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# Transcriptome analysis of predator- and prey-induced phenotypic plasticity in the Hokkaido salamander (*Hynobius retardatus*)

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## Abstract

**Predator- and prey-induced phenotypic plasticity is widely observed among amphibian species. Although ecological factors inducing diverse phenotypic responses have been extensively characterized, we know little about the molecular bases of variation in phenotypic plasticity. Larvae of the Hokkaido salamander, *Hynobius retardatus*, exhibit two distinct morphs: the presence of their prey, *Rana pirica* tadpoles, induces a broad-headed attack morph, and the presence of predatory dragonfly nymphs (*Aeshna nigroflava*) induces a defence morph with enlarged external gills and a high tail. To compare the genes involved in predator- and prey-induced phenotypic plasticity, we carried out a de novo transcriptome analysis of Hokkaido salamander larvae exposed to either prey or predator individuals. First, we found that the number of genes involved in the expression of the defence morph was approximately five times the number involved in the expression of the attack morph. This result is consistent with the fact that the predator-induced plasticity involves more drastic morphological changes than the prey-induced plasticity. Second, we found that particular sets of genes were upregulated during the induction of both the attack and defence morphs, but others were specific to the expression of one or the other morph. Because both shared and unique molecular mechanisms were used in the expression of each morph, the evolution of a new plastic phenotype might involve both the co-option of pre-existing molecular mechanisms and the acquisition of novel regulatory mechanisms.**

*Keywords*: eco-genomics, gene ontology analysis, phenotypic plasticity, salamander, transcriptome

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## Introduction

Phenotypes of individual organisms are determined not only by their genetic backgrounds but also by environmental conditions. The expression of alternative phenotypes from the same genetic background in response to environmental stimuli is called phenotypic plasticity (West-Eberhard 2003). Because surrounding environments, such as the density of predators, prey items and competitors, can fluctuate temporally, organisms must express phenotypes suitable to each environment to cope with a variety of environments and to maximize their fitness. Flexible phenotypic expression, therefore, enables organisms to rapidly adapt to changing environments (Waddington 1942; Pigliucci 2001; West-Eberhard 2003; DeWitt & Scheiner 2004).

Predator-induced plasticity, the expression of a defensive phenotype in a prey species in the presence of predators, is one of the most widely observed types of

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plasticity, and it is especially common in aquatic animals (Tollrian & Harvall 1999; Gilbert & Engel 2009). In the crucian carp (*Carassius carassius*), for instance, a deep body shape induced by the presence of predators likely prevents the carp from being eaten by gape-limited predators and also increases its escape locomotor performance (Brönmark & Miner 1992; Domenici *et al.* 2008). Prey-induced phenotypic plasticity, the expression of a predacious phenotype in a predator species in the presence of prey, is another common plasticity type (Gilbert 2001). For example, in the presence of animal prey (fairy shrimp), spadefoot toad (*Spea bombifrons* or *S. multiplicata*) tadpoles develop into cannibalistic carnivores with large horny beaks and large mouth cavities, features that likely increase their feeding efficiency (Pfennig & Frankino 1997). Although the ecological factors that cause such diverse plastic phenotypic responses have been extensively studied, we know little about the genetic basis of the evolution of phenotypic plasticity.

The great diversity of plastic phenotypic responses seen in amphibians (Kishida *et al.* 2010) makes amphibian systems particularly suitable for investigating the molecular and genetic bases of variations in phenotypic plasticity. The expression of a high tail is a predator-induced phenotypic response that is widely observed across diverse amphibian taxa, including salamanders and anurans (Van Buskirk 2002; Mori *et al.* 2009; Arendt 2010), which suggests that this phenotypic plastic responses have been evolutionarily conserved. In contrast, prey-induced plastic responses vary among amphibians. For example, the expression of a broad-headed predatory morph in the presence of prey is observed only in salamander species (Collins & Cheek 1983; Nyman *et al.* 1993; Walls *et al.* 1993; Michimae & Wakahara 2002), suggesting that this type of prey-induced plasticity is a novel trait acquired after anurans and salamanders diverged.

To understand the genetic basis of the evolution of various phenotypic plastic responses, it is crucial to identify what genes are involved in the expression of alternative phenotypes. Recent advances in massively parallel sequencing technologies make it possible to conduct extensive surveys of genes that are differentially expressed in response to environmental stimuli (Ekblom & Galindo 2011). Even without any prior knowledge about genome sequences, RNA sequencing (RNA-seq) can be used to find transcripts that show expression differences and then the functions of the differentially expressed genes (DEGs) can be inferred by performing BLAST searches of reference databases of closely related taxa (Aubin-Horth & Renn 2009).

In this study, we conducted RNA-seq to analyse genes showing expression differences between predator- and prey-induced phenotypic plastic responses in the larvae of the Hokkaido salamander (*Hynobius retardatus*). This species can exhibit either an evolutionarily conserved 'defence morph' or a relatively newly evolved 'attack morph' in response to the presence of predators or prey, respectively. The presence of predatory dragonfly nymphs (*Aeshna nigroflava*) induces a phenotype with enlarged external gills and a high tail (Iwami *et al.* 2007; Hangui *et al.* 2009). Enlarged gills may be adaptive because they enable the salamanders to absorb oxygen efficiently from the water and hide motionless underwater for a long time (Iwami *et al.* 2007), whereas the high tail may increase the larva's burst swimming ability and thus improve its ability to escape from predators (Arendt 2010). In contrast, the presence of prey, namely, *Rana pirica* tadpoles, induces the expression of an attack morph, which is characterized by a wide mouth and wide head (Michimae & Wakahara 2002). These traits likely help the salamander larvae to swallow the prey tadpoles (Takatsu & Kishida 2013). We know, however, little about the molecular mechanisms underlying the expression of these predator- and prey-induced plastic responses in the Hokkaido salamander.

The main aim of this study was to compare transcriptomic patterns in the brain and peripheral tissues between predator-exposed and prey-exposed larvae of the Hokkaido salamander using RNA-seq technologies. We first asked whether the number of genes differentially expressed after exposure varies between these two stimuli. If more genes are involved in the expression of one alternative phenotype than in the expression of the other alternative phenotype, the former plasticity may require more complicated developmental changes. Next, we asked how many DEGs were shared and how many were different between these two stimuli. Genes that show a similar response to both stimuli may play important roles in the general decision to divert the developmental trajectory from the ordinary pathway to other pathways, whereas genes that show different responses may play important roles in the expression of particular alternative phenotypes. Finally, we discuss how such information improves our understanding of the genetic basis and evolution of diverse plastic phenotypic responses in amphibians.

## Materials and methods

### *Experiments to induce phenotypic plasticity*

We collected eggs of the Hokkaido salamander (*Hynobius retardatus*) and its prey (*Rana pirica*) as well as nymphs of the hawker dragonfly, *Aeshna nigroflava*, from several ponds in the *Teshio* and *Uryu* Experimental

Forests of Hokkaido University. Each salamander egg cluster was placed into a different stock tank (33.4 cm × 20 cm × 10 cm high) filled with 2 L of aged tap water and kept in the laboratory at 4 °C until use. After they hatched, we placed each individual salamander hatchling separately into a small plastic cup with aged tap water until it developed enough to eat tadpoles (7 days). Then, salamander larvae were individually exposed to either predator or prey cues to induce the different morphs.

We carried out two different experiments, a tadpole experiment and a dragonfly experiment, each consisting of a treatment group and a control group (Fig. 1A–C). In the tadpole experiment (Fig. 1A), 7-day-old salamander hatchlings (snout–vent length, SVL = 12.09 ± 0.48; mean ± SD, $N = 6$) were individually assigned to plastic enclosures (84 mm × 57 mm × 44 mm high) containing 10 small tadpoles as inducers (weight = approximately 150 mg each) (i.e. tadpole-exposure treatments) or to enclosures without tadpoles (i.e. tadpole controls). In all, we performed 180 replicate control treatments and 180 replicate exposure treatments.

In the dragonfly experiment (Fig. 1B), the sides of plastic enclosures (84 mm × 57 mm × 44 mm high) were perforated with small holes (Ø = 2 mm) and the 10 enclosures were put into tanks (33.4 cm × 20 cm × 10 cm high) containing 2 L of aged tap water. Thus, all enclosures in the same tank shared the same water. We assigned a 7-day-old salamander larva to each of nine enclosures in every tank. In the dragonfly-exposure treatment, a dragonfly nymph was placed in the remaining enclosure. In the dragonfly control treatment, the tenth enclosure was left empty. Salamander larvae in the exposure treatments could detect the presence of the dragonfly nymph via waterborne chemicals, but the nymph was unable to prey upon the salamanders. In the dragonfly control treatments, the water carried no predator cue. We set up 18 replicate dragonfly-exposure treatment tanks and 18 replicate control tanks (i.e. each replicate was a 2-L tank containing 10 enclosures). Thus, there were 162 salamander larvae in the dragonfly-exposure treatments and 162 in the control treatments. We randomly exchanged plastic enclosures containing salamander larvae among the different tanks of each treatment type every 2 days to avoid bias in the induction experiment.

In both experiments, the salamanders were fed *Tubifex* worms ad libitum every 2 days. Rearing water of the animals was also changed every 2 days. The animals were maintained and the experiments were conducted in a laboratory maintained at 16 °C under a natural day/night regime.

We measured mouth width, head width, tail depth and SVL of several individuals ($N = 4$–8) in each of the

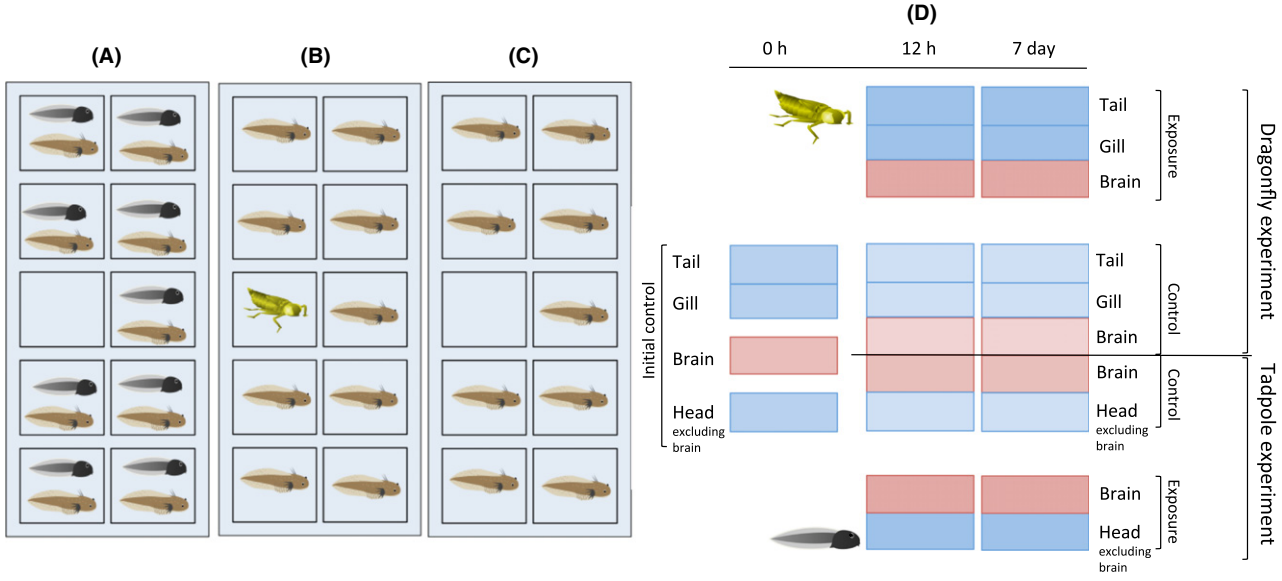


**Fig. 1** Experimental designs. The induction experiments comprised four conditions: (A) tadpole-exposure treatments, (B) dragonfly-exposure treatments and (C) dragonfly and tadpole controls. The enclosures used for the dragonfly-exposure treatments and controls had small holes in their sidewalls. (D) Tissues used for the transcriptome analysis were sampled at three time points, and three replicates were analysed each time. Red and blue indicate control and exposure treatment samples, respectively. In all, 72 samples were sequenced. Only control tissues were sampled before the induction experiment (0 h). In the tadpole experiment, brains and heads of 2–5 individuals were sampled. In the dragonfly experiment, brains, gills and tails were sampled from 2 to 5 individuals. The exposure treatments were sampled at 12 h and 7 days after the salamander larvae were first exposed to the stimulus.

treatments at several time points (0 and 12 h, and 3, 7 and 10 days after first exposure) to check for induction of the morphs, as explained in the Results. As indexes of induction, we adopted morphological ratios used in previous studies (Michimae & Wakahara 2002; Michimae & Hangui 2008).

### *RNA extraction and sample preparation for sequencing*

We collected tissue samples for the transcriptome analysis at three census time points (0 h, 12 h and 7 days), as representative of the initial gene expression (0 h control), gene expression before morphological induction (12 h) and gene expression after the morphological induction (7 days). We dissected the salamander larvae from each experiment and sampled tissues from the brain and from those body parts that were changed drastically in the experiment (tadpole experiment, head excluding the brain; dragonfly experiment, gills and tail). We pooled the sampled tissues from 2 to 5 individuals to obtain the required total amount of RNA for making a sequencing library. The tissue-sampling scheme is shown in Fig. 1D. For the transcriptome sequencing, we collected three replicate subsamples from every tissue type in every treatment at each census time point; thus, we sequenced a total of 72 tissue sample units. The tissue samples were preserved immediately in RNAlater (Life Technologies, Grand Island, NY, USA) and stored at −20 °C to about −80 °C until RNA extraction.

Total RNAs were extracted from the tissue samples using TRIzol reagent (Life Technologies) and isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA). RNA quality and quantity were analysed with a spectrophotometer (Nanodrop1000: Thermo Scientific) and the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The sequencing libraries were constructed using TruSeq RNA Sample Preparation Kits v2 (Illumina, San Diego, CA, USA). Paired-end sequencing of each 100-bp end of cDNA was performed with the Hiseq2000 sequencing system (Illumina). We used three lanes of the Hiseq2000 system (24 samples per lane).

### *De novo short-read assembly*

To reconstruct salamander cDNA sequences from the short-read data generated by the HiSeq2000 system, we carried out de novo sequence assembly. For each read, nucleotides with a low-quality score (Phred score <20; $>10^{-2}$ error rate) at sequence ends were removed using the SOLEXAQA program ver. 2.2 (Cox *et al.* 2010) and the adapter sequences were also removed using the CUTADAPT program ver. 1.2.1 (Martin 2011). The remaining sequences were subjected to de novo assembly with the TRINITY program ver. 2013-2-25 (Grabherr *et al.* 2011) under default parameter settings. We used subcomponent-level contigs. The assembly results generated by Trinity were processed with the TRANSDECODER program (part of the TRINOTATE package; Haas *et al.* 2013) to extract open reading frames and amino acid sequences.

### *Gene annotation*

To identify vertebrate orthologs among the salamander contigs, all of the predicted amino acid sequences in the obtained transcriptomes were searched against human (*Homo sapiens*), mouse (*Mus musculus*) and frog (*Xenopus tropicalis*) amino acid sequences using BLASTP. The complete amino acid sequence data sets for human, mouse and frog were downloaded from the ENSEMBL database version 72 (http://www.ensembl.org/). BLASTP searches were performed using the salamander contigs as queries and a $10^{-5}$ e-value cut-off against each human, mouse and frog data set. The top hit proteins in each database were defined as orthologs in the focal salamander.

### *Identification of differentially expressed genes*

All reads of the 72 samples were mapped to the reference transcript database that was created by de novo assembly (see above). We used Bowtie v0.12.8 (Langmead *et al.* 2009) for mapping. RSEM v1.1.6 software (Li & Dewey 2011) was then used to estimate the relative abundance and expected read counts of all contigs. The contigs count data were normalized by the trimmed mean of *M* values (TMM) method available in the EDGER software package (version 3.2.4, R version 3.01; Robinson *et al.* 2010; Robinson & Oshlack 2010; McCarthy *et al.* 2012). The TMM method estimates scaling factors between samples, and these factors are used for normalization of sample-specific effects, such as differences in sequencing depth. Briefly, log base 2 of the ratio of each gene's relative expression level between samples (its *M* value) was calculated. Then, genes with *M* values in the upper or lower 30% of all *M* values (highly differentiated between samples) were removed. Genes with expression levels in the upper or lower 30% (highly abundant transcripts and very rare transcripts) were also removed. Then, the scaling factors were calculated such that the log ratio of the expression differences between samples for the majority of the remaining genes (putatively housekeeping genes) would be zero. The adjusted count values were then used for the DEG analysis.

We analysed tissue-specific DEGs using EDGER package software (Robinson *et al.* 2010; McCarthy *et al.*

2012). In each tissue of each exposure experiment (dragonfly exposure and tadpole exposure), we were interested in DEGs between the experimental treatments (control and treatment) at each census time (from 0 h to 7 days). Because the data sets consisting of a control treatment and a number of census times did not constitute a complete two-way experimental design, we used one-way array with five levels (0-h control, 12-h control, 12-h treatment, 7-day control and 7-day treatment). We first conducted a generalized linear model (GLM) analysis of the five-level data set to test the null hypothesis, $H_0$, that the overall gene-expression pattern among the five levels was identical. Rejection of $H_0$ implies that the gene-expression pattern differed among at least some levels. We then conducted a preliminary DEG search (see results). After the preliminary search, we performed pairwise comparisons of the gene-expression pattern using a comparison scheme that we described later (see Results).

In the GLM analysis, we first sorted genes in descending order according to their likelihood values for rejection of $H_0$ and then selected those genes that contributed to the rejection of $H_0$ with a false discovery rate (FDR) cut-off of 0.05. The GLM result implied that the selected genes could be expressed differently in any of the five levels (i.e. 0-h control, 12-h control, 12-h treatment, 7-day control and 7-day treatment). In the selected genes, we reviewed the estimated log-fold changes (logFCs) between the 0-h control and the 12-h treatment or the 7-day treatment. When the logFCs at both the 12-h and 7-day time points were either both positives or both negatives, we classified the genes as DEGs and annotated them as upregulated or downregulated, respectively. When logFC at one time point was positive and logFC at the other time point was negative, we classified the expression patterns of these genes as fluctuating.

In the pairwise analysis, we again sorted genes in descending order according to their likelihood values for rejection of the null hypothesis (i.e. equal expression), selected DEGs with a FDR cut-off of 0.05 and then evaluated the signs of the DEGs' logFC values.

### *Gene ontology analysis*

We conducted a gene ontology (GO) enrichment analysis with ErmineJ software ver. 3.0.2 (Gillis *et al.* 2010) to investigate which gene functions were upregulated and which were downregulated in each exposure. GO terms of each contig were determined based on the GO terms of human homologs, which were obtained from the ENSEMBL database (http://www.ensembl.org/). *P*-values were adjusted by the multiple-test correction method of Benjamini and Hochberg (Benjamini & Hochberg 1995).

For the GLM analysis, we used hypergeometric distribution-based overrepresentation analysis. This analysis searches for GO terms that are enriched in DEGs in comparison with all annotated contigs (Mills *et al.* 2012). In the pairwise comparison, we used the gene score resampling method (Gillis *et al.* 2010). Here, the relative differences in gene scores, the negative of the log-transform of *P*-values of the pairwise comparison, were taken into account: a gene with a much lower *P*-value had more weight than one with a slightly lower *P*-value. We set gene score threshold as *P*-value <0.05 in the pairwise comparison and compared weighted DEGs with all annotated contigs to identify overrepresented GO terms.

## Results

### *Phenotypic plasticity induction experiments*

Because a larger mouth width is characteristic of the prey-induced attack morph (Michimae & Wakahara 2002), we used the mouth width/head width ratio as the index of the attack morph in the tadpole-exposure experiment. When the attack morph is induced, the mouth width becomes larger, approaching the head width, so that the index approaches 1. In our experiment, at 7 days after the initial exposure, the attack morph index in the induced larvae was significantly higher than the index in the controls ($t < -4.3925$, df > 10.232, $P < 0.0013$; control $N = 5$–8, exposure treatment $N = 5$–8; Fig. 2A).

Because greater tail depth is characteristic of the predator-induced defence morph (Hangui *et al.* 2009; Kishida *et al.* 2009), we used the tail depth/snout–vent length ratio as the index of the defence morph in the dragonfly-exposure experiment. In the presence of a predator, the salamander tail enlarges, so this index increases. The index differed significantly between the controls and exposure treatments at 7 days after the initial exposure ($t < -4.5712$, df > 7.275, $P < 0.0023$; control $N = 5$–6, exposure treatment $N = 4$–6; Fig. 2B). These results showed that our experimental protocols successfully induced the expected predator- and prey-induced phenotypic plastic responses in the Hokkaido salamander.

### *Transcriptome assembly and annotation*

We sequenced transcriptomes derived from a total of 72 samples, each of which had 6–10 million reads. To make a whole-transcript database of the salamander genome, we first carried out de novo transcript assembly using all of the reads (0.6 billion reads) and obtained 740 933 contigs with an average length of 540 bp (Table S1). Among these contigs, 174 852 open

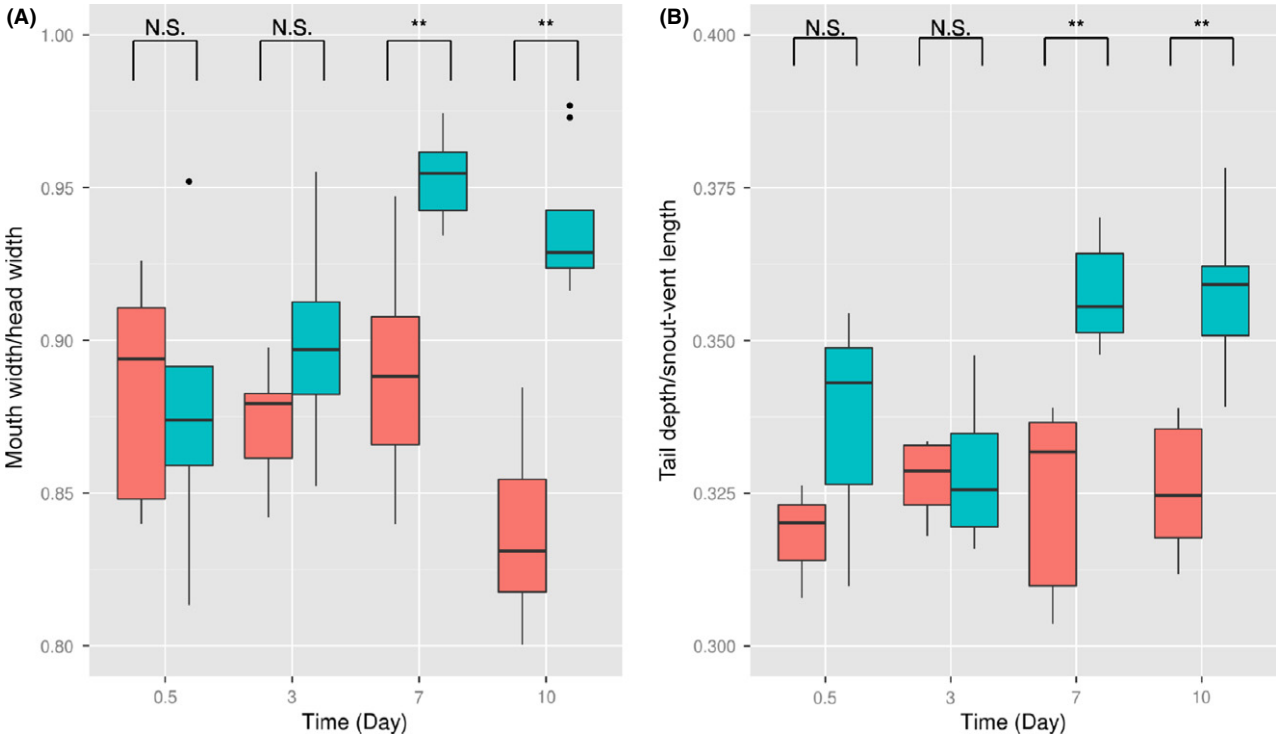


**Fig. 2** Morphological measurements of Hokkaido salamander larvae. Box-plots showing the results of the controls (red) and the exposure treatments (blue) in the (A) tadpole and (B) dragonfly experiments. On day 7 after the initial exposure, statistically significant differences between the treatments and controls were detected in both experiments ($t < -4.3925$, df $> 7.275$, $P < 0.0023$). (A) The mouth width/head width ratio was used as an index for the attack morph. (B) The snout–vent length/tail length ratio was used as an index for the defence morph. $^{**}P < 0.05$.

reading frames (i.e. protein-coding genes) were predicted and used for homology searches.

To identify orthologs among the salamander contigs, we used the predicted amino acid sequences as queries in a BLASTP homology search of human (*Homo sapiens*), mouse (*Mus musculus*) and frog (*Xenopus tropicalis*) data. The BLASTP search results were similar among the three data sets (Table S2). The salamander contigs covered 65%, 65% and 76% of the total protein sequences of human, mouse and frog, respectively. Previous comparative genomic analyses have shown that in every taxonomic group so far studied, 10–20% genes lack recognizable homologs in other species (so-called orphan genes; Khalturin *et al.* 2009). The remaining 80–90% genes are evolutionarily conserved and are detectable by BLAST searches. The salamander contigs included 60–70% of these conserved genes. Thus, by this analysis, we successfully identified orthologs in the focal salamander species.

### *Identification of differentially expressed genes in plastic phenotypes*

We first conducted a GLM analysis of each tissue sample. In brain tissue, we identified 103 DEGs in the tadpole experiment and 605 DEGs in the dragonfly experiment; among these, 39 DEGs were identified in both experiments (Fig. 3). These shared DEGs included 29 upregulated, 8 downregulated and 2 fluctuating genes against their initial controls. In head tissue (excluding brain) in the tadpole experiments, we identified 182 DEGs, and in gill and tail tissues in the dragonfly experiments, we identified 1086 and 714 DEGs, respectively; 14 of these DEGs were identified in all three responding tissues (Fig. 4). All of the genes evaluated by the GLM analysis are described in Tables S3 and S4.

The GLM analysis revealed an interesting pattern. The number of DEGs in the dragonfly experiment was significantly larger than the number in the tadpole experiment, comparing both brain and peripheral tissues (Figs 3 and 4). We conducted chi-squared tests to confirm that the number of DEGs detected in the dragonfly exposure was significantly higher than that detected in the tadpole exposure: $P < 0.001$ for tadpole brain (103/740 933) vs. dragonfly brain (605/740 933), $P < 0.001$ for tadpole head (182/740 933) vs. dragonfly gill (1086/740933) and $P < 0.001$ for tadpole head (182/740 933) vs. dragonfly tail (714/740 933).

We next performed pairwise gene-expression comparison analyses, using the comparison scheme shown in

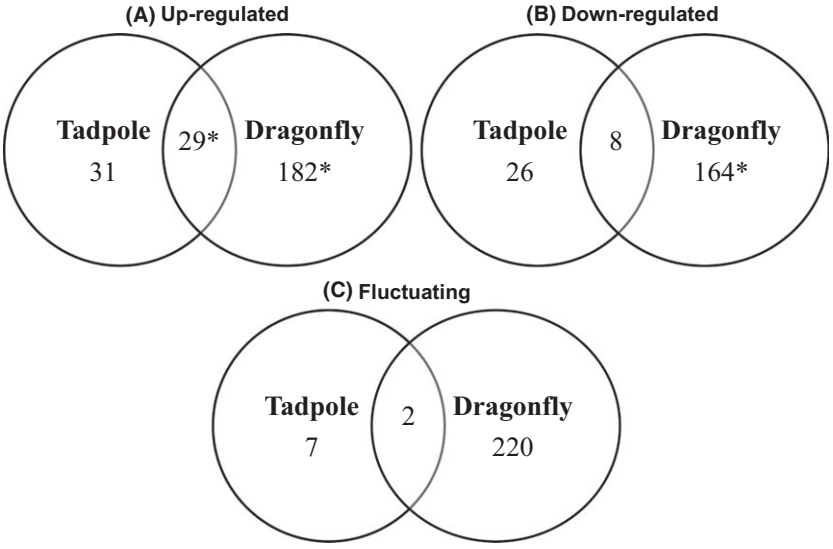


**Fig. 3** Venn diagrams showing differentially expressed genes in brain according to the experimental treatment. In the GLM analysis of the tadpole experiment and dragonfly experiment, genes that were expressed differently in any of the five levels (0-h control, 12-h control, 12-h treatment, 7-day control and 7-day treatment) were reviewed. When the estimated logFCs between the 0-hr control and the 12-hr treatment or the 7-day treatment were both positives or both negatives, these genes were classified into (A) upregulated or (B) downregulated DEGs, respectively. When just one of the logFCs was different, these genes were classified as (C) fluctuating DEGs. Asterisks represent groups showing marginally significant enrichment of some gene ontology terms (corrected *P*-value <0.1).

Fig. 5, to examine both temporal and treatment effects in each tissue. We first confirmed the pattern that the number of DEGs was generally higher in the dragonfly-exposure experiment than in the tadpole-exposure experiment. For example, in the brain at 7 days after the exposure, 76 DEGs were found in the dragonfly-exposure treatment, whereas only one DEG was found in the tadpole-exposure treatment. We also found another pattern: more DEGs were identified at 7 days after the exposure than at 12 h after the exposure for both experiments. For example, we identified a number of DEGs between controls and exposure treatments at 7 days after the exposure, whereas at 12 h, we identified only a few DEGs between controls and exposure treatments in the tail tissues, including one DEG in the head in the tadpole-exposure treatment. All of the genes evaluated by the pairwise comparisons are described in Table S5. The differences in the number of DEGs between the 12-h and 7-day time points are consistent with observations that detectable morphological changes have not yet occurred at 12 h but are completed by 7 days (Fig. 2).

### *Gene ontology analysis*

We carried out the gene ontology analysis to characterize the DEGs identified by the GLM (Tables 1 and 2). In the brain, we found that some GO terms showed similar changes between the dragonfly- and the tadpole-exposure experiments. GO terms related to reactive oxygen and hydrogen peroxide were upregulated in both the tadpole-exposure and the dragonfly-exposure experiments (Table 1). For example, genes related to reactive oxygen, such as YWHAG, HBB, HBA2 and SQLE, were upregulated in both experiments. Thus, similar sets of genes could show expression changes when different phenotypes were induced. We also found that in the dragonfly-exposure experiment, GO terms related to neuron development were downregulated, whereas GO terms related to translation and muscle-system processes were upregulated.

In the different peripheral tissues showing phenotypic changes, distinct types of genes changed their expression levels in comparison with their expression in brain tissue (Table 2). In gill, genes related to translation were upregulated, and genes related to biogenesis were fluctuating. In both tail and gill, genes related to muscle development were upregulated. In both gill and head, genes related to muscle development were also upregulated. Interestingly, although shared DEGs are different between two categories, we found enrichments of similar GO terms. These data suggest that in peripheral tissues, expression levels of genes related to morphological alteration and catabolism change, a finding that is consistent with the fact that breakdown and subsequent reorganization of the peripheral tissues occur. We also observed that genes related to visual perception were upregulated in head, mainly because of

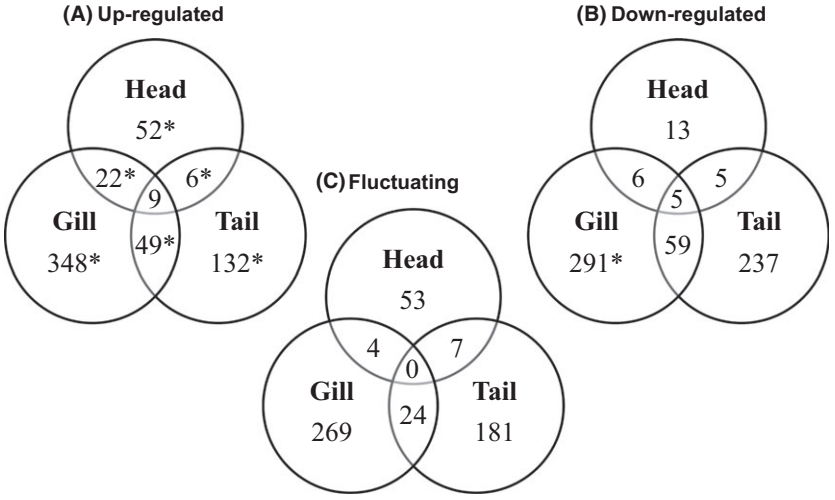


**Fig. 4** Venn diagrams showing differentially expressed genes in periphery tissues according to the experimental treatments. In the GLM analysis of the tadpole experiment and dragonfly experiment, genes that were expressed differently in any of the five levels (0-h control, 12-h control, 12-h treatment, 7-day control and 7-day treatment) were reviewed. When the estimated logFCs between the 0-h control and the 12-h treatment or 7-day treatment were both positives or both negatives, these genes were classified into (A) upregulated or (B) downregulated DEGs, respectively. When just one of the logFCs was different, these genes were classified into (C) fluctuating DEGs. Asterisks represent groups showing marginally significant enrichment of gene ontology terms (corrected *P*-value <0.1).

changes in the expression of crystallin genes such as CRYAA, CRYBB2, CRYBB3, CRYGB and CRYGD.

## Discussion

### *Differences in the number of differentially expressed genes between stimuli*

Our transcriptome analysis revealed different patterns of expression changes between predator- and prey-induced phenotypic plasticity in the Hokkaido salamander. First, the total number of DEGs was significantly higher in the dragonfly-exposure experiment than in the tadpole-exposure experiment. This difference may reflect the difference in the complexity of the developmental changes leading to the different phenotypes. Previous studies have reported that induction of the defence morph involved developmental changes in many tissues, such as enlargement of gills (Iwami *et al.* 2007), increase in tail height (Michimae & Hangui 2008), changes in tail colour (Matsunami and Kishida, unpublished data) and behavioural defence (Kishida *et al.* 2009), whereas the attack morph required the changes in only head morphology.

Differences in the number of DEGs among different stimuli have also been reported in invertebrates. For example, the carpenter ant (*Camponotus floridanus*) is a eusocial insect showing caste polyphenism with three distinct morphs, reproductives, minor workers and major workers. Recent RNA-seq and ChIP-seq analyses of this ant species have revealed that the number of DEGs related to the induction of minor workers and major workers is 79 and 42, respectively (Simola *et al.* 2013). These different numbers of DEGs may also reflect differences in their behavioural and developmental complexity. Although major workers are specialized for colony defence, minor workers perform many colony tasks, such as brood care, foraging and colony maintenance (Wilson 1976). Because the tasks of minor workers also require sensitive perception of pheromones, minor workers may need to develop more complex perception systems than major workers. Thus, the expression of distinct morphs requiring different levels of phenotypic changes may generally involve different numbers of genes.

### *Shared expression changes between different stimuli in the brain*

Our brain transcriptome analysis showed that particular sets of genes were upregulated in brain during the induction of both morphs. For example, genes related to the response to reactive oxygen and hydrogen peroxide were upregulated in both the tadpole-exposure and the dragonfly-exposure experiments. This same direction of response in the genes related to reactive oxygen and hydrogen peroxide may reflect shared molecular mechanisms of morphogenesis were used to produce distinctive morphs. When animals increase their metabolic rates and consume more oxygen for energy

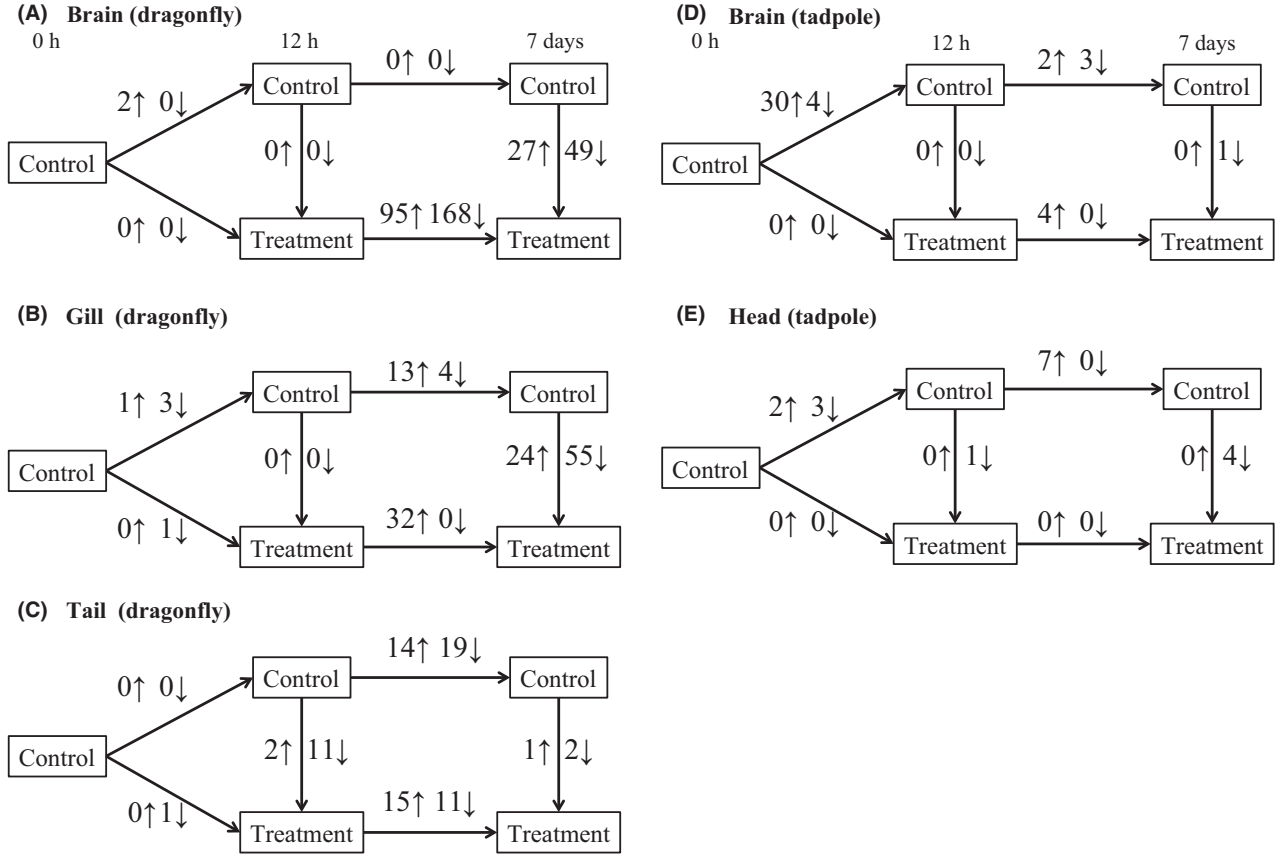


**Fig. 5** Differentially expressed genes identified by pairwise comparisons. Extensive pairwise comparisons of all examined tissues were carried out: (A) brain (dragonfly experiment), (B) gill (dragonfly experiment), (C) tail (dragonfly experiment), (D) brain (tadpole experiment) and (E) head (tadpole experiment). When contigs were upregulated in the treatment samples (treatment/control comparisons) or in the samples at later census time points (0 h/12 h, 12 h/7d comparisons), these contigs were defined as upregulated DEGs, otherwise as downregulated DEGs, with an FDR cut-off of 0.05. The numbers of upregulated (up arrows) and downregulated (down arrows) DEGs in each pairwise comparison are shown.

production, genes related to reactive oxygen and hydrogen peroxide are expected to be upregulated. Because morphological changes require more energy, the expression changes of these genes may reflect increased metabolic rate associated with morphological changes.

Although no significantly enriched gene ontology terms were found, several genes showed similar expression changes in both the dragonfly- and tadpole-exposure experiments. Gene involved in cholesterol and steroid metabolism is one of such examples: farnesyl diphosphate synthase (FDPS) was upregulated in both treatments. As steroid hormones are known to modulate phenotypic plasticity in amphibians (Denver 1997; Middlemis Maher *et al.* 2013), some of these genes may regulate the plastic expression of phenotypes through the regulation of steroid metabolism.

These data may shed light on how phenotypic plasticity is acquired along the course of evolution. Hokkaido salamanders have two alternative phenotypes, an attack morph and a defence morph. Because a higher tail as a trait of a defence morph is widely reported across amphibian lineages (Van Buskirk 2002; Mori *et al.* 2009; Arendt 2010), the evolution of defence morphs may have preceded the acquisition of attack morphs. We found shared expression changes between the two phenotypes (Fig. 3). Therefore, we can hypothesize that the genes shared by both morphs were originally used for expression of the defence morph and then became co-opted for the expression of the attack morph. On the other hand, we also detected genes expressed only in one or the other morph. Overall, our findings suggest that the evolution of a new phenotype involves both the co-option of pre-existing molecular mechanisms and the acquisition of novel regulatory mechanisms.

### *Differentially expressed genes in the peripheral tissues*

In the peripheral tissues, our gene ontology analysis showed that the expression levels of genes related to

**Table 1** Enriched gene ontology terms related to biological process in brain tissues. Significantly enriched terms (corrected *P*-value <0.05) and marginally significantly enriched terms (corrected *P*-value <0.1) with two or more DEGs with significant gene scores are shown here. For each GO term, we counted the numbers of all annotated contigs, contigs detected as DEG and contigs with higher ErmineJ gene scores than a threshold ($P < 0.05$)

| Name | ID | All annotated contigs | DEG | DEG with significant gene scores | Corrected *P*-value |
|---|---|---|---|---|---|
| (A) GO terms upregulated in both the dragonfly and tadpole exposures | | | | | |
| Single-organism catabolic process | GO:0044712 | 96 | 52 | 3 | 0.0234806 |
| Response to reactive oxygen species | GO:0000302 | 26 | 10 | 2 | 0.02186518 |
| Cellular response to oxidative stress | GO:0034599 | 26 | 10 | 2 | 0.02186518 |
| Cellular response to reactive oxygen species | GO:0034614 | 26 | 10 | 2 | 0.02186518 |
| Response to hydrogen peroxide | GO:0042542 | 26 | 10 | 2 | 0.02186518 |
| Hydrogen peroxide metabolic process | GO:0042743 | 26 | 10 | 2 | 0.02186518 |
| Hydrogen peroxide catabolic process | GO:0042744 | 26 | 10 | 2 | 0.02186518 |
| Cellular response to hydrogen peroxide | GO:0070301 | 26 | 10 | 2 | 0.02186518 |
| Reactive oxygen species metabolic process | GO:0072593 | 26 | 10 | 2 | 0.02186518 |
| Response to inorganic substance | GO:0010035 | 32 | 15 | 2 | 0.03388468 |
| Cellular response to oxygen-containing compound | GO:1901701 | 32 | 15 | 2 | 0.02541351 |
| Response to oxidative stress | GO:0006979 | 44 | 23 | 2 | 0.04869342 |
| Response to oxygen-containing compound | GO:1901700 | 48 | 27 | 2 | 0.05612651 |
| Cellular response to chemical stimulus | GO:0070887 | 55 | 33 | 2 | 0.07204221 |
| Cellular catabolic process | GO:0044248 | 246 | 165 | 3 | 0.08931512 |
| (B) GO terms upregulated in only the dragonfly exposure | | | | | |
| Translation | GO:0006412 | 317 | 173 | 10 | 0.0001215 |
| Muscle-system process | GO:0003012 | 22 | 18 | 3 | 0.06631595 |
| Muscle contraction | GO:0006936 | 22 | 18 | 3 | 0.06631595 |
| (C) GO terms downregulated in only the dragonfly exposure | | | | | |
| Axonogenesis | GO:0007409 | 253 | 157 | 11 | 0.0002697 |
| Cell morphogenesis involved in neuron differentiation | GO:0048667 | 255 | 159 | 11 | 0.0001531 |
| Neuron projection morphogenesis | GO:0048812 | 256 | 159 | 11 | 0.0001021 |
| Cell morphogenesis involved in differentiation | GO:0000904 | 256 | 160 | 11 | 0.00008154 |
| Axon development | GO:0061564 | 257 | 160 | 11 | 0.00006523 |
| Neuron projection development | GO:0031175 | 267 | 167 | 11 | 0.00008337 |
| Neuron development | GO:0048666 | 276 | 174 | 11 | 0.0001074 |
| Neuron differentiation | GO:0030182 | 281 | 179 | 11 | 0.0001242 |
| Cell development | GO:0048468 | 306 | 196 | 11 | 0.0002672 |
| Axon guidance | GO:0007411 | 231 | 139 | 9 | 0.0006032 |
| Neuron projection guidance | GO:0097485 | 231 | 139 | 9 | 0.0006032 |
| Chemotaxis | GO:0006935 | 234 | 142 | 9 | 0.000652 |
| Taxis | GO:0042330 | 234 | 142 | 9 | 0.000652 |
| Locomotion | GO:0040011 | 298 | 186 | 9 | 0.005013 |
| Response to external stimulus | GO:0009605 | 332 | 188 | 9 | 0.005022 |

morphogenesis and catabolism altered during the focal phenotypic changes. Given the morphological changes exhibited by peripheral tissues, these findings are not surprising, but they nevertheless confirm that our transcriptome analysis could successfully detect at least some of the expected sets of DEGs. Among genes not related to morphogenesis and development, one interesting DEG that was upregulated in the dragonfly-exposure experiment was HSDL1 (hydroxysteroid dehydrogenase like), which encodes a protein involved in steroid metabolism. HSDL1 is an ortholog of genes belonging to the HSD3b (hydroxysteroid dehydrogenase 3b) gene family (Meier *et al.* 2009). The upregulation of this gene, therefore, also supports the idea that steroid signalling plays important roles in morphogenesis related to the plastic expression of phenotypes (Brown & Cai 2007; Denver 2009; Middlemis Maher *et al.* 2013).

## Conclusions

The results of our transcriptome analysis have implications for the evolutionary mechanisms involved in the acquisition of a new type of plastic phenotypic responses. In a species already capable of a certain plastic phenotypic response, some molecular mechanisms involved in the expression of pre-existing phenotype

**Table 2** Enriched gene ontology terms related to biological process in the peripheral tissues. Significantly enriched terms (corrected *P*-value <0.05) and marginally significantly enriched terms (corrected *P*-value <0.1) with two or more DEGs with significant gene scores are shown here. For each GO term, we counted the numbers of all annotated contigs, contigs detected as DEG, and contigs with higher ErmineJ gene scores than a threshold ($P < 0.05$)

| Name | ID | All annotated contigs | DEG | DEG with significant gene scores | Corrected *P*-value |
|---|---|---|---|---|---|
| (A) GO terms upregulated in both gill and head tissues | | | | | |
| Muscle-system process | GO:0003012 | 22 | 18 | 3 | 0.001295 |
| Muscle contraction | GO:0006936 | 22 | 18 | 3 | 0.001295 |
| Skeletal muscle contraction | GO:0003009 | 6 | 5 | 2 | 0.006217 |
| Striated muscle contraction | GO:0006941 | 6 | 5 | 2 | 0.006217 |
| Multicellular organismal movement | GO:0050879 | 6 | 5 | 2 | 0.006217 |
| Musculoskeletal movement | GO:0050881 | 6 | 5 | 2 | 0.006217 |
| System process | GO:0003008 | 202 | 105 | 3 | 0.09164831 |
| (B) GO terms upregulated in both gill and tail tissues | | | | | |
| Tissue development | GO:0009888 | 94 | 57 | 4 | 0.01052032 |
| Striated muscle tissue development | GO:0014706 | 5 | 5 | 2 | 0.022735 |
| Cardiac muscle tissue development | GO:0048738 | 5 | 5 | 2 | 0.022735 |
| Muscle tissue development | GO:0060537 | 5 | 5 | 2 | 0.022735 |
| Muscle organ development | GO:0007517 | 13 | 8 | 2 | 0.04222006 |
| Ribosome biogenesis | GO:0042254 | 55 | 47 | 3 | 0.04404504 |
| Tissue morphogenesis | GO:0048729 | 13 | 10 | 2 | 0.04057232 |
| Ribonucleoprotein complex biogenesis | GO:0022613 | 60 | 52 | 3 | 0.03964747 |
| Epidermis development | GO:0008544 | 33 | 14 | 2 | 0.05820263 |
| Skin development | GO:0043588 | 36 | 16 | 2 | 0.06692636 |
| Organ development | GO:0048513 | 286 | 159 | 4 | 0.06227643 |
| Muscle structure development | GO:0061061 | 24 | 17 | 2 | 0.06057569 |
| (C) GO terms upregulated in both head and tail tissues | | | | | |
| Heart development | GO:0007507 | 28 | 24 | 2 | 0.006738 |
| Cardiovascular system development | GO:0072358 | 54 | 42 | 2 | 0.01049942 |
| Circulatory system development | GO:0072359 | 54 | 42 | 2 | 0.01049942 |
| (D) GO terms upregulated in gill tissues only | | | | | |
| Translation | GO:0006412 | 317 | 173 | 33 | 1.116E-21 |
| (E) GO terms upregulated in head tissues only | | | | | |
| Visual perception | GO:0007601 | 116 | 50 | 5 | 0.0001009 |
| Sensory perception of light stimulus | GO:0050953 | 116 | 50 | 5 | 0.0001009 |
| Sensory perception | GO:0007600 | 153 | 68 | 5 | 0.0002403 |
| Neurological system process | GO:0050877 | 173 | 82 | 5 | 0.0004088 |
| System process | GO:0003008 | 202 | 105 | 5 | 0.001041 |
| (F) GO terms upregulated in tail tissues only | | | | | |
| Translation | GO:0006412 | 317 | 173 | 8 | 0.001056 |
| (G) GO terms fluctuating in gill tissues only | | | | | |
| Ribonucleoprotein complex biogenesis | GO:0022613 | 60 | 52 | 7 | 0.004678 |
| Ribosome biogenesis | GO:0042254 | 55 | 47 | 6 | 0.01462698 |

may be recruited for the production of the new plastic phenotype. For example, we found that some genes showed similar expression changes in both evolutionarily old predator-induced phenotypes and evolutionarily newer prey-induced plastic phenotypes. Thus, the co-option and modification of gene networks already used for the expression of evolutionarily old plasticity may occur during the evolution of a novel plastic phenotypic response.

Because the sequencing depth of the present study was relatively low, we cannot exclude the possibility that we overlooked some DEGs that were expressed at low levels. In the future, therefore, transcriptomic analysis with more read depth should be conducted to discover such DEGs with low expression levels. An increase in the number of replicates would also increase the power of detection of DEGs. Another caveat is that we were not able to exclude the possibility that different contigs corresponded to splice variants of the same gene. The availability of whole-genome sequences for this salamander should help to resolve this problem. Despite these caveats, our transcriptome analysis

revealed several interesting patterns. An important next step is to test whether similar patterns also exist in other amphibian species that show diverse plastic phenotypic responses. Our mRNA database for the Hokkaido salamander will also be a useful resource for annotating mRNA short reads obtained from other amphibian species.

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M.M., O.K., J.K., H.M. and K.N. participated in the experimental design. M.M. and O.K. performed the phenotypic plasticity induction experiments. M.M. and J.K. prepared the samples for sequencing. M.M. carried out all computational analyses. M.M. wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript.

## Data accessibility

Transcriptome data have been deposited into GenBank/DDBJ. BioProject ID is PRJDB2409. Accession IDs of Sequence Read Archive are DRX015082-DRX015153. The transcriptome assembly, annotation of assembly, the DEGs input and output files and all morphological measurements have been deposited into Dryad (doi:10.5061/dryad.d95j4).

## Supporting information

Additional supporting information may be found in the online version of this article.

**Table S1** De novo assembly results.

**Table S2** BLASTP homology search against representative vertebrates.

**Table S3** DEG list for brain screened in the GLM analysis.

**Table S4** DEG list for peripheral tissues screened in the GLM analysis.

**Table S5** DEG list identified by pairwise comparisons.