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# Contrasting gene expression programs correspond with predator-induced phenotypic plasticity within and across generations in *Daphnia*

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## Abstract

Research has shown that a change in environmental conditions can alter the expression of traits during development (i.e., "within-generation phenotypic plasticity") as well as induce heritable phenotypic responses that persist for multiple generations (i.e., "transgenerational plasticity", TGP). It has long been assumed that shifts in gene expression are tightly linked to observed trait responses at the phenotypic level. Yet, the manner in which organisms couple within- and TGP at the molecular level is unclear. Here we tested the influence of fish predator chemical cues on patterns of gene expression within- and across generations using a clone of *Daphnia ambigua* that is known to exhibit strong TGP but weak within-generation plasticity. *Daphnia* were reared in the presence of predator cues in generation 1, and shifts in gene expression were tracked across two additional asexual experimental generations that lacked exposure to predator cues. Initial exposure to predator cues in generation 1 was linked to ~50 responsive genes, but such shifts were 3–4× larger in later generations. Differentially expressed genes included those involved in reproduction, exoskeleton structure and digestion; major shifts in expression of genes encoding ribosomal proteins were also identified. Furthermore, shifts within the first-generation and transgenerational shifts in gene expression were largely distinct in terms of the genes that were differentially expressed. Such results argue that the gene expression programmes involved in within- vs. transgeneration plasticity are fundamentally different. Our study provides new key insights into the plasticity of gene expression and how it relates to phenotypic plasticity in nature.

KEYWORDS
ecoresponsive, epigenetics, transcriptomics, transgenerational plasticity, water fleas

## 1 | INTRODUCTION

It is now clear that organisms can respond to environmental signals by altering the expression of traits during development, as well as modifying traits across multiple generations (Bonduriansky, Crean, & Day, 2012; Fox & Mousseau, 1998; Jablonka & Raz, 2009; Uller, 2008). This "within-generation phenotypic plasticity" and "transgenerational plasticity" (TGP or across-generational plasticity) has been documented in a diverse array of taxa, including plants, bryozoans, rotifers, beetles and birds (Charmantier et al., 2008; Fox & Mousseau, 1998; Galloway & Etterson, 2007; Marshall, 2008; Schröder & Gilbert, 2004) in response to numerous environmental stimuli (e.g., temperatures; Salinas & Munch, 2012; food shortages; Bashey, 2006; canopy shading; Galloway & Etterson, 2007).

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Research has shown that both within-generation plasticity and TGP are often adaptive (Agrawal, Laforsch, & Tollrian, 1999; Bashey, 2006; Dyer et al., 2010; Galloway, 2005; Galloway & Etterson, 2007; Salinas & Munch, 2012; Walsh, Cooley, Biles, & Munch, 2015) and can evolve in response to divergent ecological conditions (Walsh et al., 2016). The long-standing assumption is that underlying shifts in gene expression manifest as patterns of plasticity at the phenotypic level. Examples of environmentally induced changes in gene expression within- and across generations are rapidly accumulating (Aubin-Horth & Renn, 2009; Carone et al., 2010; Herman, Spencer, Donohue, & Sultan, 2014; Herman & Sultan, 2011; Jablonka et al., 1995; Miyakawa et al., 2010; Molinier, Ries, Zipfel, & Hohn, 2006; Simons, 2011; Tollrian & Leese, 2010). Yet, the manner in which organisms couple within-generation plasticity in conjunction with TGP in response to a change in environmental conditions, especially at the molecular level, is unclear.

Recently developed theory predicts that divergent ecological conditions will select for divergent patterns of within- vs. TGP (Kuijper & Hoyle, 2015; Leimar & McNamara, 2015; Uller, English, & Pen, 2015a). Here, variation in a key environmental selective pressure (i.e., temporal variation in environmental stability) is predicted to select for increased within-generation plasticity *or* increased TGP, but not both. Empirical research has indeed shown that organisms harbour extensive variation in the direction and magnitude of within- and across-generation plasticity (Donohue & Schmitt, 1998; Schmitt, Niles, & Wulff, 1992; Walsh et al., 2015) and that environmentally induced within- and transgenerational responses can have synergistic (Galloway, 2009; Lin & Galloway, 2010; Sultan, Barton, & Wilczek, 2009) and antagonistic (Walsh et al., 2015) effects on the traits of organisms. It follows logically that contrasting gene expression programmes may be linked to divergent patterns of within- vs. TGP. Natural selection may alter the path from gene expression to phenotypic plasticity because selection for within- or across-generation plasticity acts either upon the same genes, but drives changes in the expression levels or in the direction of expression. Conversely, selection for within- vs. TGP may act upon different genes or on different numbers of genes. These two hypotheses lie at opposite ends of a continuous spectrum and are therefore not necessarily mutually exclusive.

Studies of clonal and ecoresponsive *Daphnia* sp. offer an opportunity to examine the manner in which natural selection modulates connections between gene expression and phenotype within and across generations. *Daphnia* are an ubiquitous feature of freshwater environments (Carpenter, Fisher, Grimm, & Kitchell, 1992), and they possess characteristics that make them ideal for experimental studies, including easy culturing, short generation times, parthenogenic reproduction and many readily quantifiable traits (Miner, De Meester, Pfrender, Lampert, & Hairston, 2012; Stollewerk, 2010). *Daphnia* species are well known to respond to changes in the environment by altering the expression of a multitude of traits (Riessen, 1999; Stibor, 1992). For example, exposure to predator chemical cues elicits dramatic shifts in morphology, behaviour and life history traits (Tollrian & Harvell, 1999). Research has used these known patterns of plasticity to begin to consider the underlying molecular mechanisms for predator-induced plasticity (Rozenberg et al., 2015; Schwarzenberger, Courts, & von Elert, 2009). For example, Schwarzenberger et al. (2009) evaluated patterns of plasticity in gene expression of several candidate genes in *Daphnia magna* that were exposed to chemical cues produced by fish and invertebrate predators. This approach revealed strong upregulation of cyclophylin, involved in protein folding in the presence of fish predator cues, while exposure to invertebrate predator cues was associated with a downregulation of cyclophylin. Given that *Daphnia* differ in their life history responses to fish vs. invertebrate predator cues (Riessen, 1999; Stibor, 1992), these contrasting gene expression responses could indicate that cyclophylin is linked to the expression of life history traits (Rozenberg et al., 2015; Schwarzenberger et al., 2009; Tollrian & Harvell, 1999). Such results provide a clear connection between phenotypic plasticity and gene expression. Still, the connections between within-generation responses and TGP at the molecular level remain largely unexplored.

Our previous work quantified patterns of within and TGP at the phenotypic level in multiple clones of *Daphnia ambigua* from lakes in Connecticut, USA. We found that *Daphnia* respond to initial exposure to predator cues by shifting life history to mature slower and produce less embryos compared to the transgenerational change (Walsh et al., 2015). We classify these life history responses that occur during development as "within-generation plasticity." We also found that *Daphnia* exposed to predator cues programmed future generations for faster development. Such transgenerational responses were apparent two generations following cue removal (Walsh et al., 2015). That is, life history differences between parental *Daphnia* that were and were not exposed to predator cues were still observed in the grand-offspring. These patterns of transgenerational life history plasticity are correlated with shifts in methylation (Schield et al., 2016). More importantly, phenotypic experiments have revealed extensive variation in the direction and magnitude of phenotypic responses to predator cues within and across generations. Such variation in these two forms of plasticity provides the raw material to test for variation in gene expression programmes within and across generations. While our previous study (Walsh et al., 2015) measured life history traits, the current study complements previous work through the addition of gene expression analyses, thus providing new insight into the gene regulatory basis of these responses to environmental cues.

Here, we tested the influence of predator chemical cues on the patterns of gene expression within and across generations in a single clone of *D. ambigua*. We reared *Daphnia* in the presence and absence of fish chemical cues in first-generation individuals and tracked shifts in gene expression across two additional asexual generations. Importantly, the clone of *Daphnia* used in these experiments responds to predator cues by strongly programming future generations for rapid development (i.e., strong TGP) but exhibits weak within-generation plasticity (Walsh et al., 2015). These phenotypic data stem from our previous work (Walsh et al., 2015) and thus set the foundation for comparisons with gene expression-based

responses over multiple generations. Given these known divergent phenotypic responses to predator cues within and across generations for this clone, we predicted that the number genes that are differentially expressed across generations would exceed those that are differentially expressed within the first generation. Comparisons between patterns of predator-induced within- and TGP in gene expression responses will allow us to determine whether *Daphnia* couple within- and TGP by altering the expression of the same sets of genes, or whether these two forms of plasticity correspond with expression of distinct sets of genes.

## 2 | MATERIALS AND METHODS

### 2.1 | Empirical experimental design

We used a single clone of *D. ambigua* from Dodge Pond in Connecticut, USA (Post, Palkovacs, Schielke, & Dodson, 2008). In June 2013, we isolated ephippia from a sediment sample that was originally collected via an Ekman grab in 2009. Upon hatching, cultures from this clone were maintained in 250-ml glass jars for several months prior to the start of the experiments. During this time, *Daphnia* cultures were maintained at moderate densities (<60 adults/L) and provided with fresh media and algae weekly. It is important to note that this clone of *Daphnia* reproduces asexually under benign conditions and reverts to sexual reproduction when stressed. However, all *Daphnia* were propagated asexually in the experiments described below. *Daphnia* rearing experiments, and all molecular laboratory experiments, were conducted at the same times and in parallel where possible to minimize experimental variation.

Our experimental approach consisted of rearing the focal clone of *Daphnia* in a common-garden setting for two generations (Fig. S1), followed by three generations of experimental manipulation (Figure 1). To initiate the multiple generations of common-garden rearing, we isolated 30 adults from existing stock cultures and placed each adult in separate 90-ml containers containing COMBO media (Kilham, Kreeger, Lynn, Goulden, & Herrera, 1998) and specified, nonlimiting quantities of green algae (*Scendesmus obliquus;* concentration 0.8 mg $\times$ C L$^{-1}$ $\times$ day$^{-1}$). For each isolated adult, a single neonate was immediately pulled from the first asexual clutch and these neonates were moved to new 90-ml containers containing the same media and algae; these individuals represent the first common-garden generation. All individuals were transferred to fresh media and algae every day and were reared at 21°C and a 14-hour:10-hour light:dark schedule. To initiate the second common-garden generation, we collected neonates from the second clutch of each replicate jar and these offspring were again transferred to fresh media and algae daily (see diagrammed design in Fig. S1).

Our experiment began with third-generation laboratory born individuals. On day 1 of the experiment, we collected all neonates that were born over the previous 12 hr from each of the parental jars. This yielded ~180 newly-born *Daphnia* from the third clutch or later of the second-generation laboratory reared parents and all

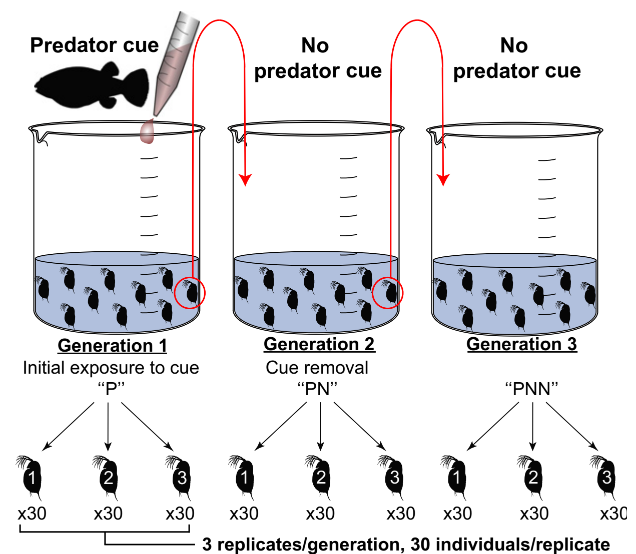


**FIGURE 1** Experimental design. A clonal generation representing the third common-garden generation of *Daphnia ambigua* was exposed to predator cues (Generation 1; "P" treatment). A single neonate from the second clutch was transferred into a new jar (Generation 2). A neonate was then collected from Generation 2 at 10 days after initiation and placed in a new jar (Generation 3). Generations 2 and 3 were not exposed to any additional predator cues ("N" treatments), so any differentially expressed genes in these generations are a product of transgenerational plasticity stemming from the initial predator cues in Generation 1. RNAseq libraries were prepared from three replicates per generation, with 30 individual *Daphnia* composing each replicate. In addition to the predator cue removal experiment shown above (P, PN, PNN generations, respectively), a second control experiment was conducted in an identical manner only differing in the absence of any predator exposure (N, NN, NNN generations, respectively) [Colour figure can be viewed at wileyonlinelibrary.com]

neonates were placed into 250-ml jars containing COMBO media at a density of 40–48 *Daphnia*/L, or 18 jars with 10 *Daphnia* per jar (Kilham et al., 1998). Each jar was randomly allocated to one of two treatments: (1) predator exposure during the first generation followed by two generations in the absence of predator cues (i.e., generation 1 = P, generation 2 = PN, generation 3 = PNN; Figure 1), or (2) three generations in the absence of predator cues (i.e., generation 1 = N, generation 2 = NN, generation 3 = NNN). All *Daphnia* were transferred to fresh media, algae and kairomones (see below details of kairomone collection) daily. We monitored jars daily for maturation (i.e., release of first clutch into the brood chamber) and for the production of new clutches. Based upon previous work (Walsh & Post, 2011, 2012), we estimated that 10 days were needed for *Daphnia* to release their second clutch. We thus initiated the second experimental generation after 10 days of exposure to predator cues. This experimental generation was again initiated by collecting newborn individuals under the same conditions described above. The third experimental generation was collected and reared in this same manner. After collecting neonates to initiate the second and third experimental generations, all adults were flash-frozen (in liquid nitrogen) for the subsequent RNAseq analyses. *Daphnia* from the third

experimental generation were frozen following 10 days of common-garden rearing.

## 2.2 | Kairomone collection

COMBO medium conditioned by the presence of planktivorous fish was collected daily from a tank containing 2 redbreast sunfish (*Lepomis auritus;* ~3 cm in total length) in 130 L of water. Each day, media containing fish chemical cues was filtered using membrane filters (47 mm diameter, 0.45 μm mesh) and added at a concentration of 0.0025 fish/L to the predator treatments. Injured *Daphnia* emit chemical cues that contribute to the magnitude of phenotypic response to predation (Laforsch, Beccara, & Tollrian, 2006). We thus added filtered, macerated *Daphnia* (100 *Daphnia*/L) every day to the appropriate predator treatments to ensure that our predator treatments contained both fish kairomones and *Daphnia* alarm cues.

## 2.3 | RNA isolation, library preparation and sequencing

We extracted RNA using the Zymo Research Duet Kit from snap-frozen samples. Each generation included three biological replicates, with each replicate comprised of 30 clonal *Daphnia* individuals (Figure 1). Appropriate amounts of RNA were not available from single individuals. We pulled 30 individuals per replicate for the purposes of library construction and sequencing, as all individuals have identical genetic backgrounds. A similar pooling approach has also been used in other studies of *Daphnia* differential gene expression (e.g., Roy Chowdhury et al., 2015; Soetaert et al., 2007). Isolated RNA was quantified using a Qubit fluorometer (Invitrogen), and mRNA-seq libraries were constructed using Illumina TruSeq library kits. A total of 3 μg of total RNA from each replicate (representing a pool of 30 individuals) was used for RNAseq library preparation. Each of these samples was uniquely indexed, and all 18 individual libraries were multiplexed into a single, pooled library and sequenced on a single Illumina MiSeq run using 150-bp paired-end sequencing reads.

## 2.4 | Assessing differential gene expression

Raw Illumina RNAseq reads were demultiplexed by index and quality trimmed using TRIMMOMATIC v. 0.36 (Bolger, Lohse, & Usadel, 2014) with default settings. We used the BWA MEM algorithm v. 0.7.13 (Li & Durbin, 2009), with default settings, to map quality trimmed reads to the complete annotated transcript set of *Daphnia pulex* (Colbourne et al., 2011) obtained from ENSEMBL. On average, about 70% of trimmed reads mapped to the reference genome transcript set. Raw gene expression counts were estimated by counting the number of reads that mapped uniquely to a particular annotated transcript using SAMTOOLS v. 1.3.1 (Li et al., 2009). Raw expression counts were then normalized using the TMM normalization method in EDGER (Robinson, McCarthy, & Smyth, 2010; Robinson & Oshlack, 2010), and all subsequent gene expression analyses used these normalized data. Using

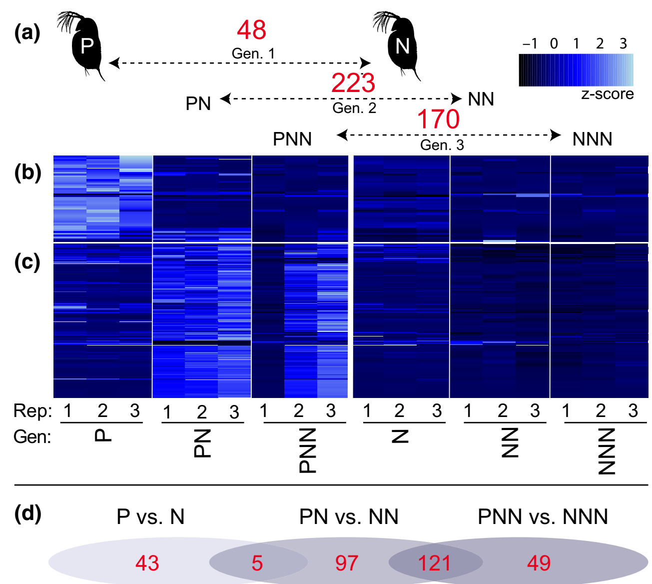


**FIGURE 2** Analysis of gene expression (RNAseq) changes in response to predator cues. (A) Numbers of significantly differentially expressed genes between the experimental and control replicates in each generation. (B) Patterns of gene expression for 48 genes differentially expressed between first-generation treatments (P vs. N). (C) Patterns of expression for 223 genes that differ significantly in expression between second-generation treatments (PN vs. NN). For B–C, lighter blue colours indicate high level of expression, while darker blue indicates low expression (this gradient is based on normalized count values); gene expression profiles are clustered by similarity. (D) Significantly differentially expressed genes that overlap between generations. There was no gene overlap between generation 1 (P vs. N) and generation 3 (PNN vs. NNN) [Colour figure can be viewed at wileyonlinelibrary.com]

these normalized data, we identified genes that were significantly differentially expressed between treatments by conducting pairwise tests between replicated time point samples using an exact test of the binomial distribution estimated in EDGER (Robinson et al., 2010), integrating both common and tagwise dispersion. To control for any responses that may be attributed to the experimental design, we only considered expression differences between experimental and control treatments within each generation (i.e., P vs. N, PN vs. NN and PNN vs. NNN). All genes with evidence of differential expression at an FDR value ≤0.05 were considered significantly differentially expressed between treatments. Significantly differentially expressed genes were visualized across all samples as heat maps that were generated in R (R Development Core Team, 2008) with genes clustered by expression pattern similarity using the R-package VEGAN (Dixon, 2003); gene expression pattern clustering was calculated using average linkage hierarchical clustering based on Bray–Curtis dissimilarity matrix (Figures 2b,c, 5 and 6). We also used principle component analysis (PCA; using core functions in R) to identify the degree to which patterns of RNAseq variation could differentiate between generations and individuals by comparing the same normalized gene expression data for all samples (using a singular value decomposition of expression matrix). Significantly differentially

expressed genes that overlap between generations were visualized in a Venn diagram (Figure 2d). To test whether the overlap of gene sets from different generations was more than expected by chance, we conducted a hypergeometric test using the STATS package in R.

### 2.5 | Analyses of trends in expression shifts and biological interpretations

Significantly differential genes were annotated using BLAST2GO v3.3.4 (Conesa & Götz, 2008; Conesa et al., 2005; Götz et al., 2011) and ENSEMBL BIOMART (Kinsella et al., 2011). From the BLAST2GO annotation outputs, we grouped genes that were functionally similar and associated with traits including digestive function, reproductive function, epigenetic modifications and proteolysis. Sequence IDs were then converted to DAPPUDRAFT IDs using the *D. pulex* gene annotation list from ENSEMBL; these IDs were then used to assign Gene Ontology (GO) term identifiers. We performed GO enrichment analyses (Mi et al., 2016) to determine if significantly differentially expressed gene sets were enriched for particular functional categories of genes (Ashburner et al., 2000). Because our annotations were based on genes orthologous to *D. pulex*, we minimized bias in the GO enrichment analysis by including a background of only the genes we observed as expressed in any of our *D. ambigua* experiments. We considered GO term categories as significantly enriched if the ratio test resulted in a Bonferroni-corrected $p$-value $\leq$.05. Enriched GO terms were summarized by removing redundancies using REVIGO (Supek, Bošnjak, Škunca, & Šmuc, 2011) with allowed similarity of terms set to 0.1.

## 3 | RESULTS

### 3.1 | Gene expression analyses

An average of 285,576 reads was mapped for each replicate. The numbers of raw reads obtained per library together with read mapping statistics are provided in Table S1 in the Supporting Information. Initial exposure to predator cues was associated with 48 significantly differentially expressed genes between experimental and control treatments in generation 1 (P vs. N; FDR $\leq$ 0.05). Following predator cue removal, we observed 223 differentially expressed genes in generation 2 (PN vs. NN), and 170 differentially expressed genes in generation 3 (PNN vs. NNN; Figure 2a). Sets of responsive genes in each generation were mostly distinct; the data for generation 1 shared only five responsive genes that were differentially expressed with the patterns observed in generation 2, and zero genes with generation 3 (Figure 2d). In contrast, of 223 responsive genes in generation 2, 121 (54%) were also differentially expressed in generation 3 (PNN vs. NNN; Figure 2d). Hypergeometric tests on the overlap revealed that the overlap between generations 1 and 2, as well as the overlap between generations 2 and 3, were significant ($p$ values of $1.77 \times 10^{-236}$ and $2.06 \times 10^{-05}$, respectively). While the results of the hypergeometic test indicated that proportions of overlapping genes were greater than expected at random, it is notable that the

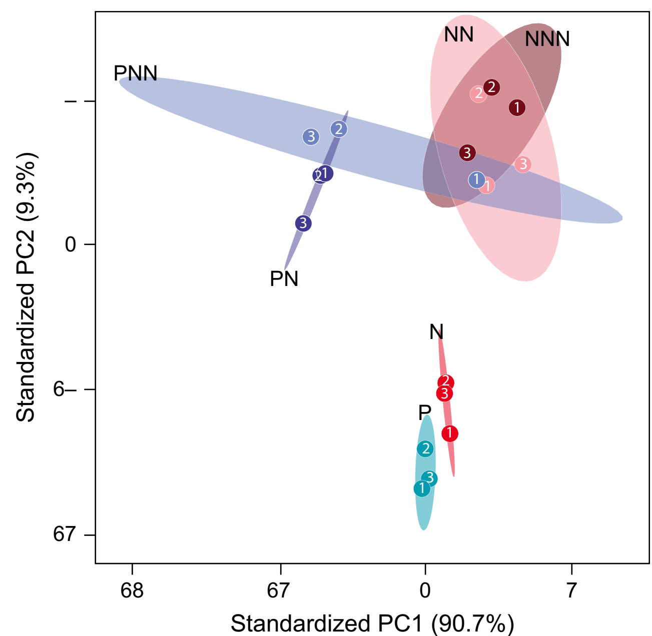


**FIGURE 3** Principle component analysis analysis of gene expression profiles for 218 genes that differ in expression between second-generation treatments (PN vs. NN). Normal data ellipses were drawn for each group using 0.98 as the size of the ellipse in Normal probability. Red-shaded ellipses represent control groups (N, NN and NNN), while blue-shaded ellipses represent the experimental group (P, PN and PNN). Numbers (1, 2 and 3) within groups represent the replicates associated with Figure 2 [Colour figure can be viewed at wileyonlinelibrary.com]

vast majority of differentially expressed genes were distinct, considering the hypothesis that they may indeed be entirely the same set.

We conducted a PCA to further explore patterns of gene expression within and across generations (Figure 3). The first principal component (PC1) explained 90.7% of the variance and clearly separated the control and experimental treatments in generation 2 and 3 (Figure 3). PC1 therefore accounts for transgenerational shifts in gene expression related to predator cue exposure. PC2 explained an order of magnitude less variation (9.3%) and primarily separated generation 1 (P & N) from generations 2 (PN & NN) and 3 (PNN & NNN); these results suggest some of the shifts in gene expression between generation 1 vs. generations 2 and 3 were similar in both the control and experimental treatments (Figure 3). However, it is important to note that none of these differences in gene expression across generations within control samples (e.g., N, NN, NNN) were statistically significant based on pairwise analyses of gene expression, and our analyses of gene expression in experimental samples take into account any such shift through comparisons with these negative controls.

Gene expression patterns tended to be consistent across all three biological replicates per generation, with the exception of a single replicate sample from the third experimental generation (PNN, replicate 1; Figure 2c). Gene expression patterns in this particular sample were more similar to those in the negative control (Figure 2c). Our PCA further confirmed this sample as having a unique replicate-specific transgenerational response compared to the other

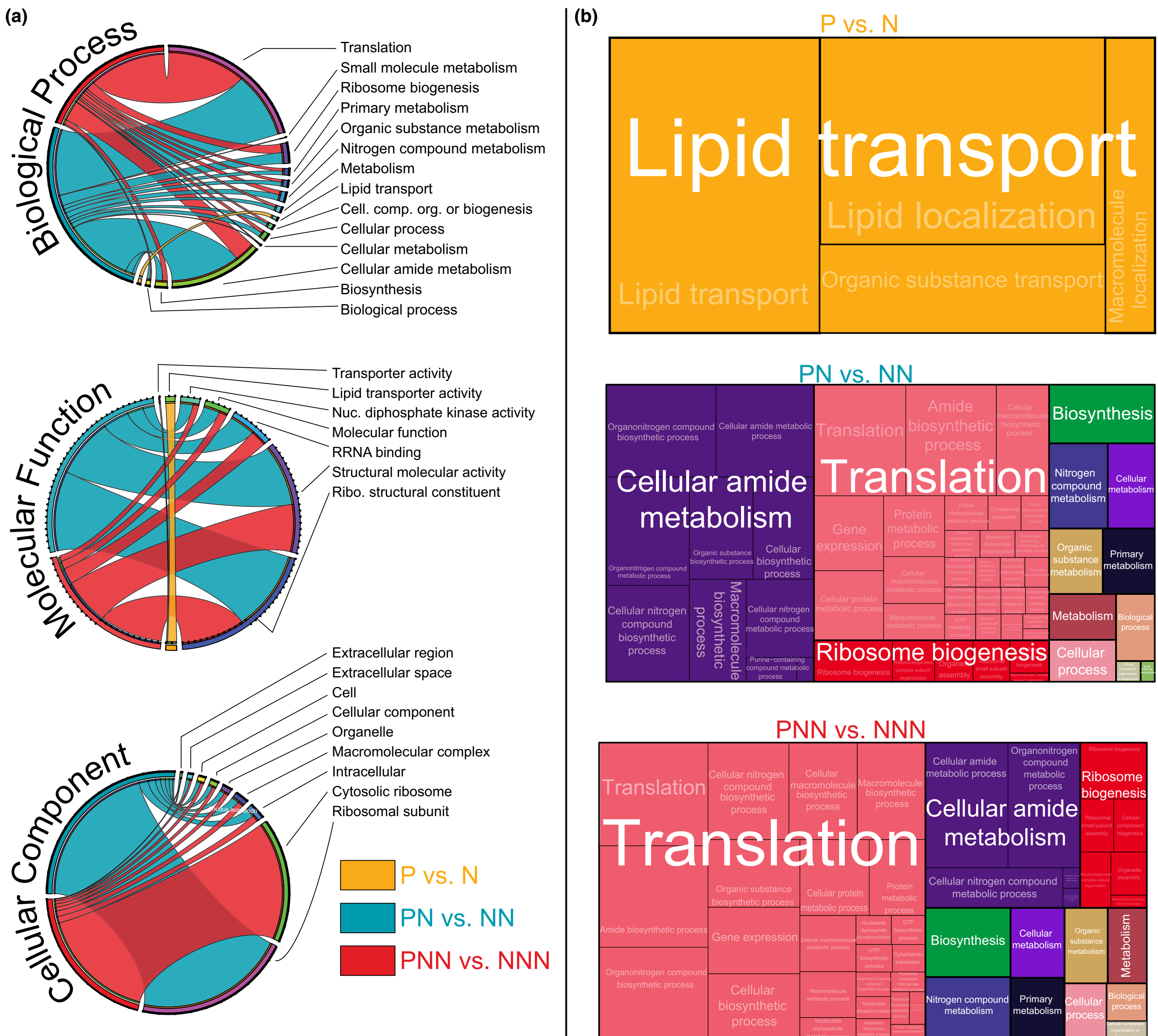


**FIGURE 4** Summarized enrichment of GO biological terms. (A) CIRCOS plots of significant GO terms for biological processes, molecular functions and cellular components (top to bottom, respectively). Yellow, blue and red ribbons represent enrichment of significantly differentially expressed genes between generations (P vs. N, PN vs. NN and PNN vs. NNN, respectively). Coloured peripheral rings represent GO terms; the larger the ribbon connecting to a peripheral ring (or GO term), the more genes described by that GO term. GO terms for Cellular component did not have any enriches terms for the first generations (P vs. N; yellow ribbon). (B) GO terms for biological process represented in a tree map provided by REVIGO. Large white letters denote the representative GO term for all the more descriptive terms (smaller, grey text) within each colour block [Colour figure can be viewed at wileyonlinelibrary.com]

two PNN treatment replicates, as this sample clustered with control samples (with NN and NNN replicates).

## 3.2 | Gene function

To dissect the biological relevance of transgenerational shifts in gene expression, we grouped responsive sets of genes into functional categories to identify how gene expression shifts might be related to transgenerational phenotypic shifts, and how within- vs. transgenerational transcriptional responses differ. Comparisons of GO terms for differentially expressed gene sets per generation highlight the uniqueness of within-generation responsive gene functions (P vs. N response), and the broad similarities of functional categories of responsive genes in generations 2 and 3 (PN vs. NN and PNN vs. NNN; Figure 4). Within-generation responsive genes were associated with few enriched GO terms, all of which were related to lipid transport and lipid transporter activity (Figure 4a,b); none of these functional classes were shared with across-generation responsive genes.

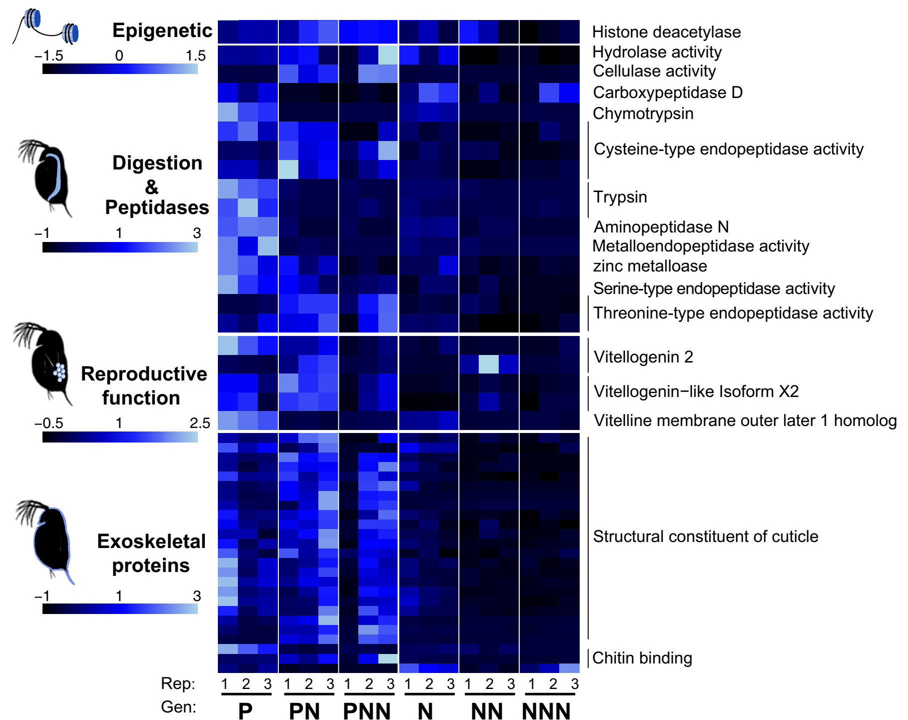


**FIGURE 5** Patterns of responsive genes involved in digestion, proteolysis, reproductive and exoskeletal function. Genes shown in heat map were found to be significantly differentially expressed across at least one pairwise time point comparison (FDR ≤ 0.05). The respective *z*-score gradient keys are provided for each heat map [Colour figure can be viewed at wileyonlinelibrary.com]

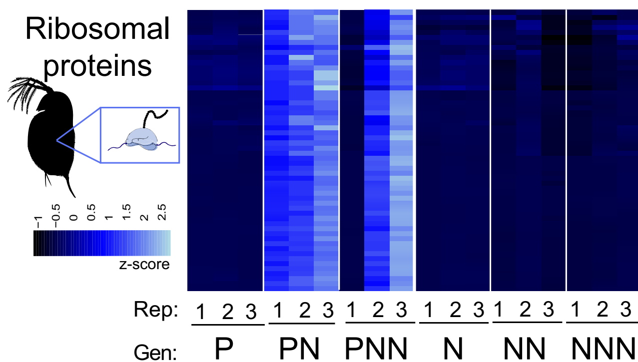


**FIGURE 6** Heat map showing patterns of gene expression in ribosomal-related proteins. Genes shown were significantly differentially expressed across at least one pairwise time point comparison (FDR ≤ 0.05) [Colour figure can be viewed at wileyonlinelibrary.com]

In contrast to limited responses in the first generation, responses in generations 2 and 3 show major shifts in the functional categories of genes responsive to predator cues, including the up-regulation of genes involved in cellular amide metabolism, translation, ribosome structure, ribosome biogenesis, biosynthesis and cellular metabolism (Figure 4a,b). GO terms enriched in transgenerational responsive gene sets were highly similar and shared many biological process and molecular function terms. While broadly overlapping, generations 2 and 3 differed in the greater response of genes related to cellular amide metabolism in generation 2, and the greater relative abundance of responsive genes related to translation and ribosomes in generation 3 (Figure 4). The only nonoverlap identified between generations 2 and 3 was in cellular component GO terms, indicating that cytosolic ribosomes are enriched in generation 2, while ribosomal subunits are enriched in generation 3 (Figure 4a—Cellular Component panel); this difference, however, may have been driven by related GO terms being derived from many of the same up-regulated genes. For these seemingly different categories, original GO enrichment terms were identical but with differing *p*-values (Tables S2 and S3), and the difference in results is due largely to the differential summarization of terms by REVIGO, which employed permissive thresholds for similarity (c = 0.10) for visualization purposes (Tables S4 and S5). Thus, functional classes of responsive genes in generations 2 and 3 are, in fact, highly overlapping.

To complement our GO analyses and further dissect the links between gene expression and phenotypes associated with TGP, we broke down sets of responsive genes into functional categories linked to key phenotypic or molecular aspects of plasticity, including: epigenetic modification, reproduction, exoskeleton structure, digestion, and ribosomal protein synthesis (Figures 5 and 6). Among genes relevant to epigenetic modifications, we identified a single responsive gene encoding a histone deacetylase (HDAC; a transcriptional silencer; Braunstein, Rose, Holmes, Allis, & Broach, 1993) that was variably expressed across treatments, and only significantly differentially expressed between experimental and control treatments in generation 3 (PNN vs. NNN; Figure 5); we provide plausible explanations for this observation in the discussion.

Genes encoding peptidases and other digestive enzymes exhibited a split pattern, with some genes in this class being responsive upon initial cue exposure (P vs. N), and others showing transgenerational responses. Genes that were significantly responsive exclusively between P and N were peptidases with serine-type endopeptidase activity (i.e., chymotrypsin and trypsin) or metalloendopeptidase activity (i.e., zinc

metalloase; Figure 5 and Fig. S2). Conversely, genes exclusively responsive in generations two and three (PN vs. NN and PNN vs. NNN) were carboxypeptidase D, peptidases functioning in cysteine-type endopeptidase activity (i.e., cathepsin and caspase) and digestive enzymes functioning in hydrolase and cellulase activity (i.e., Cel7A fusion and lysosomal alpha-glucosidase-like, respectively; Figure 5 and Fig. S1).

Up-regulation of genes involved in reproductive function (i.e., vitellogenins; VTG) was primarily associated with the within-generation response. Of the 48 responsive genes in generation 1 (Figure 2b), five encoded proteins associated with VTG were associated with within-generational responses (including VTG-like isoforms X2, VTG2, and vitelline membrane outer layer 1 homolog), and only a single gene annotated as "VTG-partial" was responsive in the third-generation treatment. GO enrichment analysis showed overrepresented genes involved in biological process terms for lipid transport and molecular function for lipid transporter activity (Figure 4), which is consistent with our finding of up-regulation of VTGs in generation 1. Genes encoding exoskeletal proteins were more responsive across generations. Multiple exoskeleton-associated genes, including genes involved in the structural constituent of the cuticle, were exclusively differentially expressed between experimental and control treatments in generations 2 and 3 (PN vs. NN and PNN vs. NNN), while only a single gene (peritrophic matrix) involved in chitin binding was significantly responsive in the first generation (P vs. N; Figures 5 and Fig. S1).

Among all responsive gene sets, the most pronounced example of a transcriptional programme of functionally related genes exclusively linked to TGP was that of ribosomal protein-encoding genes; these genes were up-regulated in response to predator cues in generations 2 and 3 (Figure 6). Of the 223 significantly differentially expressed genes in generation 2 (PN vs. NN; Figure 2c), 52 (23%) were annotated as ribosomal protein components. Similarly, 40 (23%) of 170 genes in the third generation were also annotated as ribosomal protein components (Figure 6).

## 4 | DISCUSSION

Our results provide compelling evidence that within- vs. across-generation responses may be driven by distinct gene expression programmes, indicating these programmes are likely regulated independently. Interest in the evolutionary drivers of TGP has developed slowly and largely in parallel of the study of within-generation plasticity. Initially, theory predicted that similar ecological conditions favour the evolution of plastic responses that occur within- and across generations (Day & Bonduriansky, 2011; Ezard, Prizak, & Hoyle, 2014; Fischer, Taborsky, & Kokko, 2011; Hoyle & Ezard, 2012; Jablonka, Lachmann, & Lamb, 1989, 1992; Kuijper, Johnstone, & Townley, 2014; Levins, 1968; Shea, Pen, & Uller, 2011). It is also hypothesized that varying environmental conditions that are consistent between parent and offspring generations are expected to favour simultaneous increases in phenotypic plasticity within and across generations (Ezard et al., 2014; Hoyle & Ezard, 2012), but this idea has recently been challenged. It is now becoming clear that organisms exhibit strong patterns of within-generation plasticity or across-generation plasticity but not both (Donohue & Schmitt, 1998; Schmitt et al., 1992; Walsh et al., 2015). Additionally, new theory has identified ecological conditions that may independently select for within- vs. TGP (Kuijper & Hoyle, 2015; Leimar & McNamara, 2015; Uller, English, & Pen, 2015b). These frameworks predict that high temporal variability selects for the evolution of within-generation plasticity, while low temporal variability (or high temporal stability) and slow rate of environmental change favours enhanced TGP. The decoupling of within- and across-generation responses, in turn, predicts that divergent ecological conditions favour divergent patterns of plasticity, and even divergent molecular mechanisms underlying plasticity.

Based upon recent theory and empirical work (Walsh et al., 2015, 2016) illustrating a decoupling and even antagonism of within- vs. across-generational phenotypic responses, it follows logically that within and across-generation phenotypic responses involve divergent programmes of gene expression and even fundamentally different sets of responsive genes with distinct functions. Additionally, transcriptional responses (e.g., the number of responsive genes) are expected to be generally proportional to phenotypic responses within- vs. across generations. For example, lineages that exhibit strong patterns of TGP are expected to show enhanced transcriptional responses across generations. To test these predictions, we examined transcriptional responses to predator cues using a clone of *Daphnia* that is known to exhibit strong TGP (Walsh et al., 2015, 2016). We expected to observe more extensive shifts in gene expression across generations (vs. within), especially for genes involved in phenotypically responsive life history traits (i.e., programming offspring for faster rates of development and production of larger clutch sizes; Walsh et al., 2015).

Results of our gene expression analyses indicate that highly distinct gene expression programmes may underlie within- vs. across-generation responses, and that the magnitude of transcriptional responses appears to be linked to the magnitude of phenotypic responses. In this particular *Daphnia* lineage known to exhibit strong TGP responses (Walsh et al., 2015), we found small within-generation transcriptional response upon initial exposure to predator cues (P vs. N) followed by a pronounced transgenerational transcriptional response across subsequent generations (i.e., PN vs. NN and PNN vs. NNN). Many sets of responsive genes were also linked to known phenotypic responses that have been shown to coincide with exposure to predator cues, including developmental rates, reproductive rates and shifts in growth (Riessen, 1999; Stibor, 1992; Walsh et al., 2015; Figure 5). An "informational" perspective might explain why organisms exhibiting strong TGP maintain adaptive responses to predator cues, even when the predator risk has ceased (Dall, Giraldeau, Olsson, McNamara, & Stephens, 2005). Assuming the evolution of mechanisms that allow different sources of information to be weighted differently, selection should favour some sources of information more than others (Dall et al., 2005). The overall pattern suggests that this *Daphnia* clone appears to respond more to the environment experienced by their mother/grandmother rather than

their own environment, which is logical if direct predation risk is likely to be experienced infrequently (Dall et al., 2005).

Finally, multiple aspects of our results indicate a pattern of "decay" in transgenerational programming in later generations (Figures 2c and 3). That is, the number of differentially expressed genes decreased between generations 2 and 3. Such a trend is consistent with the decay in inherited epigenetic programming of subsequent generations, supporting the view that TGP is driven by epigenetic mechanisms (Kuijper & Hoyle, 2015; Leimar & McNamara, 2015; Uller et al., 2015a).

## 4.1 | Phenotypic responses within- vs. across generations

### 4.1.1 | Within-generation patterns of gene expression

Our data indicate that predator cues lead to consistent within-generation up-regulation of genes related to digestive function, including genes encoding the enzymes trypsin and chymotrypsin (serine-type endopeptidases) as well as genes associated with serine-type endopeptidase activity, metaloendopeptidase activity, and threonine-type endopeptidase activity. In addition to genes associated with peptidase activity, within-generation responses were also observed for genes that represent major precursor proteins involved in the production of egg yolk and embryo development (VTGs). Trypsin and chymotrypsin are known to represent major digestive proteases in the gut of *D. magna* (von Elert et al., 2004). *D. pulex* have been shown to respond to metabolic shifts due to colder temperatures by down-regulating trypsins, chymotrypsins and carboxypeptidases, and up-regulating VTG (Schwerin et al., 2009). We observed up-regulation of these enzymes, which may correspond with the need to accommodate increased feeding rates to achieve increases rates of growth and development and larger reproductive investment in response to exposure to predator chemical cues (Riessen, 1999; Stibor, 1992).

### 4.1.2 | Transgenerational patterns of gene expression

Distinct changes in gene expression persisted for two generations following predator cue removal. Transgenerational responses included 223 significantly differentially expressed genes in the second generation and 170 in the third generation (Figure 2a,c), with an overlap of 121 responsive genes between these generations. These transgenerational responsive genes outnumbered those that were differentially expressed in generation 1 (i.e., within-generation responses) by twofold to fourfold. Hypergeometric tests on these areas of overlap (Figure 2d) were performed to determine whether the overlap in gene sets was more than expected by chance. In both cases, the overlap between generations 1 and 2 and the overlap between generations 2 and 3 were significant with *p*-values $<.05$. Despite this overlap, the degree to which within- and transgenerational responses were largely distinct, in terms of the genes that were differentially expressed, is notable. These contrasting gene expression responses within- and across generations are consistent with new theory regarding the decoupling of these two forms of plasticity (Kuijper & Hoyle, 2015; Leimar & McNamara, 2015; Uller et al., 2015b). Our previous phenotypic work showed that parents respond to initial exposure to predator cues by programming offspring for earlier maturation and the production of larger clutch sizes (Walsh et al., 2015). Although within-generation responses focused on up-regulating a small set of genes related to reproductive efforts (Figures 4b and 5), across-generation responses included many genes linked to components of the exoskeleton, ribosomal proteins, carboxypeptidase D and other peptidases functioning in cysteine-type endopeptidase activity, hydrolase activity and cellulose activity (Figure 5). Chitin metabolism has been extensively studied in insects and in order for development to occur, cuticles forming the exoskeleton need to be continuously replaced during ecdysis. The ability for an arthropod to undergo morphogenesis is completely dependent on the constant destruction and reconstruction of chitin-containing structures (Merzendorfer & Zimoch, 2003). Therefore, increasing the transcription of proteins involved in the cuticle in *Daphnia* is also likely indicative of more frequent moulting.

Perhaps the most remarkable transcriptional evidence for a TGP-specific gene expression programme is the observed up-regulation of 62 responsive genes encoding ribosomal proteins associated with 60S and 40S ribosomal subunits (Figure 6). Despite a sensible explanation for this observation as being linked to an increase in translation, previous studies have shown increased transcription of ribosomal proteins without increased production of ribosomes (Sun, Li, & Wang, 2015; Wang et al., 2013). Proteomic data gathered on *D. magna* in response to predator cues show similar, but less extreme responses in ribosomal protein up-regulation (Otte, Schrank, Frohlich, Arnold, & Laforsch, 2015). Furthermore, it is known that ribosomal proteins have functions outside of ribosome assembly and translation in response to stress (i.e., oncoprotein suppression, immune signalling and development; Zhou, Liao, Liao, Liao, & Lu, 2015). Although the functional significance of up-regulation of the ribosomal protein-coding genes observed in the current study unclear, it is notable that this class of responsive genes was tightly linked to TGP, and a greater understanding of this response may provide unique insight into TGP response programmes.

### 4.1.3 | Stability of transmission and epigenetic decay

Our understanding of the mechanistic basis of plasticity, especially TGP, has been historically limited. A major difficulty is that several nonexclusive mechanisms may underlie patterns of TGP (e.g., maternal effects, histone modification, RNA interference, DNA methylation; Bossdorf, Richards, & Pigliucci, 2008; Jaenisch & Bird, 2003; Vandegehuchte & Janssen, 2011). Environmentally induced epigenetic shifts in DNA methylation can influence gene expression

patterns (Kalisz & Purugganan, 2004; Turck & Coupland, 2014) including TGP in gene expression (Boyko et al., 2010; Carone et al., 2010; Kooke et al., 2015), and variation in patterns of DNA methylation among natural populations has been correlated with shifts in trait values and trait plasticity (Herrera & Bazaga, 2010; Herrera, Pozo, & Bazaga, 2012; Kooke et al., 2015; Zhang, Fischer, Colot, & Bossdorf, 2013). Evolutionary theory connects environmental variation with the expression of TGP by predicting that evolutionary divergence in TGP may be linked to differences in the patterns and duration of environmentally induced epigenetic effects (i.e., differences in rate of "epigenetic resetting," (Kuijper & Hoyle, 2015; Leimar & McNamara, 2015; Uller et al., 2015a). Additionally, our previous study of genomewide methylation using the same clone of *D. ambigua* used in the present study found evidence for significant transgenerational shifts in genomic methylation patterns (Schield et al., 2016). Collectively, available evidence supports DNA methylation as an important mechanism underlying both the transmission and evolution of TGP.

Motivated by existing links between epigenomic modification and TGP, we searched for evidence of predator cue responsive genes related to epigenetic modification in generations 2 and 3. We found distinguishable differences in HDAC mRNA expression levels across treatments; HDAC is expressed consistently higher in the "predator removal" treatments compared to controls (Figure 5) and expression of HDAC differ significantly in generation 3 (PNN vs. NNN). These transcriptional silencers (Braunstein et al., 1993) are involved in the epigenetic modifications of histones required to condense chromatin. We were somewhat surprised by this result, as we did not expect this gene to be responsive only in later generations (i.e., generation 3) because we have previously shown major epigenomic modifications resulting in shifts in genomic cytosine methylation patterns in generations 1–2 upon predator cue exposure (Schield et al., 2016). It is also notable that we observed no significant changes in gene expression for DNA methyltransferases across generations and treatments, despite evidence for shifts in methylation in response to predator cue exposure (Schield et al., 2016). We believe the most likely explanation for these findings is that HDACs and DNA methyltransferases do not necessarily require shifts in transcription to undergo epigenetic shifts (Law & Jacobsen, 2010; Vandegehuchte, Coninck, Vandenbrouck, Coen, & Janssen, 2010). An alternative explanation for the lack of transcriptional responses in genes encoding for epigenetic modifiers is that these marks occur early in development (i.e., de novo methylation), even as early as during embryonic development (Harris, Bartlet, & Lloyd, 2012; Robichaud, Sassine, Beaton, & Lloyd, 2012), and because our data were collected from adult individuals, we may not have captured substantial latent signal of transcriptional regulation of these genes.

Because our transcriptome sampling design included pooling 30 individuals per replicate, it remains an open question how much variation in gene expression exists among individuals within a treatment. Our pooled sampling design should tend to average variation across individuals within a replicate, providing an underestimate of among-individual variation. Within our pooled sampling design, gene expression was highly replicable across treatments with the exception of one replicate in generation 3 (Gen: PNN, Rep: 1; Figure 2c); this replicate more closely resembles the expression patterns of the no-predator treatments (control group; Figure 3). A plausible explanation for this replicate appearing more like the control group is that the stability of the transfer of nongenetic inheritance is variable (and/or unstable) in the generations following cue removal. In other words, failure of the mechanisms promoting a transgenerational transfer of information (i.e., epigenetic decay) could explain this discrepancy in third-generation responses among replicates, and also broadly explain shifts in TGP responses in later generations. However, more extensive tests need to be performed to confirm this possibility.

## 5 | CONCLUSION

We examined the influence of predator cues on patterns of gene expression in a clone of *D. ambigua*. Our results revealed divergent within- and transgenerational patterns of gene expression (Figures 2–6), as shifts in gene expression in response to predator exposure were largely nonoverlapping within- and across generations (Figure 2d). These contrasting gene expression programmes are correlated with previously measured differences in patterns of phenotypic plasticity within- vs. across generations in this clone (Walsh et al., 2015). These complementary data collectively indicate that the molecular mechanisms that underlie within- vs. trans-generation plasticity are fundamentally distinct. Our results foreshadow that distinct molecular pathways determine the evolution of phenotypic plasticity within and across generations. A key next step is to determine how natural selection operates on the gene expression programmes for within- and TGP in natural systems. Specifically, this poses the intriguing questions of what tradeoffs there might be in lineages with different phenotypic responses, and if these differences involve expression of fundamentally different sets of genes, or if phenotypic differences instead stem from modulation based on which generation experiences up-regulation of particular gene expression programmes.

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## DATA ACCESSIBILITY

Raw RNAseq reads: National Center for Biotechnology Information (NCBI) Sequence Read Archive BioSample Accession nos SAMN05928333–SAMN05928350 under BioProject Accession no. PRJNA349191. Raw and processed data, along with metadata, have also been archived on Dryad https://doi.org/10.5061/dryad.q8m4f.

## AUTHOR CONTRIBUTIONS

M.R.W. and T.A.C. initially conceived the project and provided funding. M.R.W. conducted rearing experiments, while T.A.C., D.R.S. and A.L.A. performed laboratory work to generate RNAseq libraries. N.R.H. and D.C.C. processed the data, and N.R.H. performed statistical analyses. N.R.H., M.R.W. and T.A.C. wrote the manuscript, with contributions from all co-authors.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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