



# Transcriptomic Changes in Hot Spring Frog Tadpoles (*Buergeria otai*) in Response to Heat Stress

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*Buergeria* frog tadpoles exhibit high thermal tolerance and are occasionally found in water pools that temporarily exceed 40°C. With the aim of understanding how they can cope with the severe heat stress, we performed RNA-seq of three heat-treated (38°C) and three control (25°C) tadpoles and compared their transcriptomic profiles. We identified 382 differentially expressed transcripts. A protein-protein interaction (PPI) network analysis of these transcripts further identified hub proteins involved in protein degradation, stress granule assembly, and global suppression of DNA transcription and mRNA translation. Along with the avoidance behavior against high water temperature, these endurance mechanisms potentially support tadpoles to survive in high temperatures for short periods of time. Similar mechanisms may exist in many other amphibian species whose habitats are prone to high temperatures.

**Keywords:** amphibia, anura, differentially expressed genes, protein-protein interaction network, RNA-seq, high thermal tolerance, heat stress response

## INTRODUCTION

In their natural environment, organisms face various environmental stressors such as ultraviolet light, low and high pH, osmotic pressure, freezing, and heat. For survival, organisms exhibit various responses to such stressors at multiple biological levels, including changes in morphology, physiology, behavior, and life history (Crespi et al., 2013; Wingfield, 2013; Gormally and Romero, 2020). Most of these responses are initially caused by intracellular regulations mediated by the neural and hormonal system (Mastrangelo et al., 2012; Crespi et al., 2013; Wingfield, 2013; Gormally and Romero, 2020). Therefore, these stress responses become the paradigm of how molecular changes influence individual survival under environmental challenges (Boonstra, 2013). Thus, a better understanding of how organisms cope with extreme stressors can reveal important molecular mechanisms enabling them to survive in such environments. In particular, thermal conditions pose significant challenges to ectotherms. Without thermoregulatory mechanisms, the survival and distribution of organisms are largely determined by temperature, whereby an appropriate response to thermal stress is essential for their survival.

Tree frog species of the genus *Buergeria* are suitable for studying how organisms cope with extreme thermal stress because several populations live in extreme hot environments. *Buergeria* frogs are distributed in a wide range of habitats, from mountain streams to coastal areas across continental islands in Eastern and Southeastern Asia (Haramura, 2004; Matsui and Maeda, 2018).

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Currently, six species have been identified, and several populations of three species, *B. otai*, *B. choui*, and *B. japonica*, are further found in geothermal hot springs (Morita, 1988; Chen et al., 2001; Wu and Kam, 2005; Komaki et al., 2016b).

In geothermal hot springs, the water temperature is non-uniform and varies temporally and spatially due to rainfall, water depth, air temperature, and distance from the hot spring source. Field and laboratory observations revealed that *Buergeria* tadpoles and adults from hot spring populations tended to avoid inhabiting and reproducing in water temperatures above  $\sim 35^{\circ}\text{C}$  (Wu and Kam, 2005; Komaki et al., 2020). Avoidance behaviors against heat stress are probably essential for populations to survive in hot spring environments. However, due to the nature of hot springs, the water temperature can fluctuate, and tadpoles must face inevitable temperature spikes in this fully aquatic life stage. Indeed, *Buergeria* frogs living in hot springs can tolerate hot water: a previous study measured 30 *Buergeria* tadpoles collected from a hot spring habitat and revealed tolerance of temperatures up to  $46^{\circ}\text{C}$ , whereas above this temperature, tadpoles lost balance and swimming ability or died (Komaki et al., 2016b). Such high thermal tolerance has been observed not only in hot spring populations but also in freshwater *Buergeria* populations across their distribution (Komaki et al., 2016a). The high thermal tolerance necessary for survival at these temperatures demands protective transcriptional and subsequent cellular responses to such stress. These responses, together with behavioral strategies, are expected to play essential roles in *Buergeria* frogs inhabiting hot spring environments.

Transcriptional and proteomic responses of anurans to cold or freezing temperatures have been previously characterized, and genes and proteins related to heat shock, ubiquitin-proteasome, antioxidation, and glycogenolysis were identified in response to low-temperature stress (Kiss et al., 2011; Nagasawa et al., 2013; do Amaral et al., 2020). However, to the best of our knowledge, transcriptome analysis related to heat stress has not been reported, presumably due to the lack of well-known heat-tolerant frog species. Therefore, *Buergeria* frogs, which are known to inhabit hot springs and exhibit high thermal tolerance, are suitable subjects for studying the transcriptional response to explicit heat stress.

Given their avoidance of high-temperature waters (Wu and Kam, 2005; Komaki et al., 2020), *Buergeria* frogs may be less exposed to long-term heat stress. Rather, it may be more important for these frogs to endure temporary heat stress while moving away from the stressor. In this study, to further understand the molecular mechanisms and strategies that function in response to heat stress, we performed a comparative transcriptome analysis of heat-treated and untreated *B. otai* tadpoles.

## MATERIALS AND METHODS

### Sample Preparations

We collected *B. otai* adult females and males in Hualien, Taiwan in August 2013. The adult individuals were kept in a laboratory where they then laid eggs. It is unclear whether the eggs were

laid by a single female-male pair or not. Eggs were transferred to approximately  $25^{\circ}\text{C}$  water tanks ( $\sim 50$  L) with filtration systems and 9:15 light-dark cycle. The water temperature was chosen according to previous studies (Chen et al., 2001; Wu and Kam, 2005). Hatched tadpoles were kept under the same condition until use in thermal experiments. The water was not replaced. Tadpoles were fed boiled spinach once a day.

Six tadpoles at Gosner stages 26–28 (Gosner, 1960) were moved into two 1000-mL glass beakers containing 500-mL of  $25^{\circ}\text{C}$  water and were maintained for a day in incubators. After an additional 12 h fasting, three tadpoles were directly moved into a glass beaker with  $38^{\circ}\text{C}$  water which is slightly cooler than their possible thermal limit ( $\sim 40^{\circ}\text{C}$ ) (Komaki et al., 2016a). We confirmed that all three tadpoles did not exhibit any abnormal behavior after a sudden temperature change. The other three tadpoles were moved into a beaker with  $25^{\circ}\text{C}$  water. We maintained the water temperatures of each beaker for approximately 4 h using incubators, and then tadpoles were euthanized by pithing immediately after they were taken out from the water. Bodies were transferred into a RNAlater Stabilization Solution (Thermo Fisher Scientific, MA, United States).

Total RNA was extracted from the whole-body using a RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA samples were shipped to BGI (Beijing, China), where RNA-seq was performed using Illumina Hi-Seq 2000 (Illumina, CA, United States).

Since amphibian tadpoles were not subject to ethical review in Hiroshima University where the experiment was conducted at that time, no institutional ethical approval was obtained. This study was carried out in compliance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.<sup>1</sup> In the experimental procedures using living tadpoles, we made an effort to minimize stress of individuals following Guidelines for Live Amphibians and Reptiles in Field and Laboratory Research developed by the American Society of Ichthyologists and Herpetologists in 2004.

### Transcriptome Analyses

Raw RNA-seq data went through read filtering based on base quality and read length using Trim Galore v0.6.4 with default settings (phred score threshold of 20 and length threshold of 20 bp). Then, *de novo* assembly was performed using Trinity v2.8.3 (Grabherr et al., 2011), and summary statistics of the assembly were calculated by SeqKit v0.13.2 (Shen et al., 2016). Completeness of the assembly was assessed using BUSCO version 5.0.0 (Seppey et al., 2019) specifying tetrapoda\_obd10 as a reference. Pseudoalignment and transcript quantification was performed using kallisto v0.45.1 (Bray et al., 2016) with 100 bootstraps. Output files of kallisto were imported into the R environment using the R package "tximport" v1.10.1 (Soneson et al., 2016), and counts of reads mapped to each contig were normalized by the trimmed means of M (TMM) values method. A differential expression analysis was then performed using the R package "edgeR" v3.24.3 (Robinson et al., 2010) in R 3.6.3 (R Foundation). A likelihood ratio test based on TMM values

<sup>1</sup><https://arriveguidelines.org>

was applied in the analysis. Transcripts with an FDR-corrected  $p$  ( $q$ -value)  $\leq 0.05$  and an absolute value of  $\log_2$  fold change  $\geq 3$  were defined as differentially expressed transcripts.

## Annotation of Differentially Expressed Transcripts

To annotate differentially expressed transcripts, we first searched candidate coding regions in each contig of differentially expressed transcripts using Transdecoder v5.5.0 with default settings and the “-single\_best\_only” option so that only the single best candidate region per transcript was considered (Haas et al., 2013). We then scanned all predicted protein sequences for homology in the Ensembl reference protein sequence of *Xenopus tropicalis* (v9.1) using local BLASTP v2.10.1 + (Altschul et al., 1990). We used the default settings and set the  $e$ -value threshold to  $10^{-5}$ , and only the top hits for each contig were considered in the downstream analyses.

## Functional Analyses

For an overview of the transcriptomic change in response to the heat stress, we performed a protein-protein interaction (PPI) analysis and functional enrichment analyses based on transcripts that were differentially expressed and annotated by BLASTP search.

A web database STRING (Szklarczyk et al., 2019) was used for the PPI analysis, specifying *X. tropicalis* as the target organism with default settings. Annotations of both upregulated and downregulated transcripts obtained from BLASTP annotation using *X. tropicalis* as a reference were analyzed together in a single PPI analysis. The obtained pairwise PPIs were further analyzed with the R package “igraph” v1.2.5 (Csardi and Nepusz, 2006) to detect hub proteins in the PPI networks. We adopted two different criteria of measurements, including degree centrality and betweenness centrality, to identify hub proteins as applied in Chen et al. (2019) and Mao et al. (2020). The degree centrality method measures the number of interactions each protein participates in, and betweenness centrality measures how often the shortest paths connecting two other proteins pass through the given protein. Proteins with a higher score of degree centrality or betweenness centrality were regarded as more important hubs in the networks.

We conducted enrichment analyses implemented in STRING database to identify gene ontologies, pathways, keywords, domains, and features overrepresented by differentially expressed and BLASTP-annotated transcripts. The available frameworks in the STRING analysis were GO (Gene Ontology) annotations, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, Uniprot keywords, Reactome pathways, Pfam protein domains, SMART (Simple Modular Architecture Research Tool) protein domains, and InterPro protein features. We performed three independent analyses: upregulated transcripts, downregulated transcripts, and all differentially expressed transcripts (upregulated and downregulated transcripts). In the analyses, strength and Fisher’s exact test  $p$ -values were calculated, and the significance threshold was set to FDR-corrected  $p$ -value  $< 0.05$ . Strength is the  $\log_{10}$ -transformed number of

proteins belonging to a certain category divided by the expected number of proteins belonging to the category when proteins were randomly extracted from the database and indicates the degree of enrichment.

## RESULTS

### De novo Assembly and Expression Analysis

In total, 106,079,672 reads were obtained from RNA-seq of six tadpoles’ whole body and were applied for *de novo* assembly (Figure 1 and Supplementary Table 1). We assembled 299,588 contigs with an average length of 979.6 bp (range: 165–60,421 bp) and N50 of 2,272 bp. Estimated completeness of the assembly was 92.1% (40.7% single-copy and 51.4% duplicated BUSCOs). Fragmented and missing BUSCOs were 2.2 and 5.7%, respectively (Supplementary Table 1).

In the quantification of transcripts, 17,679,945 reads, on average, were processed per sample, 92% of which were pseudoaligned onto any contigs. Based on the log-likelihood ratio test  $q$ -value and  $\log_2$  fold change thresholds from differential expression analyses, we found 147 and 235 significantly upregulated and downregulated transcripts in response to the heat stress, respectively (Figure 2 and Supplementary Table 2). There were 131 and 190 coding regions predicted by Transdecoder from 147 upregulated and 235 downregulated transcript sequences, respectively.

### Annotations and Protein-Protein Interactions

The local BLASTP using *X. tropicalis* as a reference protein sequence annotated 86.7% (113 upregulated and 165 downregulated transcripts) of Transdecoder-predicted

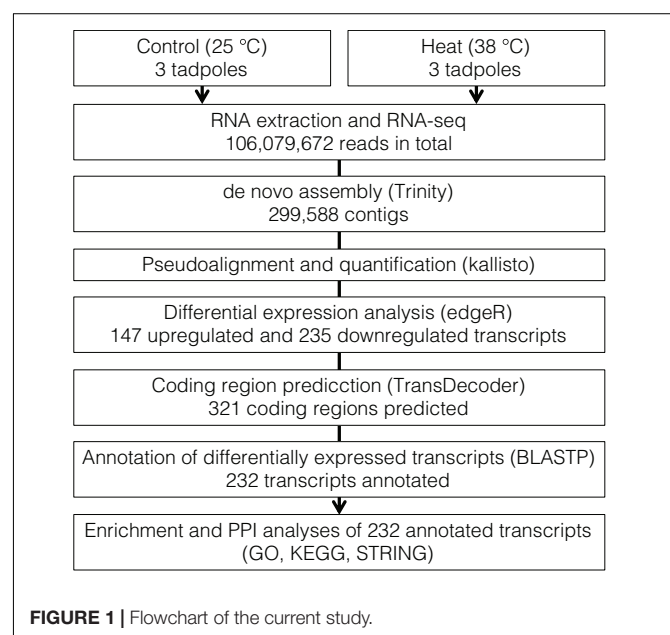
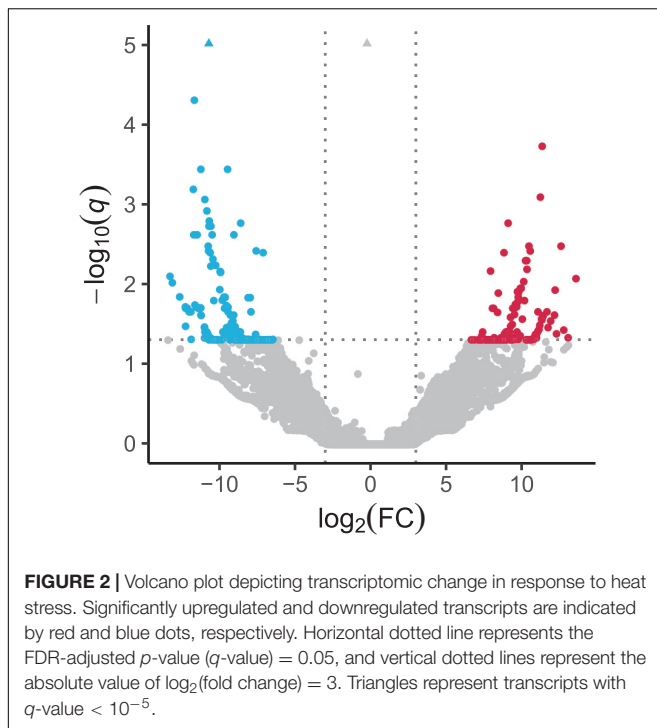


FIGURE 1 | Flowchart of the current study.



coding sequences. There were overlapped annotations among transcripts, and the unique number of annotations was 232.

In the STRING PPI analysis, among 232 unique genes with differentially expressed levels and BLASTP annotations, 64 had protein interactions (**Figure 3**). The number of protein interactions was significantly larger than expected according to the STRING network stats (PPI enrichment  $p = 2.2 \times 10^{-3}$ ). In total, 13 networks were formed. Members of proteins with the highest degree centrality and betweenness centrality were similar to each other; GAK, DDX3X, and KMT2B exhibited degree centrality and betweenness centrality scores within the top five (**Supplementary Table 3**) and belonged to the largest network. The second-largest PPI network consisted of 10 proteins, and FBXW4 served as a hub protein.

## Function of Hub and Related Proteins

Integrating the gene functions, six subgroups were identified from the PPI networks: heat shock protein (HSP), mitochondrial fission, translation regulation ( $\times 2$ ), methyltransferase, and ubiquitination.

Heat shock protein subgroup contained three proteins of GAK, HSPA1B, DNAJA1. GAK, a hub protein in the network, is a member of the DnaJ/HSP40 family. DNAJA1, closely located to GAK on the network, is also a member of the family. HSPA1B, which had PPI interactions with GAK and DNAJA1, belongs to the HSP70. mRNA expression levels of these proteins were downregulated in response to the heat stress.

DNM1L, which had an interaction with GAK, mediates mitochondrial fission. Upregulation of this gene was also reported in a cow and mouse in response to heat stress (Yu et al., 2018; Chen et al., 2020). Under the heat stress, DNM1L-induced

mitochondrial fission does not play a protective role for the cell but leads to apoptosis (Yu et al., 2016).

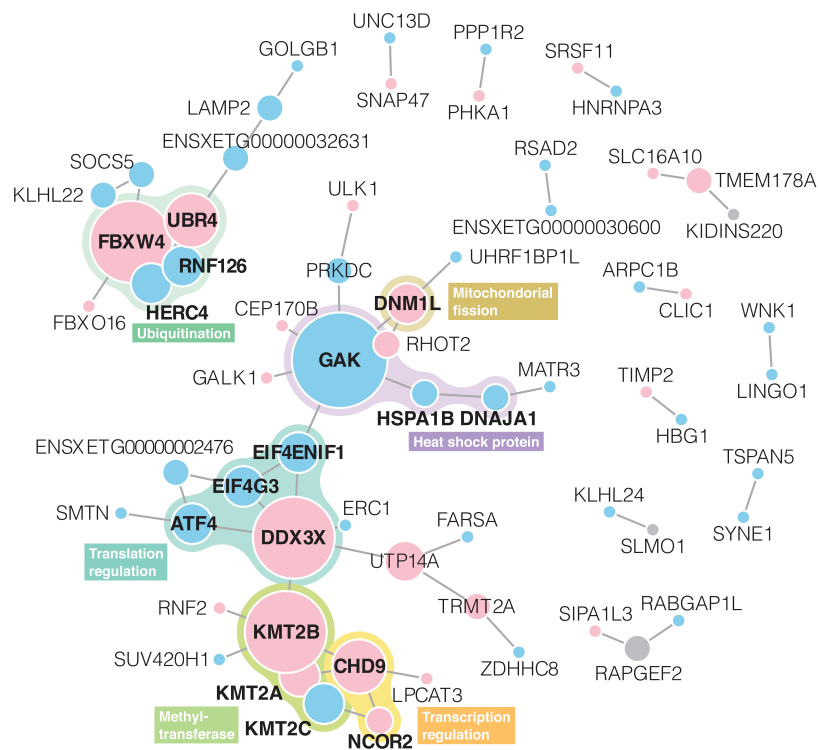
The translation regulation subgroups were found in two non-directly linked regions. One subgroup contained DDX3X, ATF4, eIF4G3, and eIF4ENIF1. DDX3X which was identified as a hub protein of this subgroup is an important component for stress granule assembly and molecular dynamics (Hilliker et al., 2011). Stress granules are dense aggregations of RNAs and RNA-binding proteins. It is formed in the cytosol when the cell is exposed to stress and is expected to have a protective function for the cell through mRNA translation regulation including, sequestration, degradation, and translation re-initiation of mRNA (Lake et al., 2016). ATF4 is a master transcription factor during the stress response. For the mRNA expression of *ATF4*, the binding of DDX3X to the eIF4F complex is required (Adjibade et al., 2017). The downregulated expression level of *ATF4* detected in this study thus can be involved in the possible suppression of the eIF4F complex formation under the heat stress. eIF4F (eukaryotic initiation factor 4F) is a translation initiation factor, and consists of eIF4E, eIF4G, and eIF4A. eIF4G3 is an isoform of eIF4G, and its expression level was downregulated in *B. otai* tadpoles. eIF4ENIF1, a transporter of eIF4E, also exhibited a downregulated expression level in response to the heat stress. It was demonstrated that eIF4F formation is disrupted, and subsequently, mRNA translation is inhibited in mice under heat stress so that protein synthesis and subsequent possible misfolding can be prevented (Cuesta et al., 2000). The other translation regulation subgroup contained CHD9 and NCOR2, which were closely located to the KMT2 family in the PPI network, are known as transcriptional regulators.

In the methyltransferase subgroup, three proteins (KMT2A–C) of the lysine methyltransferase 2 (KMT2) family were included. This family mainly functions as a histone methyltransferase and is thus known as a transcriptional regulator. In response to the heat stress, *KMT2A* and *KMT2B* were upregulated whereas *KMT2C* was downregulated.

Ubiquitination subgroups including FBXW4, UBR4, RNF126, and HERC4 formed an independent PPI network. They are ubiquitin-related proteins and known to promote ubiquitination and degradation of proteins (O'Leary et al., 2016; UniProt Consortium, 2019). All of these proteins are E3 ubiquitin-protein ligases, however, expression patterns were different: *FBXW4* and *UBR4* were upregulated and *HERC4* and *RNF126* were downregulated in response to the heat stress.

## Enrichment Analyses

Enrichment analyses performed on upregulated proteins demonstrated that three Pfam, four InterPro, and four SMART categories were enriched, which are involved in methylation and other expression regulations, and no GO term and KEGG pathway were enriched (**Supplementary Table 4**). Methyltransferase genes, such as *KMT2A/B*, were involved in the enriched categories. In the downregulated protein analyses, only two UniProt categories (coiled coil and kelch repeat), which were involved in expression regulation and ubiquitination, were enriched (**Supplementary Table 4**). Three proteins, ENC1.2, KLHL22, and KLHL24, and 32 proteins (e.g., eIF4G3, *KMT2C*,



**FIGURE 3 |** Protein-protein interaction networks derived from the STRING web database. Node size represents degree centrality (**Supplementary Table 2**). Gene symbols obtained in BLASTP annotation using *X. tropicalis* as a reference are presented in each node. Red and blue nodes represent proteins with upregulated and downregulated expressions, respectively. Gray nodes indicate that both upregulated and downregulated transcripts shared the annotations. Proteins written in bold letters are those mentioned in the main text.

and HSPA1B), were involved in the coiled-coil and kelch repeat, respectively. There was no overlapping category between the upregulated and downregulated protein analyses. The enrichment analyses performed on all differentially expressed proteins demonstrated 16 overrepresented pathway, keywords, domains, and features while no enriched GO term was detected (**Table 1**). All of them, except for the coiled-coil and kelch repeat, can be directly or indirectly linked to methyltransferase activity.

## DISCUSSION

Through a comparative transcriptomic analysis, we identified hundreds of transcripts differentially expressed between heat-treated and control tadpoles of *B. otai*. Knowledge-based analyses imply that our transcriptome analyses detected sets of genes with important functions in coping with the heat stress. Specifically, the PPI network analysis identified hub proteins with functions such as HSPs, stress granule assembly, methylation, and ubiquitination.

### Transcriptional and Translational Regulation Mechanisms

First, the transcriptional response to the environmental stimuli is led by epigenetic regulation mechanisms. This study of comparative transcriptional analyses identified

genes, pathways, domains, and features involved in different epigenetic mechanisms like DNA and histone methylation. Methyltransferase itself does not have a specific target of genes but is involved in the global change of DNA and histone methylation status. Subsequently, it can cause the transcriptional change of genes. It is not clear how many differentially expressed genes identified in this study are regulated by the methyltransferase activity. However, expression levels of all genes mentioned in the results section, except for *DDX3X* on the X chromosome, are associated with DNA methylation levels of neighboring DNA methylation sites in humans (Komaki et al., 2018).

Expression levels of *KMT2A* and *KMT2B* were upregulated in response to heat stress, whereas that of *KMT2C* was downregulated. The contrasting expression patterns may be explained by the structural and functional differences between them. For example, the KMT2 enzymes can be distinguished by the number of PHD fingers: *KMT2A/B* contain four PHD fingers and have a similar overall domain structure. *KMT2C*, on the other hand, contains eight PHD fingers, which is the largest number among the known KMT2 enzymes (Ali et al., 2014). These structural differences will lead to functional differences. It is known that *KMT2A/B* and *KMT2C* have unique subunits, which are known to be associated with differences of binding regions: *KMT2A/B* are suggested to bind to the promoter and enhancer regions, while *KMT2C* binds to the enhancer

**TABLE 1** | Results of STRING functional enrichment analyses based on combined dataset of upregulated and downregulated proteins.

Framework	ID	Description	Strength	q-value
KEGG pathways	xtr00310	Lysine degradation	1.04	0.0339
Annotated keywords (UniProt)	KW-0489	Methyltransferase	0.90	0.0233
	KW-0949	S-adenosyl-L-methionine	0.84	0.0304
	KW-0175	Coiled coil	0.24	0.0048
	PF05964	F/Y-rich N-terminus	1.93	0.0041
Protein Domains (Pfam)	PF05965	F/Y rich C-terminus	1.93	0.0041
	PF00856	SET domain	1.27	0.0075
	IPR003888	FY-rich, N-terminal	1.93	0.0070
Protein domains and features (InterPro)	IPR003889	FY-rich, C-terminal	1.93	0.0070
	IPR003616	Post-SET domain	1.49	0.0269
	IPR034732	Extended PHD (ePHD) domain	1.31	0.0463
	IPR001214	SET domain	1.16	0.0269
	SM00542	FY-rich domain, C-terminal region	2.03	0.0010
Protein domains (SMART)	SM00541	FY-rich domain, N-terminal region	1.93	0.0010
	SM00508	Cysteine-rich motif following a subset of SET domains	1.49	0.0048
	SM00317	SET [Su(var)3-9, Enhancer-of-zeste, Trithorax] domain	1.25	0.0034

Strength represents the degree of enrichment, and the q-value is the FDR-corrected p-value.

region (Rao and Dou, 2015). The CXXC domain, unique to KMT2A/B, also contributes to the binding of the enzymes to CpG sites (Rao and Dou, 2015). Taken together, the contrasting expression pattern of *KMT2* genes implies that specific regulatory mechanisms, such as DNA methylation, may be responsive to heat stress.

In addition to methyltransferase activity, we identified differentially expressed genes involved in the activation and suppression of transcription and translation. NCOR2 is a transcriptional co-suppressor, and it was upregulated under heat stress. Conversely, genes involved in forming the eIF4F complex, a translation initiation factor, were downregulated. These results imply that gene transcription and translation are globally suppressed in *B. otai* tadpoles under heat stress. Presumably, the mechanisms contribute to the prevention of misfolding protein synthesis under heat stress. The upregulation of *DDX3X*, a stress granule component, is representative of the hypothesis. Stress granule formation is a well-known cellular stress response and prevents RNA from being translated.

*CHD9* upregulation is also notable. The gene belongs to the chromodomain helicase DNA-binding (CHD) family. CHD is a chromatin remodeler that contributes to chromatin structural change and thus, affects chromatin accessibility. Different from the above-mentioned proteins that can change the global DNA transcription and RNA translation pattern, *CHD9* is suggested to have a specific target of genes related to osteogenesis, such as *RUNX2* (Newton and Pask, 2020). Indeed, it is well documented that heat stimulus promotes osteogenesis (Chen et al., 2013; Ikuta et al., 2015), and it is conceivable that *CHD9* participates in the process. However, it is unclear whether the *CHD9*-mediated osteogenesis can be linked to the protective function against heat stress. Since water temperature is known to affect the timing of metamorphosis (Maciel and Juncá, 2009; Goldstein et al., 2017), the upregulation of *CHD9*

possibly be related to the body plan change associated with accelerated metamorphosis.

## Protein Degradation

We also identified ubiquitination-related proteins that formed an independent PPI network. Proteins damaged by denaturation, such as heat stress are degraded by proteasomes, and the process is usually mediated by ubiquitination (Hershko, 1996). Transcriptional upregulation of ubiquitination-related genes was also identified in two comparative transcriptome studies in which amphibians were exposed to cold stress (Nagasawa et al., 2013; do Amaral et al., 2020). Thus, in combination with above-mentioned protein synthesis suppression, ubiquitination-mediated protein degradation may contribute to reducing dysfunctional proteins.

There were four differentially expressed genes of E3 ubiquitin-protein ligases, although *RNF126* and *HRC4* were downregulated, while *FBXW4* and *UBR4* were upregulated in response to the heat stress. There are hundreds of known E3 ligases and each ligase is thought to have specific substrate, however, the relationship is still not well understood, and it is also not known how ligases recognize misfolded proteins. In addition, only a limited number of E3 ligases are known to participate in the misfolded protein ubiquitination (Chhangani et al., 2013), and various other functions have been proposed for ubiquitination, such as DNA repair, signal transduction, and transcriptional regulation (Wilkinson et al., 2005). Therefore, we cannot provide a clear explanation for the up- and downregulations of each E3 ligase gene in response to the heat stress. However, the contrasting expression patterns strongly suggest that these genes play different roles under heat stress.

## Heat Shock Proteins

The HSP family is the most famous protein family produced under heat stress. Three genes belonging to HSP40 and

HSP70 were differentially expressed in *B. otai* tadpoles but were all downregulated in heat-treated individuals. Since HSP40 is a co-chaperone of HSP70, it is probable that they demonstrated the same transcriptional change pattern. However, we have no clear insights to explain why they were downregulated in response to heat stress. Contrary to the current study, several frog species exposed to cold stress exhibited upregulation of HSP70 (Kiss et al., 2011; do Amaral et al., 2020). Although the upregulation of HSP70 in those species is expected to function in the degradation of proteins misfolded due to the stress, it cannot explain the downregulation of HSP70 under the heat stress, which also leads to protein misfolding. The downregulation of HSP genes in response to heat stress (21°C compared to 11°C) was also observed in a salamander species (Clay et al., 2019). However, upregulation of the other HSP genes were simultaneously observed and the responses of these specific genes have not been discussed in the previous study. HSPs have various functions, several of which change the responsiveness according to the duration of the stress (Gormally and Romero, 2020). Furthermore, they can be controlled by negative feedback. These gene-specific functions and regulations may underlie the suppression of HSP genes in response to heat stress.

## Suppressive Transcriptional Response Against Heat Stress

It has been demonstrated that tadpoles prefer cooler water (Wu and Kam, 2005; Komaki et al., 2020) and the water temperature in hot springs can drop at night due to the lower air temperature; thus, tadpoles can avoid long-term exposure to heat even in hot spring environments. Rather, it probably important for them to tolerate the short-term heat spike. The present study showed that the majority of hub proteins identified in this study had functions related to suppression of protein synthesis and subsequent prevention of protein misfolding. These responses should negatively impact tadpole development if the suppression persists. Therefore, although not verified, the mechanisms are not likely to be sustained under long-term heat stress. That is, *Buergeria* tadpoles may adopt a combination of suppressive transcriptional responses to tolerate occasional heat stress and avoidance behavioral response to prevent long-term heat exposure.

Suppression of protein synthesis under heat stress is not a mechanism specific to hot spring tadpoles but a widely conserved mechanism. Thus, the mechanisms identified in this study were not evolutionarily acquired in *Buergeria* lineages. Many anuran species lay eggs in temporally formed shallow water pools, such as paddy fields, whereby water temperatures can reach 36°C (Maruyama et al., 2017). Therefore, although the present study employed rapid and acute heat stress and did not take into account acclimation effects that may occur in the field, a wide range of anuran species may have a similar transcriptomic response to heat stress as that observed in *B. otai* tadpoles.

## Limitations

It should be noted that the sample size in this study is small. Therefore, it is possible that the set of differentially expressed genes may change when the sample is increased. To reduce the uncertainty arising from the small sample size, we mainly focused on the summarized characteristics of upregulated and downregulated gene sets obtained from enrichment and PPI network analyses.

It is also important to mention that this study only focused on annotated transcripts. This means that even if there are novel heat stress response-related transcripts specific to *Buergeria* frogs, they cannot be identified in this study design. Since RNA-seq data obtained in this study is publicly available, it will possible to approach this point in future studies.

In addition, RNA was extracted from the whole body. Thus, genes showing tissue- or cell type-specific heat stress response patterns are unlikely to be detected in this study. The differentially expressed genes identified in this study are genes whose expression patterns do not conflict among tissues or cell types.

## DATA AVAILABILITY STATEMENT

RNA-seq data obtained in this study are deposited in DDBJ Sequence Read Archive (DRA) (BioProject accession number: PRJDB12187). Codes used in this study are available on GitHub ([https://github.com/RolyPolyCoily/BuergeriaOtai\\_2021](https://github.com/RolyPolyCoily/BuergeriaOtai_2021)).

## ETHICS STATEMENT

Ethical review and approval was not required for the animal study because amphibian tadpoles were not subject to ethical review in Hiroshima University where the experiment was conducted at that time; therefore, no institutional ethical approval was obtained.

## AUTHOR CONTRIBUTIONS

SK designed the study, carried out field and laboratory works and data analyses, and led the writing of the draft. MM carried out data analyses and participated in the writing of the draft. J-WL, K-HL, Y-PL, and YL participated in sample collection and critically revised the manuscript. S-ML coordinated the field work, participated design of the study, and critically revised the manuscript. TI designed the study, carried out laboratory works and data analyses, and revised the manuscript. All authors gave final approval for publication.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.706887/full#supplementary-material>

**Supplementary Table 1** | Summary of RNA-seq and subsequent analyses.

**Supplementary Table 2** | List of transcripts differentially expressed between heat-treated and control tadpoles with analytical results and annotations.

**Supplementary Table 3** | Scores of degree centrality and betweenness centrality for each protein mapped on STRING protein-protein interaction networks.

**Supplementary Table 4** | Results of STRING enrichment analyses.

**Supplementary Text 1** | Methods and results of additional annotations.

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