tools, techniques or the knowledge that can be used to integrate multiple data sets: (1) ecological surveys/knowledge, (2) genomic tools (microarrays, sequencing), (3) toxicity tests, (4) retrieval of diapausing eggs from sedimentary cores, (5) evolutionary theories/knowledge, and (6) phylogenetic reconstruction techniques.

2000, 2004; Lynch et al. 2008; Keightley et al. 2009), whereas little work has been conducted on the transcriptional changes in MA lines (Rifkin et al. 2005). Because of the uniform original genetic background for all the MA lines, it is easy to compare the mutational and transcriptional changes among the MA lines exposed to normal and challenging environments (e.g., combinations of anthropogenic stressors). Analyses of the genomes and transcriptomes of treated and untreated MA lines using the next generation of sequencing technologies allows the investigation of fitness and gene-expression changes, as well as the spectrum of genomic mutations deleterious to overall fitness. The results arising from such studies can have important evolutionary and ecotoxicological implications, allowing us to understand if multiple stressors cause unique molecular stress responses compared to a single stressor, and to clarify the genetic basis of the organism's response to single and multiple stressors.

Although aquatic organisms now commonly face multiple environmental stressors such as decline of calcium, rise of temperature, and metal toxins, few data are available for understanding the combined

effects of multiple stressors. This is largely because the effects of single stressors are already difficult to characterize from the angles of physiology, gene expression, and ecology. However, with the growing possibilities of having an improved perspective of single stressors, we envision a better understanding of how aquatic organisms deal with multiple stressors. We can attempt to understand the effects of multiple stressors by integrating diverse fields of biology (Fig. 1). First ecotoxicogenomic and ecological genomic studies aim to integrate gene-expression data with ecological knowledge to reveal how organisms alter their gene expression in response to environmental and anthropogenic stressors. For example, gene transcription and translation may be altered in animals exposed to metals (Shaw et al. 2007), predator-born kairomones (Schwarzenberg et al. 2009), toxic chemicals (Watanabe et al. 2007), hypoxia (Zeis et al. 2009), change in temperature (Schwerin et al. 2009), microcystin-producing cyanobacteria (Schwarzenberg et al. 2009; Miyakawa et al. 2010), and hormone derivatives that trigger the production of males (Eads et al. 2008). The results from these studies constitute baseline data that would

become more useful in the context of mutation-accumulation experiments in which treatment with single/multiple stressors are used to examine how mutation rates and transcriptome profiles are altered in a stressful environment. Secondly, integration of genetic and toxicological work can indicate if biotic ligand models would need to be developed for multiple *Daphnia* genotypes or species to be effective at predicting environmental risk and toxicity of contaminants (Janssen et al. 2000). When combined with ecological surveys this ecotoxicogenomics approach can predict if it is metals that are regulating the recovery of daphniids from historical stress, such as in heavily contaminated lakes near the Sudbury, Ontario, smelters region (Valois et al. 2010). Lastly, because *Daphnia*'s dormant eggs are preserved in sediments; historic generations can be hatched via methods of resurrection ecology (Kerfoot and Weider 2004). Therefore, we can use this paleolimnological approach in combination with genomic and phylogenetic techniques to illuminate how animals dealt with historic environmental challenges (Eads et al. 2008; Orsini et al. 2011), providing perhaps excellent clues to the future of these keystone pelagic herbivores.

## Supplementary data

Supplementary data are available at ICB online.

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