Received: 18 January 2018 | Revised: 23 April 2018 | Accepted: 26 April 2018

DOI: 10.1002/jez.2175

RESEARCH ARTICLE

WILEY JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY

Developmental plasticity in reptiles: Insights from temperature-dependent gene expression in wall lizard embryos

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Abstract

Many features of the development of reptiles are affected by temperature, but very little is known about how incubation temperature affects gene expression. Here, we provide a detailed case study of gene expression profiles in common wall lizard (*Podarcis muralis*) embryos developing at stressfully low (15°C) versus benign (24°C) temperature. For maximum comparability between the two temperature regimes, we selected a precise developmental stage early in embryogenesis defined by the number of somites. We used a split-clutch design and lizards from four different populations to evaluate the robustness of temperature-responsive gene expression profiles. Embryos incubated at stressfully low incubation temperature expressed on average 20% less total RNA than those incubated at benign temperatures, presumably reflecting lower rates of transcription at cool temperature. After normalizing for differences in total amounts of input RNA, we find that approximately 50% of all transcripts show significant expression differences between the two incubation temperatures. Transcripts with the most extreme changes in expression profiles are associated with transcriptional and translational regulation and chromatin remodeling, suggesting possible epigenetic mechanisms underlying acclimation of early embryos to cool temperature. We discuss our findings in light of current advances in the use of transcriptomic data to study how individuals acclimatize and populations adapt to thermal stress.

KEYWORDS

embryo, epigenetics, gene expression, lizards, thermal plasticity, transcriptome

1 | INTRODUCTION

Temperature profoundly influences nearly all developmental processes and biological functions. While the embryos of live-bearing endotherms experience a constant temperature throughout their developmental period, ectothermic embryos often have to cope with a wide range of temperatures (Birchard, 2004). For example, embryos of egg-laying reptiles are likely to be exposed to thermal stress in the nest. This includes daily fluctuations and prolonged exposure to very high or low temperatures, the latter sometimes for weeks in a row as a result of low ambient temperature or limited solar irradiation in temperate regions (Berglind, 2000; Löwenborg, Shine, Kärvemo, & Hagman, 2010; While et al., 2015). This is important, because low temperature reduces the speed of biochemical reactions, and thus slows down growth and differentiation (Angilletta, 2009; Gillooly, Charnov, West, Savage, & Brown, 2002). Consequently, cool-incubated eggs hatch later than warm-incubated eggs and will therefore experience a shorter first growing season (Le Henanff, Meylan, & Lourdais, 2013; Löwenborg, Gotthard, & Hagman, 2012; While et al., 2015). Incubation at low temperatures can also lead to the disruption of biological processes or, in extreme cases, embryonic mortality (e.g., Shine, Madsen, Elphick, & Harlow, 1997).

While there is evidence that temperature stress impairs ectotherm development, we still know very little about the underlying mechanisms and the extent to which physiological changes may buffer such damage. Studies of short-term thermal stress indicate that gene expression can be modulated to minimize damaging effects of extreme temperatures. For example, zebrafish larvae exposed to both critically cool or hot temperatures upregulate a number of heat shock protein (hsp) genes, including *hsp90* and *hsp70* (Long, Li, Li, He, & Cui, 2012), which assist the folding of proteins that would otherwise be denatured. In addition to this general stress response, critically cool temperatures also cause expression of specific pro-inflammatory cytokines in zebrafish and common carp, such as *cirbp* (cold inducible RNA binding protein) and *hmgb1* (high-mobility group box 1; Chou et al., 2008; Gracey et al., 2004; Long et al., 2012). Studies on the impact of more prolonged exposure to sub-critical temperatures are currently lacking, but it is reasonable to assume that such temperatures also have the potential to trigger adaptively plastic responses that ensure that development can be sustained.

Despite the fact that developmental plasticity has been reported for a diverse range of functional traits across a wide range of reptile taxa (reviewed in Noble, Stenhouse, & Schwanz, 2018; Warner, 2014), surprisingly little is known about how gene expression varies with incubation temperature in reptilian embryos. The one exception to this is sex determination, where existing transcriptomic data come from studies aiming to reveal the genetic basis of temperature-dependent sex determination in turtles, crocodiles, and lizards (Czerwinski, Natarajan, Barske, Looger, & Capel, 2016; Deveson et al., 2017; Radhakrishnan et al. 2017; Yatsu et al., 2016). However, since these studies targeted exclusively gonadal tissue, their findings do not allow conclusions to be drawn about systemic responses to incubation temperature.

To address this, we used RNA sequencing (RNA-seq) to characterize the transcriptomic responses of common wall lizard (*Podarcis muralis*) embryos exposed to sustained cool temperature. Wall lizards have a long history in studies of responses to thermal extremes. Indeed, adult wall lizards were among the first vertebrates demonstrated to be capable of freeze-tolerance (Claussen, Townsley, & Bausch, 1990; Weigmann, 1929). While this suggests substantial cold tolerance in the adult life stage, embryos are strongly affected by low temperature (Cooper, 1958, 1965; While et al., 2015). As in many other lizards, wall lizard embryos begin their development before the eggs are laid, which means that early thermal conditions reflect maternal thermoregulation. However, following oviposition in spring, soil temperatures can be substantially lower and embryos may need to maintain biological function and develop at temperatures well below 20°C. For example, thermal profiles of nests in non-native populations in England frequently average below 20°C, with only brief periods of time at the higher temperatures typically used to incubate eggs in captivity (While et al., 2015). Similar climatic conditions occur at high altitude or latitude in their native range and, even within the warmer parts of the species' distribution, soil temperatures could stay well below 20°C for days on end.

Here, we used animals from four native populations of wall lizards, two from France and two from central Italy, to assess the effects of low incubation temperature on gene expression in early embryos, and to test how conserved temperature-specific gene expression is across populations. We found that a large proportion of the transcriptome is differentially expressed at 15°C versus 24°C, and that this response is highly conserved across the geographic scale of our study. We identify four main types of responses: gene expression regulation, protein folding, epigenetic modification, and cell-cycle regulation. Thus, our transcriptomic study allows the formulation of more precise hypotheses for further studies on the molecular mechanisms underlying thermal acclimation and adaptation.

2 | MATERIALS AND METHODS

2.1 | Experimental design and egg incubation

The common wall lizard (*Podarcis muralis*) is widely distributed in central Europe. Oviposition occurs from spring to early summer and females typically bury their eggs into soil between 5 and 10 cm deep (Gruschwitz & Böhme, 1986). We selected four populations from the species' native distribution, two from France and two from central Italy. These two regions are inhabited by lizards belonging to two of the main genetic lineages of this species, which diverged about 2.5 million years ago (Gassert et al., 2013; Michaelides, While, Zajac, & Uller, 2015). In April 2015, we collected 13 gravid females from Western France (around Pouzagues [46.788 N, −0.837 E] altitude: 258 m; will be referred to as Fr1), 15 from the French Pyrenees ([42.860 N, 1.105 E]; altitude: 1396 m; Fr2), 18 from lowland Tuscany (Greve in Chianti [43.588 N, 11.318 E], Colle di Val d'Elsa [43.428 N, 11.118 E], Certaldo [43.548 N, 11.042 E]; altitude: 83–245 m; It1), and 15 from the Tuscan Apennines (around Saltino [43.727 N, 11.538 E]; altitude: 966 m; It2). Although these populations experience different climatic conditions, partly as a result of differences in latitude and altitude, we did not necessarily expect any of them to be adapted to very low temperature, in contrast to the previously studied non-native populations in England (Feiner, Rago, While, & Uller, 2018; While et al., 2015).

Gravid females were transported to the animal facility at the Oxford University, UK, and all procedures were conducted according to the University of Oxford's Local Ethical Review Process and the UK Home Office (PPL: 30/2560). Females were kept individually in cages under standard conditions (cage size: 590 × 390 × 415 mm; sand as substrate; 12L:12D light cycle; access to basking light [60 W] for 8 hr per day and UV light [EXO-TERRA 10.0 UVB fluorescent tube] for 4 hr per day; access to mealworms, crickets, and water *ad libitum*). We inspected cages twice a day for laid clutches to retrieve eggs within 12 hr after oviposition. Since variation of developmental stages within a clutch is negligible (T.U., pers. obs.), we immediately dissected one egg per clutch to determine the developmental stage of its siblings at laying. Remaining eggs were divided into two groups and incubated in small plastic containers filled two-thirds with moist vermiculite (volume ratio 5:1 vermiculite:water) sealed with clingfilm. One half of the eggs were incubated at 15°C (cool) and the other half was incubated at 24°C (warm). The cool temperature is likely to be commonly experienced for sustained periods in parts of the species' distribution and is above the critical minimum temperature for development. This exact temperature has not been systematically addressed in *P. muralis* embryos, but it is likely to be below 12°C (Cooper, 1965). However, if embryos are incubated constantly at 15°C, they are not able to hatch, even if they develop full-term (Cooper, 1958, 1965; While et al., 2015). The warm treatment is within the optimal incubation temperature judged from high hatching success, low variance in incubation duration, and low incidence of scale abnormalities (Braña & Ji, 2000; Van Damme, Bauwens, Braña, & Verheyen, 1992; While et al., 2015).

2.2 | Embryo collection

To compare gene expression profiles across temperatures and populations, it is necessary to keep ontogenetic variation in gene expression to a minimum. We therefore applied somite counts to determine developmental age instead of the more coarse-grained conventional classification of developmental stages (e.g., Dufaure & Hubert, 1961). Since somites are added at a constant rate at a given temperature (Pourquie, 2003), the count of somites at oviposition can be used

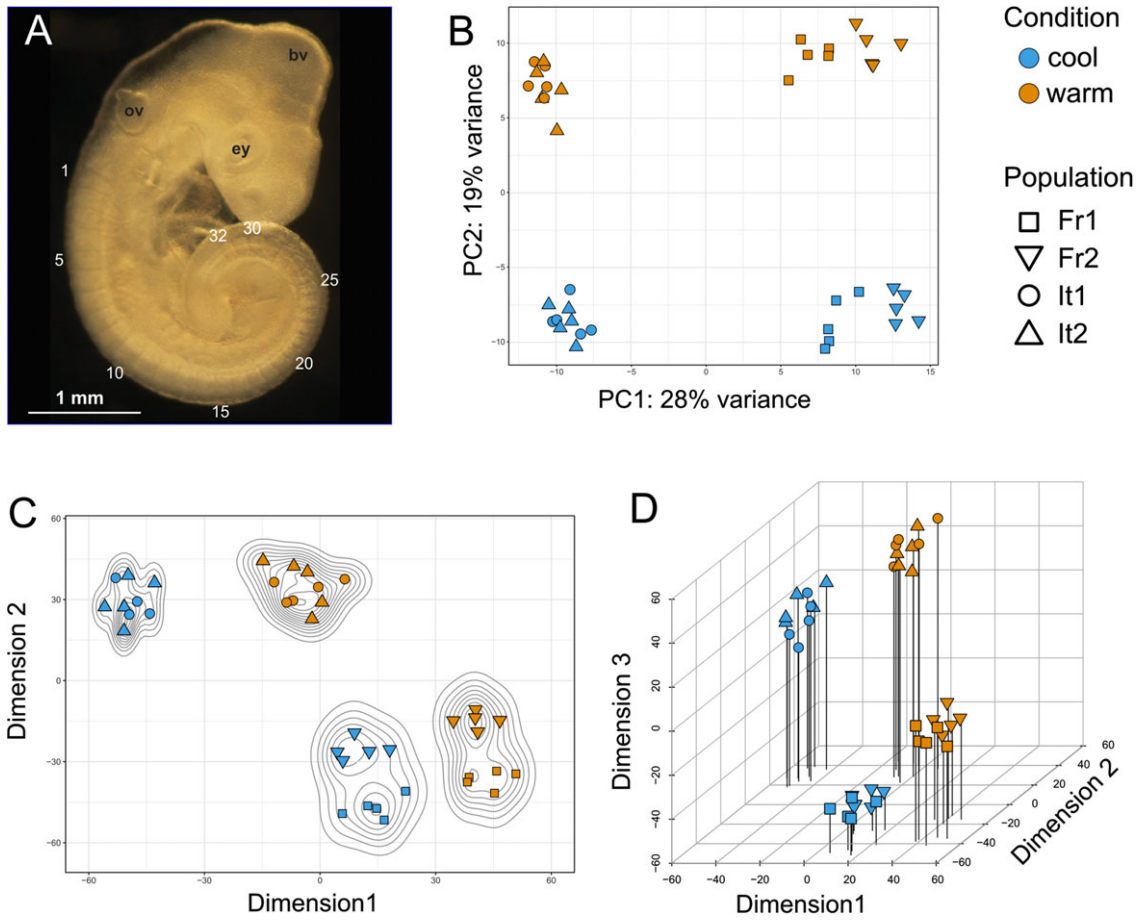

FIGURE 1 Targeted embryonic stage and broad patterns of variation in the dataset. (A) A common wall lizard embryo with 32 somites, corresponding to the pharyngula stage. (B) Plot representing the first and second principal components of expression levels. Major differences are seen between incubation temperatures (cool in blue, and warm in orange color), and between the four populations (highlighted by different shape outlines). The first principal component captures geographical differentiation and represents 28% of the total variance, and the second principal component marks differences due to incubation temperature explaining 19% of the total variance. (C and D) Sample distances reduced to a simplified 2D (C) and 3D (D) transcriptome space using tSNE. Populations group together and separate clearly between cool and warm incubation temperatures in each. French populations 1 and 2 form distinct clusters, whereas both Italian populations show similar expression patterns in panels (B) and (C). bv, brain vesicle; ey, eye; ov, otic vesicle [Color figure can be viewed at wileyonlinelibrary.com]

to predict the time required to reach the desired somite count. The somite 'clock' rate for our study species was experimentally determined to be four somites per day at 24°C and four somites per 7 days at 15°C. We deemed a somite count of 32 ± 1 (corresponding to stage 27 according to Dufaure & Hubert, 1961; Figure 1a) appropriate for our purposes since this allowed the warm incubated embryos to develop for at least 12 hr (average 43.7 ± 3.1 hr SE), while requiring the cool incubated embryos to develop for an average of about 2 weeks (16.8 ± 1.1 days). We reasoned that 12 hr is a sufficient amount of time to acclimatize gene expression patterns because (i) the magnitude of temperature change that eggs experience from the warm nesting site to the 24°C incubation treatment is very small (average temperature of nest sites in laboratory conditions, 25°C, While et al., 2015), and (ii) gene expression has been shown to adjust rather quickly, in the order of 3–4 hr (Jovic et al., 2017). Based on our predictions, we selected eggs for dissection at regular intervals to ensure that a sufficient number of embryos of the targeted developmental age would be available for sequencing.

We further decreased the confounding variation among our samples by following a strict protocol. First, we performed dissections between paired embryos of a clutch at the same time of day (within a 1-hr interval) to minimize variation caused by diurnal patterns of embryonic gene expression (Seron-Ferre, Valenzuela, & Torres-Farfan, 2007). Second, all eggs were processed within 5 min of removal from the incubator to avoid changes in gene expression patterns. We dissected embryos from yolk and extraembryonic membranes in nuclease-free phosphate-buffered saline using sterile forceps under a dissecting microscope, counted their somites, and submerged them in RNAlater (Qiagen) to stabilize RNA for storage. Total RNA from a total of 80 individual embryos at the targeted developmental age was extracted by using the RNeasy Micro Kit (Qiagen). The quantity of harvested total RNA was measured with the Qubit® 2.0 Fluorometer system and the 'RNA BR Assay Kit' (Thermo Fisher Scientific), and RNA integrity was determined to score above an RNA quality indicator value of 9 with the Experion system using the 'Eukaryote Total RNA StdSens Analysis kit' (Bio-Rad).

2.3 | Transcriptome sequencing, assembly, and quantification of expression levels

For each of the eight groups (four populations, two incubation temperatures), we sequenced five samples consisting of a single embryo each ('single embryo'), and in addition one sample consisting of a pool of equimolar amounts of total RNA from five non-sibling embryos ('pooled embryos'), resulting in 48 samples. Including a pooled sample is useful for the *de novo* assembly of transcriptomes since they contain more sequence variation than samples from individual embryos (which in turn provide more sequencing depth). Two microgram high-quality total RNA per sample was submitted to RNA sequencing, which was performed by the Welcome Trust Centre, Oxford, UK, whose service included library preparation with the 'TruSeq Stranded mRNA Library Prep' kit (Illumina). Size selected and multiplexed libraries were paired-end sequenced (100 bp) over a total of 16 lanes on a HiSeq2000 Sequencing System (Illumina) by applying a balanced block design (Auer & Doerge, 2010). This produced on average 39.3 million raw reads per sample, and visualization of the quality of sequencing data using FastQC (Andrews, 2010) indicated high quality throughout the dataset.

For a detailed description of the bioinformatics pipeline applied, including lines of code that should facilitate analyzing RNA-seq data for inexperienced users, please refer to Supporting Information File 1. In brief, we removed low quality reads and low quality bases at the ends of reads using Trimmomatic version 0.32 (Bolger, Lohse, & Usadel, 2014). Since the French and Italian lineages have diverged over 2 million years ago (Gassert et al., 2013), we assembled two separate reference transcriptomes that were merged into one before we quantified gene expression levels. Each assembly was performed using the Trinity software version 2.3.2 (Haas et al., 2013). The resulting assemblies were de-duplicated using CD-HIT-EST version 4.6.5 (Li & Godzik, 2006). After pseudo-mapping all reads with kallisto (Bray, Pimentel, Melsted, & Pachter, 2016), lowly expressed transcripts (lower than one transcript per million reads [tpm]) were removed from the assemblies, and only the transcripts with the highest expression levels per gene were retained. To make expression profiles of French and Italian lizard embryos comparable, we assigned orthologs using the Proteinortho software version 5.15 (Lechner et al., 2011) and merged the two *de novo* assemblies into one shared assembly using a custom script.

To determine levels of gene expression for all 48 samples, we applied the pseudo-alignment strategy in kallisto that uses a probabilistic framework (Bray et al., 2016). Prior to analyzing abundances, we removed genes that had zero variance in gene expression across samples, and genes with fewer than five tpm in more than four samples. This resulted in a transcriptome comprising 22,074 transcripts with a N50 value of 2.764 bp. We refer to expressed sequences as transcripts in the technical sense but use the term 'genes' in more general discussions of the data and the results. Similarly, we equate quantified numbers of transcripts with levels of gene expression, although RNA degradation also shapes the pool of transcripts (implicitly assuming that the latter process is rather nonspecific with respect to the kind of transcript).

2.4 | Principal component analysis and clustering based on expression similarity

For visualizing broad pattern of variation in our dataset and confirming cohesion among our treatment groups, we applied two different approaches: a conventional global principal component analysis (PCA) and a local machine learning approach using a tree-based algorithm (t-distributed stochastic neighbor embedding, or tSNE). Both approaches use variance-stabilized expression data as input (Love, Huber, & Anders, 2014). We plotted the first and second principal components (Figure 1b) using the 'plotPCA' function as implemented in DESeq2 (Love et al., 2014). The machine learning approach was implemented using the R package 'Rtsne' (van der Maaten, 2014) which projects samples into 2D and 3D spaces maintaining distances between them similar to the original high-dimensional space (Figures 1c and 1d).

2.5 | Differential gene expression

To take full advantage of the probabilistic quantifications produced by the kallisto software, we applied sleuth (Pimentel, Bray, Puente, Melsted, & Pachter, 2017) for analyzing differential gene expressions. This software is considered superior to other methods (e.g., DESeq2) because it decomposes inferential variance (noise stemming from random sequencing and computational analysis) from biological variance and thus leads to a higher sensitivity in identifying differentially expressed genes (Pimentel et al., 2017). In addition to performing differential gene expression analysis using sleuth, and for ease of comparison to previous studies, we confirmed our results by using the more conventional software DESeq2. For this, we imported quantification data produced by kallisto using the R package tximport (Soneson, Love, & Robinson, 2015). Since sleuth and DESeq2 do not allow mixed effects, we fitted generalized linear models with 'clutch' as a fixed effect in order to take relatedness between siblings into account. Neither of the statistical frameworks allows nested factors, and we therefore treated the populations as four levels of the factor 'population', without specifically modeling effects of the genetic lineage. For all likelihood-ratio tests (LRTs) in sleuth, we applied a false discovery rate (FDR) adjusted q-value of 0.01, and an FDR adjusted P-value of 0.01 in DESeq2.

2.6 | Transcriptional cluster analysis

Compared with single-gene linear models, cluster analyses can help overcome the intrinsic problems of multiple-hypothesis testing, effectively increasing the power of a study. Clustering analyses also allow us to detect which expression patterns are prevalent in an experiment, as well as explicitly describe the relationships between genes in each cluster. Therefore, we applied weighted gene co-expression network analysis (WGCNA), a widely used tool to cluster genes based on their expression similarity (Langfelder & Horvath, 2008). Briefly, WGCNA generates pairwise correlations across transcripts in a dataset, which are then power-transformed to an exponent sufficient to make pairwise correlations power-distributed (13 in our case,

retaining sign information for further clustering). This distribution conforms to a scale-free network topology, which is generally expected in natural networks (Barabási & Oltvai, 2004), and effectively penalizes weak correlations. WGCNA then hierarchically clusters individual transcripts according to their topological overlap, which measures the similarity between their connections. We set the 'deepSplit' parameter (roughly analogous to cut height in tree-based clustering methods) to 0, which biases the algorithm to merge similar clusters, and we imposed a minimal cluster size of 20 transcripts. Transcripts whose expression patterns are too dissimilar from their nearest cluster were assigned to the 'grey' cluster (the color terminology for module IDs is default in the WGCNA software). We represent the average expression pattern of each cluster by extracting its first principal component (or eigengene). We tested each eigengene for significant responses to temperature, population, and their interaction using a linear mixed model, which allowed us to estimate and control for the variance explained by between-clutch effects.

2.7 | Functional annotation of transcripts

Functional annotation of *de novo* assembled transcriptomes of non-model organisms can be achieved by associating transcripts with annotated homologs of other species. We used the Trinotate pipeline (https://trinotate.github.io/), which automates homology searches and summarizes the resulting annotations using a SQLite database. We performed homology searches both on the level of nucleotides as well as putative peptide sequences to increase the chances of finding an annotated homolog. Putative amino acids are predicted by Transdecoder version 2.0.1 (https://transdecoder.sourceforge.net/; minimum length set to 50 amino acids). These putative peptides (and original transcripts) were used as queries in blastp (blastx) searches against the UniProtKB/Swiss-Prot database (release "November 22, 2017"). Furthermore, putative peptides were used in hmmscan searches (hmmer version 3.1b2) against PFAM, a database of protein domains (Finn et al., 2014). Trinotate retrieves Gene Ontology (GO) annotations (Ashburner et al., 2000) from significant blast hits and PFAM hits (E-value cutoff 10^{-5}), and summarizes the results in table format. We obtained significant blast hits for 10,567 (43.42%) and PFAM hits for 8,630 (35.46%), resulting in GO terms assigned to 9,809 (40.31%) of all transcripts.

2.8 | Gene ontology signatures of incubation temperature

Since a considerable proportion of the transcriptome showed significant expression changes in response to incubation temperature (see below), we deemed it inappropriate to apply gene enrichment analysis probing which GO terms are enriched in differentially expressed transcripts. We therefore applied the R package 'GOexpress' (Rue-Albrecht et al., 2016) for the identification of GO terms that best discriminate between the cool and warm incubation treatment using a random-forest algorithm.

In brief, GOexpress attempts to separate samples incubated in cool and warm temperatures by picking a single gene and an expression threshold so that this gene exceeds the threshold in as many warm samples as possible and in as few cool ones as possible, or vice versa. This process generates one group of samples on each side of the expression threshold, which represent the best separation one can achieve with a single gene. If any of those sample groups contains a mixture of cool and warm samples, the algorithm selects a new gene-threshold pair to separate them. This produces a ranked list of genes whose expression levels can be used to separate incubation temperatures.

To avoid relying on a few genes that are highly correlated with temperature, we restrict the search to a random subset of 211 genes at each split, which corresponds to the default value of twice the square root of the entire dataset. We repeat the entire algorithm a total of 5,000 times and average the rank of each gene across the entire set. We average the score of all genes within each GO term and compare them with those produced by 5,000 random permutations of the dataset to generate empirical *P*-values. These *P*-values represent the odds that the same GO term will be identified as significantly enriched when using random sample groups. We excluded GO terms that were assigned to less than 30 transcripts in our dataset from the final evaluation. To further characterize the nature of GO terms discriminating between incubation temperatures (*P*-values < 0.01), we visualized their semantic similarities using the software REVIGO (Supek, Bošnjak, Škunca, & Šmuc, 2011) that uses multidimensional scaling and graph-based visualizations.

We were not able to apply this approach to detect enrichment of KEGG pathways. While 4,901 KEGG pathways were assigned to at least one transcript, the largest majority of them had less than 10 annotated transcripts in the final set, resulting in very low power to identify strongly discriminant KEGG pathways.

3 | RESULTS

3.1 | Global levels of gene expression

The assessment of global levels of gene expression revealed that warm incubated embryos have significantly greater amounts of total RNA than cool incubated ones (mean total RNA per embryo: 8.97 μg for warm and 7.11 μg for cool incubation temperature; *P*-value < 0.01, paired *t*-test). All results below refer to sequencing and analyses of a standardized amount of total RNA (see methods).

3.2 | Patterns of variation in gene expression profiles

Principal component analysis showed that the greatest proportion of variation is attributed to differences between populations (Figure 1b). This first principal component discriminates broadly between the Italian and French lineages, but also to a lesser extent between the two French populations. The second principal component separates between cool and warm incubation temperatures. The first and second principal components together explain 48% of the variation in the dataset, while the third and fourth explain

10 and 6%, respectively. Notably, the two French populations separate parallel to the dimensions that discriminate between Italian and French lineages and perpendicular to the dimensions that separate cool and warm incubation temperatures (Figure 1b). This suggests that the gene expression differences between the two French populations map to the same genes that differentiate between Italian and French ones, rather than to genes involved in the temperature response.

These global patterns of variation are confirmed by the complementary analysis using tSNE projection, which allows low-dimensional representation of all variation within the dataset. tSNE projection highlights that gene expression separates clearly between four main groups of samples corresponding to French versus Italian populations and cool versus warm incubation temperatures (Figures 1c and 1d).

3.3 | Differential gene expression

To address whether populations respond differentially to the cool incubation treatment, we used sleuth to compare a full model with 'temperature' and 'population' and their interaction term to a reduced model lacking the interaction term ('clutch' was included as main effect in both models). A likelihood-ratio test (LRT) between the two models revealed that only three transcripts had a significant interaction term (Supporting Information Table S1). Thus, the vast majority of transcriptional responses to different incubation temperatures are conserved across all populations in this study. Next, we compared the reduced model (both temperature and population as main effects, but no interaction term) to models that include only one of both terms. LRT showed that 11,797 transcripts (53% of the transcriptome) are differentially expressed between the two temperatures after correcting for FDR, and 4,632 transcripts (21% of the transcriptome) are differentially expressed between at least two of the four populations (most differences naturally fall between the two broad geographic regions). As illustration, the 20 most differentially expressed transcripts and their putative homologs in model organisms are given in Table 1 (the full list can be found in the Supporting Information File 2). The identity of these transcripts reveals that they are putatively involved in transcriptional or translational regulation ('TATA-binding protein-associated factor', 'Transcription factor', 'Eukaryotic translation initiation factor 3 subunit J'), nuclear import ('Importin-5'), or chromatin remodeling ('Chromobox protein homolog').

We cross-validated these results with a corresponding analysis using the DESeq2 software. Applying a full model as described above, we find seven transcripts with a significant interaction term (including the three transcripts identified by sleuth; Table S1). When applying a reduced model without interaction terms (see above), we find that 8,941 transcripts (41%) are differentially expressed at different incubation temperatures after correcting for FDR, and 3,682 (17%) are differentially expressed between populations. The identification of differentially expressed transcripts between the two temperature regimes is highly consistent between the analyses using sleuth and DESeq2 (Figure 2a).

3.4 | Transcript clustering and differential cluster analysis

WGCNA clustering generated a total of 30 modules, plus an improper 'grey' module containing 2,570 transcripts whose expression profile did not match that of any module. We summarize the expression profiles of each module via their first principal components or eigengenes (Supporting Information Figure S1).

Consistently with single-gene testing, we find that no cluster shows significant interactions between population and temperature effects (Supporting Information Table S2). We find significant responses to temperature in 22 modules, encompassing 16,503 transcripts (75% of all transcripts included), and significant differences between populations in 13 modules, encompassing 7,198 transcripts (33% of all transcripts). Nine modules (4,825 transcripts) show significant expression differences between both incubation temperature and populations. We find that this overlap between transcripts that respond to temperature and transcripts that show population-specific expression differences is slightly lower than the expected product of the two categories, and this lack of overlap is highly significant (Fisher's exact test, P-value < 0.001).

The estimated contribution of between-clutch (i.e., between-family) variation to total expression variance is highly variable from module to module, ranging from almost zero (clusters 'cyan' and 'darkgreen') to 0.9 (clusters 'lightcyan' and 'darkpurple'; Supporting Information Figure S2). Despite variation between modules, we find that the variance explained by clutch is similar across temperature-sensitive, population-specific, and non-differentially expressed modules (Mann–Whitney U test, P-values 1 and 0.17, respectively).

3.5 | Functional differences in transcripts associated with temperature-induced expression changes

GOexpress analysis revealed that the transcripts that best discriminate between the temperature regimes over-represent 74 GO terms using our significance criteria (see Methods). Of these, 51 GO terms are assigned to the category 'biological processes', 10 to 'molecular functions', and 13 to 'cellular components'. 'Biological process' terms mainly belong in six distinct areas (Figure 2b): chaperone-mediated protein folding (e.g., GO:0061077), cell cycle regulation (GO:1901990, GO:1901987), histone modification (GO:0016571), chromatin organization (GO:0006333), transcriptional regulation (GO:0006354, GO:0032784), and translation elongation (GO:0070125, GO:0006414). Enriched 'molecular function' terms (Figure 2c) include HSPs (GO:0051082), histone acetylation (GO:0008080, GO:0016407), and transcriptional and translational regulation (GO:0070063, GO:0003743) but also helicases (GO:0003678), which may be involved in unwinding double-stranded DNA during either transcription or mitosis-related processes. Significant 'cellular component' terms (Figure 2d) indicate an overrepresentation of transcripts localized in histone acetylation complexes (GO:0000123), as well as kinetochores and spindle poles (GO:0000776, GO:0000922), which are necessary for cell-division, and pre-ribosomes (GO:0030684), which are required for translation. We report the complete list of

TABLE 1 Top 20 most differentially expressed genes between incubation temperatures as detected by sleuth

Most similar gene in blast search	Gene ID	*q*-Value	Beta value[a]	Blast hit metrics	Gene description
Human RBP56	TRINITY_DN15646_c0_g1	2.22×10^{-28}	−1.59	63% identity, e-value 1.65×10^{-11}	TATA-binding protein-associated factor 15
Chicken HTF4	TRINITY_DN23728_c0_g1	2.43×10^{-26}	−0.86	86% identity, e-value 0	Transcription factor 12
Human ZN219	TRINITY_DN20059_c0_g1	9.65×10^{-26}	0.69	48% identity, e-value 3.1×10^{-88}	Zinc finger protein 219
Human TM269	TRINITY_DN11001_c0_g1	3.10×10^{-25}	1.50	50% identity, e-value 3.25×10^{-52}	Transmembrane protein 269
Mouse CBX1	TRINITY_DN5704_c0_g1	8.57×10^{-25}	−0.64	95% identity, e-value 2.38×10^{-82}	Chromobox protein homolog 1
Bovin SGT1	TRINITY_DN16388_c0_g1	1.42×10^{-24}	−0.70	71% identity, e-value 1.85×10^{-150}	Protein SGT1 homolog
Human IPO5	TRINITY_DN13982_c0_g1	4.94×10^{-24}	−0.60	95% identity, e-value 0	Importin-5
NA	TRINITY_DN19928_c0_g1	6.47×10^{-24}	2.48	NA	NA
Chicken EIF3J	TRINITY_DN7313_c0_g1	1.40×10^{-23}	-0.77	85% identity, e-value 1.26×10^{-71}	Eukaryotic translation initiation factor 3 subunit J
NA	TRINITY_DN12540_c0_g1	1.40×10^{-23}	2.06	NA	NA
RCC2_HUMAN	TRINITY_DN19643_c0_g1	1.59×10^{-23}	−0.70	93% identity, e-value 0	Regulator of chromosome condensation 2
NA	TRINITY_DN12445_c0_g1	1.66×10^{-23}	−1.24	NA	NA
TYB4_MOUSE	TRINITY_DN13484_c0_g1	1.73×10^{-23}	−0.68	96% identity, e-value 1.06×10^{-20}	Thymosin beta 4, X-linked
DNLI3_HUMAN	TRINITY_DN17106_c0_g1	1.73×10^{-23}	−0.71	78% identity, e-value 0	DNA ligase 3
NA	TRINITY_DN12902_c0_g1	1.87×10^{-23}	1.77	NA	NA
RBP56_HUMAN	TRINITY_DN15332_c0_g1	3.24×10^{-23}	−1.08	85% identity, e-value 3.54×10^{-35}	TATA-binding protein-associated factor 15
NA	TRINITY_DN21991_c0_g1	3.83×10^{-23}	0.66	NA	NA
NA	TRINITY_DN8597_c0_g1	4.99×10^{-23}	2.22	NA	NA
NA	TRINITY_DN14534_c0_g1	6.10×10^{-23}	1.81	NA	NA
LYPA1_BOVIN	TRINITY_DN6006_c0_g1	6.25×10^{-23}	−0.97	76% identity, e-value 1.95×10^{-51}	Lysophospholipase I

[a]Negative beta values indicate that the transcript is downregulated at cool compared to warm incubation temperature.

GO terms and their enrichment scores in Supporting Information Table S3.

4 | DISCUSSION

We describe for the first time how different incubation temperatures affect the transcriptome of reptilian embryos. We find that around half of all transcripts are differentially expressed at the pharyngula stage of wall lizard embryos following sustained exposure to sub-optimally cool incubation temperatures. This magnitude of expression differences is comparable to those reported in similar studies of *Drosophila* (Sørensen, Schou, Kristensen, & Loeschcke, 2016). Interestingly, these temperature responses are largely independent from geographic (and genetic) differentiation in gene expression observed across populations (one quarter of transcripts showed significant expression differences across populations). Indeed, the transcripts that differ between populations are involved in temperature responses less frequently than expected by chance, showing that genes whose expression is up- or downregulated with temperature are less likely to be divergent between populations. Even transcripts with significant effects of both temperature and population lack population-specific responses to temperature, as indicated by the lack of significant interaction terms. Further, PCA and differential expression results reveal that population differences are mainly caused by fewer genes showing greater expression differences, whereas temperature responses involve a much greater number of transcripts undergoing individually smaller changes. Taken together, these findings suggest a pervasive impact of temperature on gene expression during lizard development.

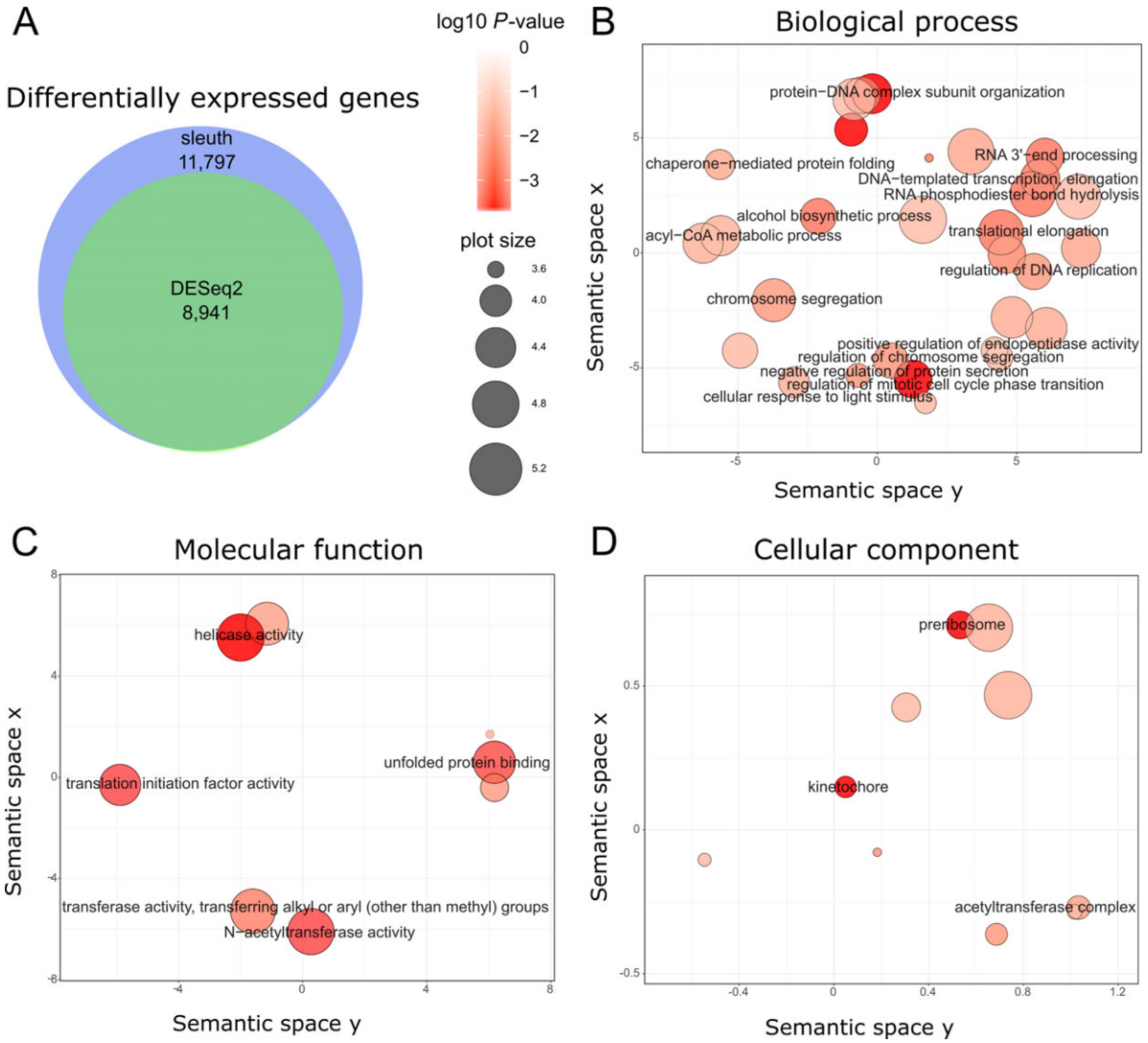

FIGURE 2 Differentially expressed genes and GO terms associated with most discriminant genes. (A) Proportional Venn diagram showing the concordance between the analyses of differential gene expression performed with sleuth and DESeq2. The vast majority of differentially expressed transcripts were concordantly identified by both analyses (8,874 transcripts). A total of 2,923 and 67 transcripts were identified by sleuth and DESeq2 alone, respectively. Proportionate Venn diagram was produced using BioVenn (Hulsen, de Vlieg, & Alkema, 2008). (B–D) Visual representation of semantic similarities in GO terms that were identified to be significantly (*P*-value < 0.01) associated with temperature-responsive transcripts by the GOexpress analysis. Plots were produced with customized R-scripts obtained from REVIGO (Supek et al., 2011). Each panel summarizes non-redundant sets of GO terms from the three main GO categories biological process (B), molecular function (C), and cellular component (D). Each circle represents a GO term with its color corresponding to the *P*-value assigned to temperature sensitivity (deep red highly significant and white less significant, but still lower than 0.01), and its size indicates the frequency of the GO term in the UniProt database (smaller size indicates less frequently assigned GO term). Names are only given for GO terms with 'dispensability' values smaller than 0.5 [Color figure can be viewed at wileyonlinelibrary.com]

These results have a number of important biological implications. First, they suggest a strong influence of incubation temperature on physiological processes as evidenced by the reduced level of total RNA in cool-incubated embryos (reduced by approximately 20% compared to benign temperature). At the molecular level, we find that terms associated with ribosome production and functioning (e.g., GO:0006364; e.g., RRP5, NOL6, and UTP4) are enriched in embryos exposed to cold temperature. This suggests that the rate of translation of mRNA into protein is affected by temperature, possibly counter-acting decreased transcriptional rates. Differential expression of ribosomal proteins and RNAs have been observed in a wide variety of animals in response to both cold (Long et al., 2012; Xiao, Wang, Cao, & Zhang, 2016) and warm (Liu et al., 2017; Prado-Lima & Val, 2016; Quinn, McGowan, Cooper, Koop, & Davidson, 2011) temperatures, yet only one study (Long et al., 2013) provides indirect evidence that they may increase resistance to temperature stress. In that study, zebrafish larvae acclimated to cold temperatures for 24 hr show both increased expression of ribosomal genes and increased survival to acute cold stress. Interestingly, increased expression of ribosomal genes is not found in zebrafish embryos which experience only acute cold stress (Long et al., 2012; Long et al., 2013), supporting the hypothesis that ribosomal genes may be involved in acclimation rather than stress response.

If we assume that this explanation is valid and applicable to other vertebrates, we can explain the increase in ribosomal expression we observe in lizard embryos as a form of acclimation to cold temperature. However, further studies are necessary in order to determine if increased ribosomal expression increases the survival of lizard embryos in cold environments and, if so, which molecular mechanisms are involved.

Second, the putatively assigned functions of differentially expressed transcripts also point to a large impact of temperature on transcription. For example, we consistently identified transcriptional regulators and elongation factors as discriminant factors between the temperature regimes. Temperature stress also triggered changes in the expression of genes involved in chromatin remodeling (nucleosome organization and histone modification), which have the potential to affect gene regulation on a global scale (for an introduction to epigenetics see Allis, Caparros, Jenuwein, & Reinberg, 2015). These results suggest that chromatin remodeling may play a role in the repertoire of epigenetic changes in response to stressfully low incubation temperatures. In support of this hypothesis, a study on wall lizards found reduced DNA methylation levels in the brain tissue of hatchlings that were incubated at stressfully cool, compared to benign, warm temperatures (Paredes, Radersma, Cannell, While, & Uller, 2016). We thus hypothesize that DNA methylation, histone (tail) modification, and chromatin organization are all affected by incubation temperature, potentially contributing to the long-lasting effects of incubation temperature on morphology, physiology, and behavior (Noble et al., 2018).

Several additional functions could also be assigned to differently expressed transcripts. For example, we found molecular chaperones (GO:0051082; e.g., Hsp90, Hsp72, Hsp40, and NMP1) were upregulated under thermal stress. This is consistent with previous studies, which have found that molecular chaperones are reliable predictors of whether an embryo has been exposed to cold stress (e.g., Long et al., 2012). Within those, we found that the Hsp90 family was the most reliable predictor (GO:0051879, P-value < 0.001), whereas the Hsp70 family (GO:0030544, P-value $= 0.96$), exhibited very limited discriminatory power between the temperature treatment. There is overwhelming evidence that HSPs assist the folding of other proteins under stressful thermal conditions (Horvath, Multhoff, Sonnleitner, & Vigh, 2008; Vabulas, Raychaudhuri, Hayer-Hartl, & Hartl, 2010), thus increasing the organism's operational range. It is therefore likely that these proteins may represent compensatory transcriptomic changes to the thermal challenge.

Enrichment analyses also revealed a consistent overrepresentation of gene ontologies associated with mitotic cell-cycle control among temperature-responsive genes (i.e., negative regulation of mitotic cell-cycle transition, GO:0045930 [e.g., MD2L1, HDC, ABL1, and PTPN3], and positive regulation of cell cycle phase transition, GO:1901989 [e.g., CDC7 and TIM]). These findings can be explained in at least two ways. It is possible that lower temperatures disrupt cell-cycle regulation, leading to an increased production of regulators to maintain cell division. Alternatively, an increase in the rate of mitotic division could partly offset the slow-down of developmental rates induced by cooler temperatures, helping embryos to complete development. While we cannot rule out the alternative of buffering dysregulation without functional genomics data, our analyses are in agreement with a previously suggested role of the TOR signaling pathway (GO:0032007; e.g., UBR1 and -2, TSC2, and DEPD5) as the potential modulator of cell-cycle acceleration in response to stressful conditions (Long et al., 2012).

With the possible exception of heat shock proteins, we cannot establish if the relative up- or downregulation of genes at low temperature is the result of compensatory changes that help to sustain development, or if they result from unbuffered thermal sensitivity. Even if we assume (conservatively) that most of the observed responses belong to the latter, our data show a substantial amount of variation both between populations and clutches. Whether this variation can contribute to adaptation will depend on whether it is heritable and how much it affects fitness. It is also worth noting that changes in gene expression profiles accompanying acclimation to sustained sub-optimally low temperatures are qualitatively different from those associated with fluctuating temperatures in both *Drosophila* flies (Sørensen et al., 2016) and killifish (Podrabsky & Somero, 2004). This indicates that the mechanisms underlying quick responses to stressful temperatures are qualitatively different from the mechanism underlying more long-term acclimation to suboptimal temperatures. Extending to evolutionary timescales, it is not obvious if adaptation to extreme temperatures mainly capitalizes on expression changes of genes involved in long-term acclimations or short-term stress responses. Our previous study detected local adaptation to cool temperature in non-native lizard embryos. We found that genes responding to selection in the non-native lizards were enriched for genes whose expression levels changed with long-term acclimation to cool temperatures in the native populations (Feiner et al., 2018). This is expected if natural selection capitalizes on the ability of individuals to acclimatize to low temperatures, but alternative hypotheses cannot be ruled out at present (see Feiner et al., 2018 for discussion).

In summary, this study is a first step in the systematic characterization of transcriptomic responses of reptilian embryos exposed to sustained low incubation temperatures. While around half of all transcripts responded to incubation temperature, we identified key components of transcriptional and translational regulation, as well as chromatin remodeling components among the most highly differentially expressed genes. We propose that differentially expressed chromatin modifiers modulate gene expression and allow embryos to acclimate to stressful incubation temperatures. Studies that combine experiments of developmental thermal plasticity with an examination of their underlying molecular mechanisms have a role to play in furthering our understanding of how individuals acclimate and populations adapt to thermal stress.

ACKNOWLEDGMENTS

This research was supported by an Early Career Research grant and a University Research Fellowship from the Royal Society of London, and a Wallenberg Academy Fellowship from the Knut and Alice Wallenberg Foundations (all to T.U.), a Humboldt Foundation Fellowship and a Wenner-Gren postdoctoral fellowship to N.F., and a research

grant from the John Templeton Foundation (60501). We thank Dan Warner, Arthur Georges, and Wei-Gu Du for inviting us to contribute to this theme issue, and Roberto Sacchi, Marco Zuffi, Fabien Aubret, and Jérémie Souchet for logistical support. Three reviewers provided helpful comments on the manuscript. We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics for the generation of sequencing data. The computations were performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under Project b2015260. Fieldwork was carried out under licenses and permits from Natural England (20146129, 20144863), Direction Régionale de l'Environnement, de l'Aménagement et du Logement (No. 2010/DDEA/SEPR/175, No. 11/2012, No. 2010-DDEA-SE-105, No. 29/2012, No. 11/DDTM/657-SERN-NB), and Ministero dell'Ambiente e della Tutela del Territorio del Mare–DG Protezione della Natura e del Mare (DPR 357/97) and Societas Herpetologica Italica (prot. ISPRA 9139 T/-A31).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Feiner N, Rago A, While GM, Uller T. Developmental plasticity in reptiles: Insights from temperature-dependent gene expression in wall lizard embryos. *J Exp Zool*. 2018;1–11. https://doi.org/10.1002/jez.2175