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LETTER

The evolution of salinity tolerance in *Daphnia*: a functional genomics approach

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Abstract

One route to genetic adaptation in a novel environment is the evolution of ecological generalisation. Yet, identifying the cost that a generalist pays for the increased breadth of tolerance has proven elusive. We integrate phenotypic assays with functional genomics to understand how tolerance to a salinity gradient evolves, and we test the relationship between the fitness cost of this generalisation and the cost of transcription that arises from evolved differences in patterns of gene expression. Our results suggest that a salt-tolerant genotype of *Daphnia* is characterised by constitutively expressed genes, which does not incur a loss of fitness or a cost of transcription relative to a salt-intolerant genotype in low saline environments. We find that many genes whose expression pattern evolved in response to salinity are also involved in the response to predators, suggesting that the cost of generalisation may be due to trade-offs along other environmental axes.

Keywords

Daphnia, generalist, microarray, salinity, tolerance curve.

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INTRODUCTION

Organisms with broad tolerance to environmental factors (i.e. generalists) may have considerable advantage transitioning between, or coping with, changing environments. A consequence of genetic adaptation to a novel environment may be alteration of the breadth of tolerance (Hutchinson 1978). Adaptation that leads to increased breadth in environmental tolerance is defined as the evolution of ecological generalisation, whereas adaptation that leads to the narrowing of environmental tolerance is defined as the evolution of ecological specialisation. Implicit in arguments on the evolution of generalisation is that it entails a cost: a loss of fitness elsewhere along the environmental gradient that leads to a fitness trade-off between generalists and specialists (Levins 1968). Although there is a well-developed body of knowledge that documents the cost of adaptation in general, the cost of generalisation remains unclear (Palaima 2007).

An empirical approach to examine the cost of generalisation estimates the fitness of genotypes in multiple environments and tests for trade-offs by calculating genetic correlations of fitness across environments. Negative correlations indicate a trade-off, whereas a positive or zero correlation indicates no trade-off. A number of studies using this approach in diverse organisms (insects: Via & Hawthorne 2002; Fry 2003; crustaceans: Palaima & Spitze 2004; unicellular organisms: Elena & Lenski 2003; and viruses: Turner & Elena 2000) have found that cross-environmental genetic correlations for fitness are either zero or positive, indicating no genetic constraints on the evolution of generalists.

Recent studies have established a direct connection between individual tolerance curves and patterns of gene expression (Takahashi *et al.* 2004) and between gene expression and degrees of phenotypic plasticity (Schadt *et al.* 2003; Seki *et al.* 2003; Promislow 2005). Together with studies connecting plasticity and adaptation to novel environments (Latta *et al.* 2007; Scoville & Pfrender 2010) these observations imply that generalisation is partly determined by the effect of phenotypic plasticity on the multi-environmental fitness of an organism, and the control of this plasticity can be at the level of gene expression. As changes in gene regulation alter the patterns of transcription, one potential cost of generalisation may arise from the cost of transcription.

There is good evidence that transcription is costly, and that this cost is important during evolution. Several lines of evidence show that increases in the level of gene expression can impact the fitness of an organism [e.g. lower intron content minimising the cost of transcription in humans and nematodes (Castillo-Davis *et al.* 2002); selection operating on levels of gene expression in *Drosophila* to maximise fitness (Bedford & Hartl 2009); the cost of transcription related to mating efficiency in yeast and a trade-off with growth rate (Lang *et al.* 2009); trade-off in constitutive expression of *lacZ* operon affecting growth rate in *E. coli* under varying energy sources (Dekel & Alon 2005)]. These costs may be the result of an energetic cost that occurs during the polymerisation of pre-mRNA (i.e. two adenosine triphosphate (ATP) molecules are hydrolysed per ribonucleotide) and the subsequent processing of the pre-mRNA by the spliceosome into mature mRNA, which requires the hydrolysis of numerous ATP molecules per splice site.

We envision two scenarios in which the cost of transcription may influence the evolution of generalisation. Generalists may be capable of increased dynamic regulation of genes related to coping with environmental gradients. Here, the generalist displays optimal expression levels across the environmental gradient that facilitates the maintenance of fitness across environments, thereby incurring

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no fitness or transcriptional cost of generalisation. Alternatively, a generalist may constitutively maintain high levels of gene expression across environments, affording it a broader tolerance, but resulting in gene products produced at inappropriately elevated levels in some environments. Under this scenario, suboptimal over-expression of gene products could result in fitness trade-offs among generalists and specialists; hence the cost of transcription provides a mechanistic explanation for a fitness trade-off. Otherwise, there could be no evidence for a fitness trade-off, and the cost of generalisation is the energetic cost of transcription itself.

Evolutionary changes in the patterns of gene regulation in response to environmental gradients can result in a transcriptional cost of generalisation (Fig. 1). These changes can be described in four alternative patterns based on differences between specialist and generalist genotypes in the level, and degree of dynamic modulation, of transcription across environments. One pattern is that the level of expression in both the specialist and generalist is invariant across environments [i.e. canalisation (Fig. 1a)]. In this case, constitutive changes in gene expression induce a cost to the generalist when genes are up-regulated with respect to the specialist across all environments. Alterations of the environmental sensitivity of genes can also incur a cost. When the level of gene expression in the specialist is plastic and varies across the environmental gradient, the level of gene expression in the generalist is canalised (Fig. 1b) the form of regulatory evolution is referred to as genetic assimilation (Pigliucci *et al.* 2006). A transcriptional cost of generalisation will arise when the generalist assimilates a level of gene expression that is higher than the level a specialist experiences in its optimal environment. Regulatory evolution can lead to an increase in plasticity when the levels of gene expression in the specialist are canalised across the environmental gradient, but the levels in the generalist are sensitive to the environment (Fig. 1c). Finally, when both the specialist and generalist have dynamic patterns of gene expression across environments, the degree of plasticity (e.g. slope of the reaction norm) is different (Fig. 1d), regulatory evolution is described as genetic accommodation (Braendle & Flatt 2006). A cost from plasticity or genetic accommodation, will only arise when the level of expression in the generalist exceeds that of the specialist in its optimal environment

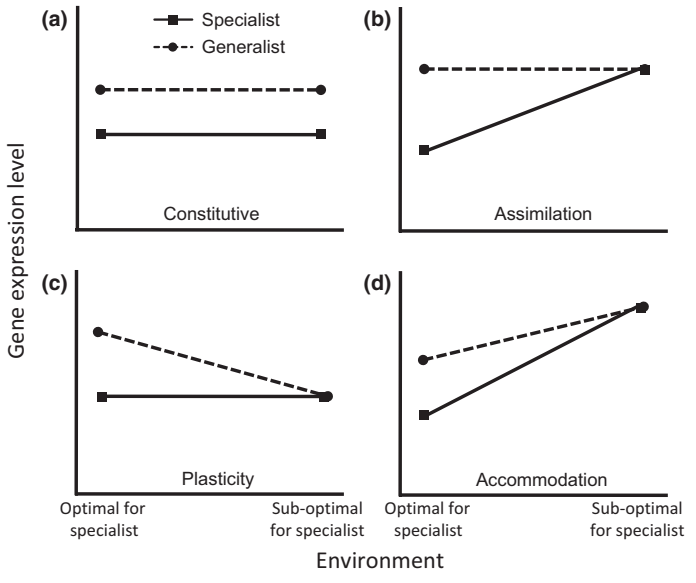

Figure 1 Forms of regulatory evolution that may result in a cost of generalisation due to a cost of transcription. (a) Constitutive up-regulation; (b) genetic assimilation; (c) plasticity; and (d) accommodation. The environment axis depicts two environments relevant for the specialist, one which is optimal for the specialist and would result in maximal fitness, and one that is sub-optimal and would result in reduced fitness.

The goal of this study was to first examine the relationship between the breadth of tolerance curves and the evolved differences in patterns of gene expression across genotypes with varying degrees of generalisation, and then determine if a cost of generalisation occurs as a result of the cost of transcription. As a model for this investigation of phenotypic plasticity and its relationship to the evolution of generalisation we use the crustacean *Daphnia pulex*. Naturally occurring populations of *Daphnia* inhabit ponds along salinity gradients providing genotypes that are genetically differentiated for salinity tolerance (Weider & Hebert 1987). *Daphnia* can reproduce asexually, which allows replication of genotypes and evaluation of genotype specific fitness across a range of environmental conditions. This property enables us to generate salinity tolerance curves to characterise the evolutionary transition between freshwater and saline environments at the phenotypic level. Finally, the availability of genomics tools (i.e. microarrays) permits a functional genomic assessment of salt-induced changes in the patterns of gene expression.

MATERIALS AND METHODS

Study system

The invasion of saline habitats by the freshwater crustacean *D. pulex* in the Churchill region of northeastern Manitoba, Canada, has been a subject of investigation over the last quarter century (Weider 1987; Weider & Hebert 1987; Weider *et al.* 2010). Several studies have documented significant differences in salinity tolerance among populations and genotypes of *D. pulex* (Weider & Hebert 1987; Weider 1993). For this study, we utilised two genotypes from the 'polar pulicaria' clade (Colbourne *et al.* 1998) whose ecology, distribution and physiological adaptations have previously been characterised. One genotype, referred to as 'clone 2' in previous studies, lives in ponds with salinities ranging from 0.11 to 0.58 g L^{-1}, while the other genotype, 'clone 4' in previous studies, resides in ponds that range in salinity from 0.60 to 9.95 g L^{-1}. In the light of the unique salt-related attributes of these genotypes, we refer to clone 2 as a 'specialist' genotype and clone 4 as a 'generalist' genotype.

We caution that because we employ only two genotypes the ability to extrapolate our results to other salt-affected systems and organisms may be limited. However, only 17 unique genotypes of *D. pulex* in the Churchill region have been identified over the last 25 years, and only five genotypes inhabit Bluff A, the location from which our genotypes were collected. Thus, the genotypes we use here may approximate well the assemblage of clones in the Churchill area.

Generation of salinity tolerance curves and estimation of niche breadth

Salinity tolerance curves for the specialist and generalist genotypes were generated by exposing replicate clonal populations to a range

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of NaCl concentrations. Five genetically identical replicate populations of 10 adult egg-bearing female individuals per genotype were placed in a 250-mL beaker containing 100 mL of a standard culture medium (Kluttgen *et al.* 1994). Starting from this baseline salinity each genotype was evaluated across a gradient of increasing NaCl concentrations from 0 to 8 g L^{-1}, in increments of 1 g L^{-1}. For each genotype, a total of 450 adult *Daphnia* distributed among nine NaCl levels were assayed. Survivorship in each beaker was measured every 24 h for 72 h. Individual mortality was determined by examining movement in the compound eye, second antennae, post-abdominal claw and thoracic appendages. Individuals were considered dead when movement in these four morphological features was absent.

Tolerance curves were constructed by fitting four-parameter logistic functions to the dose-response data. These curves were then used to generate estimates of the amount of NaCl required to kill 0.1% of the population (LC0.1). The LC0.1 describes the range of salinities over which a genotype maintains essentially 100% fitness, and provides an estimate of the niche breadth and degree of generalisation for each genotype.

Measuring gene expression profiles and costs

To assess patterns of gene expression, we used the *D. pulex* multiplex long-oligonucleotide microarray (Colbourne *et al.* 2011). The platform is a high-density NimbleGen (Roche-NimbleGen, Inc., Madison, WI, USA) microarray that accommodates 12 experiments per glass slide, with each experiment interrogating 137 000 probes. Each predicted and experimentally validated gene is represented by as many as three probes, whereas the remaining probes are designed from transcriptionally active regions whose gene models are not yet known.

RNA used in the microarray experiment is derived from 50 adult egg-bearing female individuals of each genotype within 3 L of media at each of two concentrations of NaCl (0 and 5 g L^{-1}). After a 24-h exposure, total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA, USA), and purified using the Qiagen (Venlo, Netherlands) RNeasy protocol with on-column DNase treatment (Lopez & Colbourne 2011). Because we were interested in the expression patterns that describe the adaptive differences between the genotypes and the plastic differences within a genotype, we used four competitive hybridisations that define these axes (generalist at 0 g L^{-1} vs. specialist at 0 g L^{-1}; generalist at 5 g L^{-1} vs. specialist at 5 g L^{-1}; generalist at 0 g L^{-1} vs. generalist at 5 g L^{-1}; specialist at 0 g L^{-1} vs. specialist at 5 g L^{-1}) in a loop design where each sample was used in four hybridisations with one dye-swap for each hybridisation (see Figure S1 in Supporting Information).

Microarray data were imported into an in-house analysis pipeline using Bioconductor for normalisation and analysis (Gentleman *et al.* 2004). All probes including random probes were quantile-normalised across arrays, samples and replicates. Differential expression was assessed using LIMMA and EBarrays (Kendziorski *et al.* 2003; Smyth 2004) using the median signal of probes representing genes. EBarrays uses a parametric mixture model to calculate the posterior probability of differential expression for arbitrarily complex experimental designs. To determine the significance of expression differences, and to adjust for multiple testing, we calculated the False Discovery Rate (FDR) using the Benjamini–Hochberg method (Benjamini & Hochberg 1995) for each gene using the Bioconductor LIMMA package. These data are deposited at NCBI GEO under the accessions GSM634870–GSM634877.

The first goal of this study was to infer the form of regulatory evolution a particular gene may have undergone in response to increasing salinity. Therefore, only genes that are differentially expressed in at least two conditions (e.g. a gene that differs along one axis of adaptation must also be plastic in at least one genotype) are informative. Genes not meeting this requirement were excluded from the analysis. To increase the statistical power of our study, we also only selected presently annotated genes from the subset of total genes on the arrays that had three independent probes (multi-probe gene set of 22 076 loci). This subset of genes increases the reliability of significance values because it adds another level of replication by ensuring that estimates of relative expression within a gene for a given condition are based on three independent replicates.

For each of the genes identified as having undergone regulatory evolution, we simultaneously evaluated the *M*-values, which describe the $\log_2$ transformed differential expression ratio, for all four conditions to construct reaction norm plots. These plots were then used to assign a gene to a specific form of regulatory evolution. For a particular gene, if the FDR was > 0.05 for a condition, then we inferred that the levels of expression were equal in that condition, whereas if the FDR was < 0.05 for a condition, then we used the sign and magnitude of the *M*-value to position points on the plot. For example, a gene with significant and positive *M*-values in the conditions describing adaptation, but not in the conditions describing plasticity, implies that gene expression has evolved via constitutive up-regulation.

We also examined whether or not regulatory evolution was targeting genes that control important biological processes. We particularly examined genes that were annotated with Enzyme Commission classification numbers (EC number) and used these as input for iPATH v2.0, a programme that maps genes to global metabolic pathways (Yamada *et al.* 2011). We created metabolic pathway maps and used colour-coding to distinguish the form of regulatory evolution each gene in a pathway step underwent to examine the relationship between metabolic networks and regulatory evolution.

We assessed the cost of transcription that arises due to evolved regulatory changes in the generalist vs. the specialist. A cost of transcription may be incurred if more genes are up-regulated in the generalist than in the specialist, if the level of transcription of a single gene is higher in the generalist than the specialist or if the length of the DNA being transcribed into mRNA is longer in the generalist relative to the specialist. These costs are related to the energetic cost of transcription which occurs due to the polymerisation of mRNA, but are not indicative of energetic costs that may arise during splicing. Using the microarray data, in conjunction with the *Daphnia* genome (http://genome.jgi-psf.org/Dappu1/Dappu1.home.html), we calculated four metrics that define costs related to the energetics of transcription for each genotype in each environment tested: (1) the relative number of genes that were up- or down-regulated; (2) the average *M*-value of the set of significantly differentially expressed genes; (3) the average protein length of the set of significantly differentially expressed genes; and (4) the number of ATP molecules hydrolysed per gene. Our rationale for using protein length, which is directly proportional to the length of the mRNA, instead of estimates of pre-mRNA length is twofold. First, the genotype sequenced for the *Daphnia* genome is not one of the

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genotypes used here, so estimates of protein length should provide more accurate estimates of transcript length than pre-mRNA because protein lengths are more evolutionarily conserved than pre-mRNA lengths. Second, evidence that selection may reduce the number or size of introns (Castillo-Davis *et al.* 2002), and that there is substantial intron polymorphism among populations of *Daphnia* (Li *et al.* 2009), suggest that in the absence of sequence data from these particular genotypes, pre-mRNA lengths may vary between them, whereas protein length should be conserved.

The number of ATP molecules hydrolysed for each gene was estimated by calculating the total number of ribonucleotides polymerised and then multiplying this value by two, which is the number of ATP molecules required to extend a transcript by one ribonucleotide. The total number of ribonucleotides was calculated using the protein length multiplied by three (three ribonucleotides per amino acid), and the antilog of the absolute value of the *M*-value which gives an estimate of relative transcript abundance (e.g. a threefold increase in gene expression).

Finally, to identify a potential cost of generalisation along other environmental axes, we ranked ordered 20 772 genes represented by three probes on the array – based on their *M*-values calculated by contrasting the condition × genotype interactions using linear modelling in LIMMA (Smyth 2004) – and tested for the enrichment of genes identified by a prior microarray experiment to be responsive to kairomones of the dipteran predator *Chaoborus* (NCBI GEO accession nos GPL11200–GPL11201; Colbourne *et al.* 2011) using Gene Set Enrichment Analysis (GSEA; Subramanian *et al.* 2005).

RESULTS

Salinity tolerance

The specialist experienced lower survivorship in response to increasing NaCl levels relative to the generalist (Fig. 2a). After 24 h, a majority of the specialist individuals had died at 6 g L^{-1} of NaCl. All specialist individuals perished at 7 g L^{-1} of NaCl. In contrast, the generalist had *c.* 75% survivorship at 6 g L^{-1}, and 20% survivorship at 7 g L^{-1}. This pattern of reduced survivorship in the specialist, including reduced estimates of LC0.1 (Fig. 2b) was consistent over all three sampling intervals. The difference in LC0.1 between the specialist and generalists was most pronounced after 24 h, with the generalist displaying an LC0.1 that was *c.* 2 g L^{-1} NaCl higher than the specialist. Both genotypes showed a progressive decline in LC0.1 as time of exposure increased.

Patterns of regulatory evolution

We obtained a set of 560 genes that were reliably and significantly differentially expressed in two or more tested conditions. Our initial cut-off value, FDR < 0.01, also produced a set of 596 genes showing significant differential gene expression in only a single condition (see Table S1 in Supporting Information). Because we could not unambiguously assign these genes to a specific form of regulatory evolution we did not retain them for analysis. Although these genes may be involved in the evolution of salinity generalisation a lack of statistical power precludes any evaluation of the form of regulatory evolution that these genes may have undergone. We suggest that increased replication of specialist and generalist genotypes may help increase the statistical power of future studies.

The 560 genes were assigned to one of four possible classes of regulatory evolution based on their *M*-values among conditions (Fig. 3). The most common forms of regulatory evolution were constitutive changes in gene expression (393 cases) and assimilation (113 cases). Accommodation and plasticity were rare, comprising only 22 and 18 cases respectively. A set of 14 genes had significant differential expression in the two conditions that describe plasticity, but not in either condition that describes adaptation. This result is consistent with the idea that plasticity in gene regulation has been evolutionarily conserved in both genotypes, but as these genes have not undergone regulatory evolution, they were not included in subsequent analyses. The specific nature of these regulatory changes is further supported by the reaction norm plots that show that the generalist has a predominantly canalised response to changes in NaCl levels (Fig. 4).

Of the 546 original genes identified as undergoing regulatory evolution, 348 are functionally annotated on the JGI release of the *D. pulex* genome based on sequence homology. The remaining 198 genes are lineage-specific genes with no known homology to genes of other sequenced organisms. Of the 348 functionally annotated genes, 108 were identified as enzymes having EC numbers and members of metabolic pathways. These genes were used as input for iPath v2.0 to generate metabolic pathway maps (see Figure S2 in Supporting Information). The match statistics for these genes produced 119 matches to metabolic pathways and 45 matches to the synthesis of secondary metabolites.

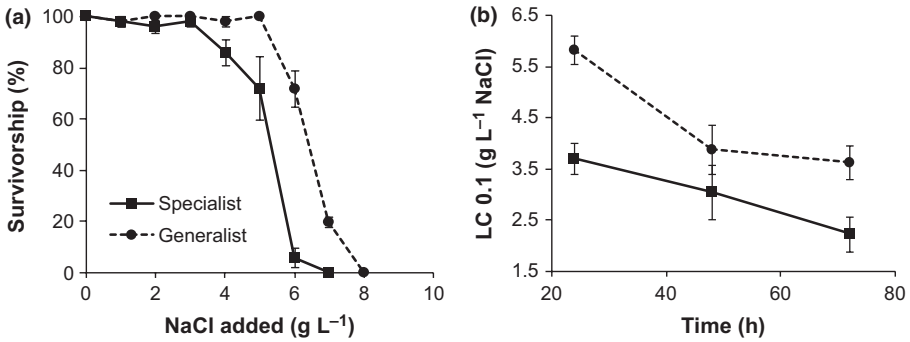

Figure 2 Fitness response to salinity in a specialist and generalist of *D. pulex*. (a) Survivorship curves for each genotype after 24 h of exposure to NaCl. Microarray samples were taken after 24 h of exposure to 0 and 5 g L^{-1}. (b) The amount of NaCl required to cause 0.1% mortality of the two genotypes for three sampling intervals. Estimates of LC0.1 provide an assessment of the degree of generalisation. Error bars in panels (b–d) are ± 2 SE.

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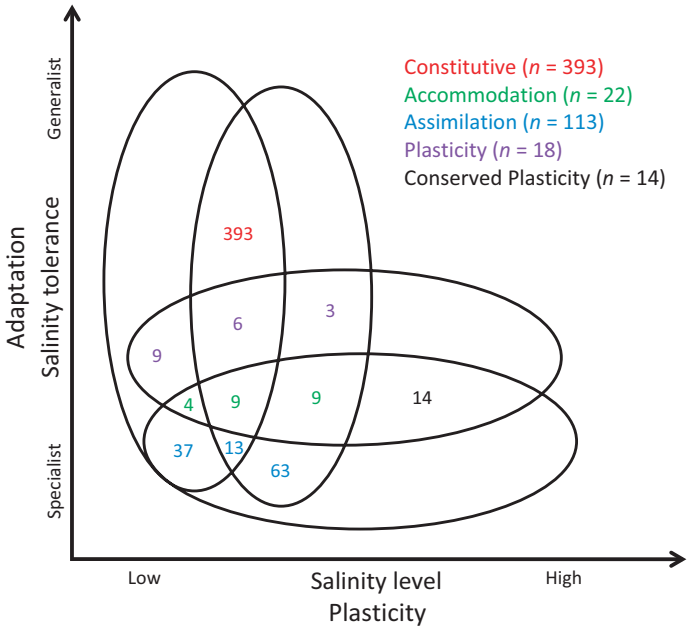

Figure 3 The distribution of differentially expressed genes and form of regulatory evolution inferred from microarrays. The adaptation axis indicates the salt-tolerance of the genotype (specialist is low, generalist is high), and the plasticity axis indicates the amount of NaCl (0 g L^{-1} is low, 5 g L^{-1} is high). Conditions that compare genotypes within a NaCl level are vertical ellipses along the adaptation axis. The relative position along the plasticity axis indicates the NaCl level. Conditions that compare genotypes across NaCl levels are horizontal ellipses along the plasticity axis. The relative position along the adaptation axis indicates the genotype used. Numbers in intersections indicate number of genes, whereas the number of genes for each type of regulatory evolution is denoted by *n*.

The cost of generalisation

A total of 471 genes were differentially regulated between the genotypes at 0 g L^{-1} NaCl and 496 genes were differentially regulated at 5 g L^{-1} NaCl. The generalist showed more down-regulated and fewer up-regulated genes than the specialist at both NaCl levels (Fig. 5a). This difference was most pronounced at 5 g L^{-1} NaCl. There were no differences between the specialist and generalist with respect to the amount of transcription, the protein length and the number of ATP molecules hydrolysed per gene (Fig. 5b–d). Overall, at 0 g L^{-1} NaCl the specialist hydrolyses 130 227 more ATP molecules than the generalist, whereas at 5 g L^{-1} NaCl the specialist hydrolyses 792 667 more ATP molecules than the generalist.

A total of 2672 genes (13%) were significantly differentially expressed (FDR < 0.05) when contrasting the condition × genotype interactions. These genes, among the ranked list of the total gene set based on their measured *M*-values, were found to be significantly enriched [Enrichment Score (ES) = 0.7; normalised ES = 2.73; $P < 0.0001$] by 564 genes that respond to kairomones produced by the common dipteran predator *Chaoborus* (Fig. 6).

DISCUSSION

Identifying the costs of ecological generalisation using phenotype-based approaches has proven elusive. However, the advancing frontier of genomics offers new tools with which to investigate potential costs associated with the evolution of niche breadth. We integrated phenotype-based assays with a functional genomics approach to examine the regulatory changes that occur during the evolution of salinity generalisation in *Daphnia*, and tested the hypothesis that the cost of the evolution of salinity generalisation involves evolutionary

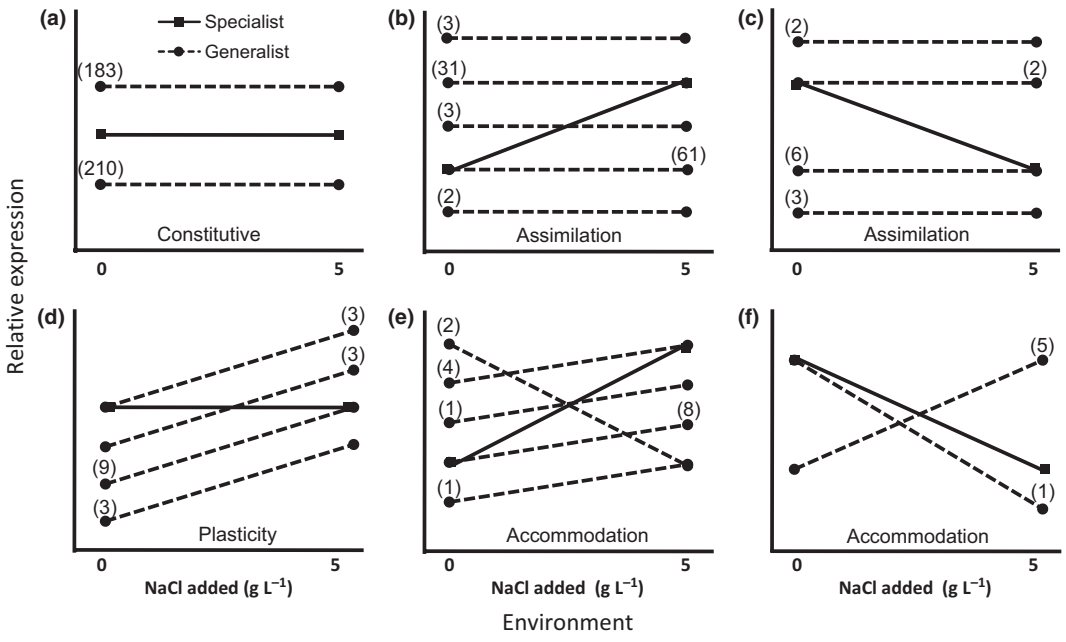

Figure 4 Gene expression reaction norms for each model of regulatory evolution from microarrays. The specialist pattern of gene expression (solid line) can evolve in response to a new environment by undergoing: (a) constitutive up- or down-regulation; (b, c) assimilation, a specialist plastic response becomes canalised; (d) plasticity, specialist canalisation becomes plastic; or (e, f) accommodation, the specialist and generalist both show plastic responses to salinity, but the slopes differ. The numbers in parentheses above points for a particular line indicate the number of genes that underwent the specified form of regulatory evolution. Note that some genes that underwent accommodation, assimilation or plasticity also have undergone constitutive changes in regulatory evolution.

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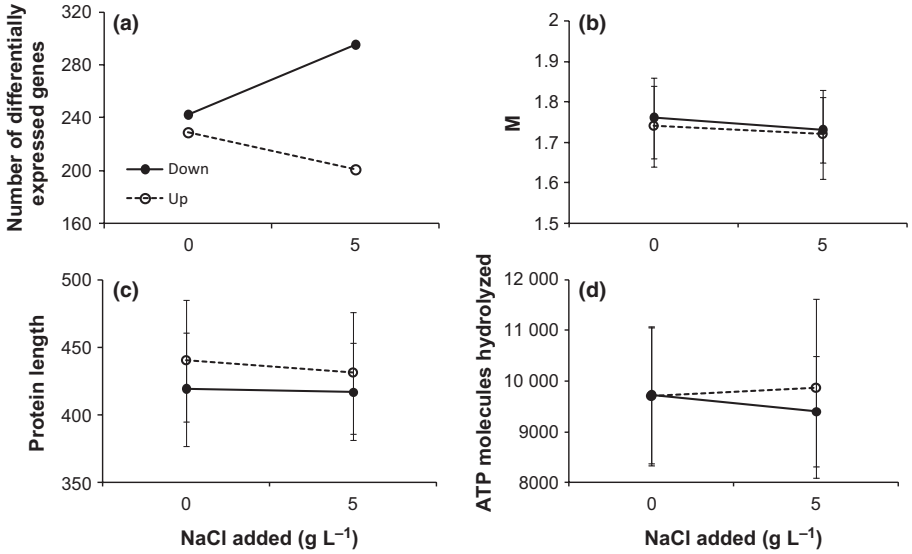

Figure 5 The energetic cost of generalisation. All values are changes in the generalist relative to the specialist. 'Up' indicates genes that are up-regulated in the generalist relative to the specialist, 'Down' refers to genes that are down-regulated in the generalist relative to the specialist. (a) The generalist down-regulates more genes at both levels of NaCl added. (b) The average *M*-values do not differ between the specialist and generalist at either level of NaCl added. (c) The average protein length does not differ between the genotypes. (d) The number of ATP molecules hydrolysed per gene does not differ between the genotypes. Error bars for panels (b), (c) and (d) are ± 2 SE.

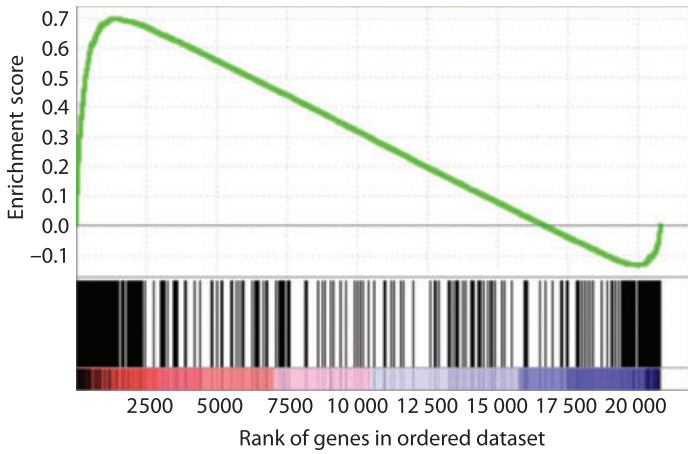

Figure 6 The environmental cost of generalisation. Gene enrichment analysis of the 564 *Daphnia pulex* gene set responding to the predator *Chaoborus* in relation to the ranked set of condition × genotype interacting genes based on *M*-values. The maximum enrichment score is reached at the 1483rd ranked gene. Fifty-four per cent (54%) of *Chaoborus* responsive genes are located within the leading edge of this enrichment score interval.

changes in gene expression that results in the inappropriate expression of genes in some environments.

Daphnia genes that underwent regulatory evolution in response to saline environments are characteristic of other investigations of the regulatory basis of salinity tolerance and gene expression. Organisms face two problems with increasing salinity: osmotic stress and ion cytotoxicity. Osmotic stress arises when the external environmental salt concentration is higher than the internal cellular salt concentration, resulting in the movement of water out of the cell. To combat the loss of water, organisms up-regulate genes involved in the biosynthesis of osmoprotectants, and intracellular osmoprotectant concentrations tend to accumulate (Zhu 2002). Osmoprotectants stabilise proteins and cellular structures to increase osmotic pressure (Yancey *et al.* 1982), and act as free radical scavengers that protect cells from oxidative stress (Smirnoff & Cumber 1989). Our metabolic pathway analysis supports the pattern that salt-tolerant organisms undergo regulatory evolution in pathways related to osmoprotectant biosynthesis (Figure S2; see Table S2 in Supporting Information). For example, various pathways for amino acid biosynthesis and metabolism, which are generally higher in salt-tolerant than salt-intolerant organisms (Ashraf & Tufail 1995), have undergone numerous constitutive changes in the generalist *Daphnia* genotype. These regulatory changes include production of the amino acid proline, which contributes to membrane stability (Mansour 1998), and accumulates in larger amounts than other amino acids in response to salt stress in other crustaceans (Burton & Feldman 1982).

The adverse effects of salt stress include ion cytotoxicity, which disrupts the hydrophobic and electrostatic forces that maintain protein structure and cause inhibition of enzyme function (Wyn Jones & Pollard 1983). One class of molecules that contributes to an adaptive response to salinity stress is ion transport proteins, particularly those involved in sodium and potassium transport. This class of proteins, which includes Na+/K+ ATPase, confers salt-tolerance when gene expression or protein activity is increased in a broad range of organisms including crustaceans (Ituarte *et al.* 2008). Our results confirm that modified sodium and potassium transport is a conserved evolutionary response to salt stress (see Table S3 in Supporting Information). We identified two copies of the Na+/K+ ATPase alpha subunit that were up-regulated in both genotypes in response to increasing salinity, with one copy undergoing regulatory evolution of accommodation. We also found a Na+/dicarboxylate cotransporter, Na+/K+ symporter, Na+/Pi cotransporter, and one Na+ channel that were constitutively down-regulated or underwent genetic assimilation in the generalist. Concomitant with decreases in the expression of the non-Na+/K+ ATPase transcripts were constitutive increases in the expression of two K+ channel proteins in the generalist. Genes involved in glycan biosynthesis, which facilitate the proper folding of newly synthesised proteins (Helenius &

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Aebi 2001), also showed altered regulatory patterns (Figure S2; Table S2).

Previous life history assessments of the genotypes utilised in this study suggest that the generalist used here does not incur a loss of fitness at low-salinity levels (Weider 1987). When these genotypes were assayed in low-salinity water, fitness traits, including clutch sizes and intrinsic rate of natural increase, were either equal or higher, respectively, in the generalist. Our estimates of survivorship corroborate these results and show that at low salinities, survivorship of the generalist is equal to or higher than survivorship in the specialist, whereas at high salinities the generalist enjoys a distinct fitness advantage over the specialist (Fig. 2a). Overall, these results suggest that, at least in the *Daphnia* system at Churchill (Weider & Hebert 1987), the evolution of salinity tolerance does not incur a loss of fitness along the environmental axis of salinity.

The patterns of gene expression evolution we observed initially suggest that the generalist, which shows predominantly canalised patterns of gene expression in response to varying salinity as opposed to plastic patterns of gene expression, may experience a cost of transcription in some environments. However, we find no evidence suggesting that the generalist experiences a higher cost of transcription. In fact, it is the specialist that displays a higher cost of transcription at both levels of NaCl tested. Considering that the generalist and specialist display equal survivorship at 0 g L^{-1} NaCl, the difference in ATP hydrolysis between the specialist and generalist in this environment appears insufficient to reduce fitness. In contrast, at 5 g L^{-1} NaCl the difference in ATP hydrolysis is 600% higher in the specialist than the difference at 0 g L^{-1}, and there is a 30% reduction in survivorship in the specialist relative to the generalist. Therefore, while we do not observe a cost of generalisation, our general hypothesis that differences in the cost of transcription may underlie differences in fitness appears supported based on the high cost of transcription the specialist displays when exposed to salinities outside its tolerance range.

The lack of fitness or transcriptional costs in the generalist would suggest that when these genotypes co-occur in low-salinity ponds in the Churchill region the frequency of the generalist should be approximately equal to the specialist. However, surveys of the distribution of the generalist and specialist over the last 25 years show a different trend; when the specialist and generalist co-occur, the specialist is most abundant (Weider *et al.* 2010).

One difference between these genotypes is their ecological context, not as it relates to salinity, but to the presence of invertebrate predators. The specialist generally inhabits ponds with invertebrate predators, whereas the generalist predominantly occupies predator-free ponds (Weider 1987). Therefore, another explanation for the limited distribution of the generalist is that the evolution of adaptive plasticity in response to salt inhibits the ability to mount an adaptive response to predators and the cost of generalisation involves a loss of fitness along a separate environmental axis. Specifically, these genotypes may differ in traits related to fitness in an ecological arena that contains invertebrate predators.

An environmental axes cost is supported by differences in morphological and life history traits between specialist and generalist genotypes. These genotypes differ in size at birth, with the generalist displaying a smaller body size at birth than the specialist (Weider 1987). Aquatic invertebrate predators preferentially select small-bodied individuals (Zaret 1980), thus, the generalist may experience higher juvenile mortality than the specialist in predator-rich environments. The assemblage of genotypes in the Churchill area differ in several other morphological traits that impact the susceptibility to invertebrate predation including tail-spine length (Wilson & Hebert 1993), and cuticle strength (Dodson 1984). Although the specific genotypes we employed here have not been tested for these traits, results from our microarray experiment in which we find 10 genes involved in chitin metabolism that have evolved differences in expression between the generalist and specialist support the hypothesis that these genotypes may differ for combinations of morphological traits that influence fitness only in the context of invertebrate predation (Table S3).

A second possibility is that the evolution of adaptive plasticity in response to salt resulted in the loss of adaptive phenotypic plasticity in response to predator kairomones. Freshwater *Daphnia* are notorious for their ability of adaptive phenotypic modification through phenotypic plasticity in response to chemical cues produced by invertebrate predators (Tollrian & Harvell 1999), and several recent studies have examined the transcriptional basis of these adaptive changes (Miyakawa *et al.* 2010; Colbourne *et al.* 2011). A comparison between the genes we identified here and genes identified in other studies implicated in the plastic response to invertebrate predator kairomones reveals that of the 546 genes we identified as having evolved differential regulation in response to salinity, 64 are also involved in the plastic response to invertebrate predators (Table S3). This number of genes corresponds to 11.3% of the total genes suspected to be involved in the kairomone response based on tiling path microarrays (Colbourne *et al.* 2011). Moreover, the gene set enrichment analysis suggests that 54% of the *Chaoborus* responsive genes are enriching the set of genes that are most different in the responses of the specialist and generalist genotypes to the salinity treatments.

If the evolved changes in these kairomone-responsive genes have a correlated effect of reducing the ability of a genotype to mount the appropriate plastic response to the presence of invertebrate predators, then the generalist genotype would be at a selective disadvantage. Recent evidence that *Daphnia* morphological defences induced by fish kairomones results in higher susceptibility to parasitism (Yin *et al.* 2011) suggests that the evolution of adaptive phenotypic plasticity in response to one environmental factor can have negative fitness consequences in the context of multiple environmental factors.

In this study, we have integrated functional genomics and phenotypic assays to ascertain the cost of salinity generalisation in *Daphnia*. Our results suggest that salt-generalists evolve via predominantly constitutive changes in gene expression, and also genetic assimilation (albeit to a lesser degree), which results in a lower overall energetic cost to the organism and no loss in fitness with respect to the salinity environmental gradient. Therefore, the cost of salinity generalisation does not lie along the salinity environmental gradient in the Churchill system, but may involve separate environmental gradients including the local predation regime. Understanding the cost of generalisation may require examination of the multivariate environmental axes that determine the realised niche of specialists and generalists.

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AUTHOR CONTRIBUTIONS

LL and MP conceived and designed the experiments; LL, LW, JC and MP performed research; LL, JC, and MP analysed data; and LL, LW, JC and MP contributed to the manuscript.

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