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Signatures of selection in embryonic transcriptomes of lizards adapting in parallel to cool climate

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Populations adapting independently to the same environment provide important insights into the repeatability of evolution at different levels of biological organization. In the 20th century, common wall lizards (*Podarcis muralis*) from southern and western Europe were introduced to England, north of their native range. Nonnative populations of both lineages have adapted to the shorter season and lower egg incubation temperature by increasing the absolute rate of embryonic development. Here, we tested if this adaptation is accompanied by signatures of directional selection in the transcriptomes of early embryos and, if so, if nonnative populations show adaptive convergence. Embryos from nonnative populations exhibited gene expression profiles consistent with directional selection following introduction, but different genes were affected in the two lineages. Despite this, the functional enrichment of genes that changed their expression following introduction showed substantial similarity between lineages, and was consistent with mechanisms that should promote developmental rate. Moreover, the divergence between nonnative and native populations was enriched for genes that were temperature-responsive in native populations. These results indicate that small populations are able to adapt to new climatic regimes, but the means by which they do so may largely be determined by founder effects and other sources of genetic drift.

KEY WORDS: **Climate, convergent evolution, lizard, nonnative, transcriptomics, thermal adaptation.**

Populations inhabiting similar environments often evolve similar phenotypes. Birds and mammals living at high altitudes commonly exhibit higher haemoglobin-oxygen affinity (Natarajan et al. 2016; Storz 2016), sticklebacks colonizing freshwater predictably lose their body armour (Colosimo et al. 2005), and reptiles expanding into cool climates often become live-bearing (Webb et al. 2006). Convergent evolution of phenotypes is sometimes underpinned by convergence of its underlying molecular mechanisms (e.g., evolution of toxins and resistance; Jensen et al. 2011; Ujvari et al. 2015), but similar phenotypes can also be produced by very different processes (e.g., wing shape in *Drosophila*; Huey et al. 2000). Revealing the patterns of convergence at different levels of biological organization, and understanding the causes of those patterns, represent major challenges for evolutionary biologists (Agrawal 2017).

Adaptive evolution often involves changes in gene regulation, suggesting that populations with similar phenotypes may have convergent gene expression profiles. For example, a study of 900 genes expressed in the liver of juvenile brown trout (*Salmo trutta*) found that gene expression profiles clustered according to whether the populations are migratory or resident rather than the populations' genetic similarity (Giger et al. 2006). Despite this, the evidence that selection plays a major role in divergence in gene expression profiles is limited. For example, more recent studies of whole transcriptomes have revealed that, although differences in gene expression between populations can be substantial, usually

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only a small number of genes exhibit convergent expression in populations that share the same environment (Dayan et al. 2015; Ghalambor et al. 2015; Zhao et al. 2015).

A weak signature of convergence in gene expression profiles may suggest that most of the variation in transcriptomes observed between populations accumulates under neutrality (e.g., Khaitovich et al. 2005). Furthermore, there are many developmental routes to the same phenotype (Wagner 2011). Most characters are not only polygenic, but developmental pathways are often highly redundant and harbor substantial genetic variation (Paaby and Gibson 2016). This suggests that selective history, founder effects, and other sources of genetic drift will influence the extent to which populations evolving in the same environment converge with respect to gene expression. For example, abundant standing genetic variation at a key locus in marine sticklebacks appears to have facilitated the repeated evolutionary loss of body armour following colonization of freshwater (Colosimo et al. 2005).

A well-known example of convergent evolution in ectotherms is that individuals from cool climates grow and develop more rapidly than individuals from warmer climates, in particular at low temperatures ("counter-gradient adaptation," Conover et al. 2009). For example, lizard embryos from populations at high altitude or latitude grow absolutely faster than conspecific embryos from populations in warmer climates (e.g., Oufieroi and Angilletta 2006; Du et al. 2010; Rodriguez-Diaz and Brana 2012). This implies that populations colonizing cooler environments evolve changes in gene regulation that counter-act the direct effect of temperature on growth and metabolism. It has been hypothesized that this mode of adaptation will primarily affect genes responsive to temperature in ancestral populations (Ghalambor et al. 2007); reducing the maladaptive direct effects of temperature on gene expression while exaggerating existing plastic expression patterns that facilitate maintenance of growth and development at low temperature. However, despite the contemporary focus on thermal adaptation in vertebrates, almost nothing is known about the mechanism by which embryos adapt to incubation temperature.

Here, we test for adaptive divergence and convergence of gene expression profiles in lizard embryos, using two genetically distinct lineages of wall lizards adapting to cool climate following their introduction from Europe to England. Embryos in English, nonnative, populations face drastically cooler soil temperatures during incubation than do embryos in native populations (While et al. 2015a). While low temperature slows down growth and development, strong natural selection for early hatching has made embryos of nonnative populations develop absolutely faster, in particular at low temperature (While et al. 2015a). We compared gene expression of early embryos incubated at harsh and benign temperatures, and tested for signatures of adaptive divergence between native and nonnative populations and adaptive convergence of nonnative populations.

Materials and Methods

STUDY SYSTEM AND EXPERIMENTAL DESIGN

The common wall lizard is a small (approx. 50–70 mm snout-to-vent length), egg-laying lacertid, widely distributed in Europe. Here, we focus on two main genetic lineages inhabiting western Europe and Italy, which diverged from each other approximately two million years ago (Gassert et al. 2013). Lizards from each of the two lineages have repeatedly and independently been introduced to England over the last 100 years (Michaelides et al. 2013). There are currently more than 25 populations across southern England, and the introduction histories have been reconstructed in detail (Michaelides et al. 2015).

Our aim was to analyze differences in gene expression profiles in early embryos, at three different levels of comparison: (1) harsh (15°C) versus benign (24°C) temperatures, (2) French versus Italian lineage, and (3) native versus nonnative populations. This resulted in a $2 \times 2 \times 2$ experiment in which we refer to the main factors as (1) "temperature," (2) "lineage," and (3) "introduction." We used a split clutch design in which embryos from each clutch were divided between the two thermal treatments (see below) allowing us to control for variation in the response to temperature caused by genetic similarity due to relatedness (Fig. S1A).

In April 2015, we collected 13 gravid females from France (Fr; Pouzagues [46.788 N, 20.448 E]) and 18 from Italy (It; 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]), as well as 12 each from nonnative populations of both lineages in England (Italian origin: Ventnor Town [50.598 N, 21.218 E], Ventnor Botanical Garden [50.588 N, 21.228 E]; French origin: Cheyne Weare [50.538 N, 22.438 E] and East Portland [50.548 N, 22.428 E]). The nonnative Italian and French populations were introduced in the 1930s and 1980s for the Italian and French lineages, respectively (Michaelides et al. 2015). The native populations were chosen because they fall within the approximate geographic origin of the nonnative populations (Michaelides et al. 2015). Once in the laboratory, females were housed individually in cages ($590 \times 390 \times 415$ mm) with sand as substrate, bricks as shelter, and a water bowl. They were kept at a light cycle of 12 L:12 D, and given access to basking lights (60 W) for 8 h per day and a UV light (EXO-TERRA 10.0 UVB fluorescent tube) for 4 h per day. Mealworms and crickets were provided *ad libitum*. Females were inspected in the morning and in the afternoon for signs of egg-laying to ensure that eggs were collected within a maximum of 12 h after oviposition. Within each clutch, one egg was dissected immediately upon laying to determine the developmental stage at laying and the remaining eggs were divided into two groups. One group of eggs was incubated at 15°C (cool) and the other group was incubated at 24°C (warm). The cool incubation

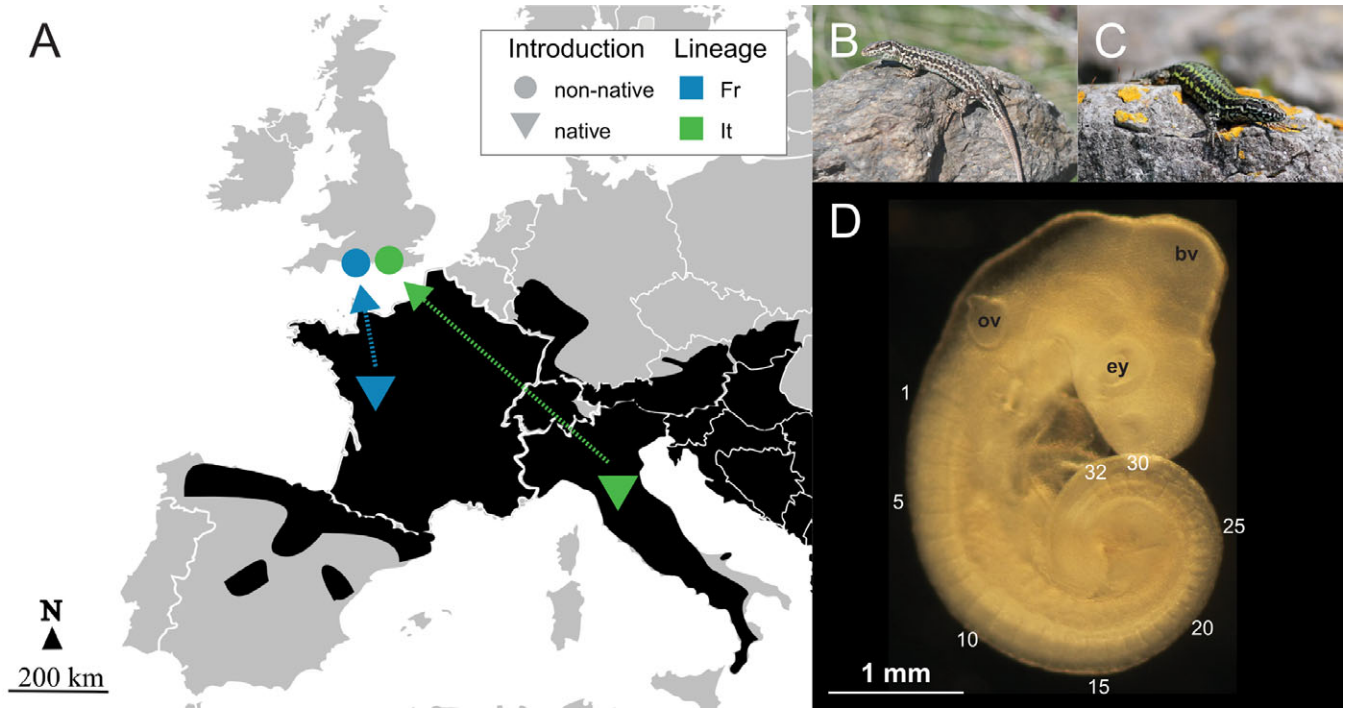

Figure 1. **Overview of the study design. (A) Marked in black is the native distribution of *P. muralis* in continental Europe (truncated at the Eastern range). Triangular symbols mark the approximate geographic origin of lizards introduced to the South coast of England (round symbols), and the color codes illustrate the genetic lineage. (B, C) A French and an Italian common wall lizard female. The lineages diverged in the Pleistocene, approximately 2 million years ago (Gassert et al. 2013) and differ in a suite of characteristics (While et al. 2015b). (D) A representative embryo at the developmental stage that was subjected to transcription profiling (stage 27, 32 somites). Somite counts are indicated with white numbers. Abbreviations: bv, brain vesicle; ey, eye; ov, otic vesicle. Picture in panel (B) courtesy of Guillem Pérez i de Lanuza.**

treatment represented temperatures frequently encountered by eggs in nests under English climatic conditions, but below the constant temperature conditions that allow successful hatching (While et al. 2015a). The warm incubation treatment represented temperature within the range encountered in natural nests of both lineages, and within the optimum thermal range for the species as estimated from the incidence of scale malformations (While et al. 2015a). All eggs were incubated in small plastic containers filled two-thirds with moist vermiculite (5:1 vermiculite/water volume ratio) and sealed with clingfilm.

To compare gene expression patterns at a precise developmental stage, we needed to account for the increase in developmental rate with temperature by adjusting the incubation duration. We estimated developmental rates of *P. muralis* embryos from native and nonnative populations at 15 and 24°C based on While et al. (2015a). We chose to target the embryonic stage 27 (Dufaure and Hubert 1961), which roughly corresponds to the pharyngula stage, since this allowed the warm incubated embryos to develop for at least 12 hours at 24°C, and thus acclimatise their gene expression, while allowing the cool incubated embryos to reach that stage in less than 4 weeks (approximate developmental rates at 24°C: four somites per 1 day and at 15°C: four somites per 7 days). Based on this prediction, we selected eggs for dissection at regular intervals to ensure that a sufficient number of embryos of the targeted developmental stage were obtained. Since embryonic stage 27 encompasses a range of 29–34 somites, we further narrowed the developmental time point for the subset of embryos subjected to gene expression by selecting only embryos with 32 ± 1 somites (Fig. 1D).

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 one-hour interval) to minimize variation caused by diurnal patterns of embryonic gene expression (Seron-Ferre et al. 2007). Second, all eggs were processed within five minutes of removal from the incubator to avoid changes in gene expression patterns. Embryos were separated from yolk and extraembryonic membranes in DEPC-treated PBS (phosphate-buffered saline) by using sterile forceps under a dissecting microscope. Each embryo was photographed, staged (including somite count), and submerged in RNAlater (Qiagen) to stabilize RNA. Total RNA from a total of 96 embryos (40 for single- and 56 for pooled embryo-sampling strategy) at developmental stage 27 (31 ± 1 somites) was extracted by using the RNeasy Micro Kit (Qiagen). The yield

of total RNA was measured with the Qubit® 2.0 Fluorometer system using the Qubit RNA BR Assay Kit (Thermo Fisher Scientific) and determined to be on average 7.45 (±0.38) μg per embryo. RNA integrity was assessed with the Experion system using the Eukaryote Total RNA StdSens Analysis kit (Bio-Rad).

TRANSCRIPTOME SEQUENCING

An overview of the applied bioinformatics pipeline is provided in Fig. S1B. For each of the eight experimental groups, five samples, each consisting of the total RNA of a single, whole embryo, were used for expression analysis. In addition, we pooled equimolar amounts of RNA from 4 to 6 (4.63 ± 1.19) embryos from different mothers per study group to obtain one gene expression dataset with minimal individual variation. These pooled samples were used in *de novo* transcriptome assembly to ensure a maximally complete reference set (see below). Thus, a total of six samples per study group were subjected to library preparation, resulting in a total of 48 samples. Per sample, 2 μg of purified, high-quality total RNA (RQI values >9) was subjected to RNA sequencing. In brief, the mRNA fraction was converted into cDNA, end-repaired, A-tailed, and adapter-ligated. 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 and Doerge 2010).

We obtained on average 39.3 million raw reads per sample. Quality control was performed using FastQC software (URL: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low quality reads were removed and a sliding window approach was used to trim low quality bases at the ends of the reads using Trimmomatic Version 0.32 (settings: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36; Bolger et al. 2014). The French and Italian lineages of *P. muralis* are sufficiently genetically differentiated to warrant *de novo* assembly of separate reference transcriptomes. For this purpose, we pooled eight representative samples for each lineage: each one consisting of a randomly selected single embryo sample and the pooled sample for cool and warm incubation treatments for native and nonnative populations. Prior to assembly, using the Trinity software Version 2.3.2 (Haas et al. 2013) and strand-specific information, the redundancy in the French and Italian pooled datasets were removed by performing *in silico* normalization as part of the Trinity pipeline. The raw assemblies were further filtered in three steps: first, duplicates were removed by clustering the assembly at 95% sequence similarity using CD-HIT-EST version V4.6.5 (Li and Godzik 2006). Second, pools containing all French and Italian reads, respectively, were mapped to the respective raw assembly using Bowtie 2 (Langmead and Salzberg 2012), and all transcripts with a FPKM coverage of <1 were discarded by using the RSEM algorithm (Li and Dewey 2011) implemented in the Trinity wrapper. Third, if several isoforms of a given transcript were present in the assembly, only the longest isoform was retained. To make expression profiles of French and Italian lizard embryos comparable, we merged the two *de novo* assemblies by using the Proteinortho software Version 5.15 (Lechner et al. 2011) and a custom script, and created a shared wall lizard reference transcriptome (divergent nucleotides are masked as "N"s). To exclude the possibility of biotic contamination of our samples, we excluded transcripts with bit scores at least 10 times higher in blastx searches against invertebrate metazoan peptides (NCBI: taxid 33208 and excluding taxid 89593) than against vertebrate peptides (NCBI: taxid 89593) using NCBI's Entrez Direct. Trimmed reads of all 48 samples were mapped to the shared reference transcriptome using Bowtie 2 and raw counts per sample were estimated using the RSEM algorithm. To avoid spurious effects from lowly expressed transcripts, we retained only transcripts with more than 10 reads in more than 50% of the samples. Our substantial filtering methodology resulted in a transcriptome comprising 20,221 transcripts with a N50 value of 2894. We refer to expressed sequences as transcripts in the technical sense but use the term "genes" and "gene expression" in more general discussions.

FUNCTIONAL ANNOTATION OF TRANSCRIPTS

The *de novo* assembled transcriptome of embryonic *P. muralis* lizards was functionally annotated using the Trinotate pipeline (https://trinotate.github.io/). The longest open reading frames of a minimum of 50 amino acids in length were predicted using Transdecoder v.2.0.1 (http://transdecoder.sourceforge.net/). These putative peptides (and original transcripts) were used as queries in blastp (blastx) searches against the UniProtKB/Swiss-Prot database (release "2017_02_15"). From accepted blast hits (*E*-value cut-off 10^{-5}), Trinotate retrieves Gene Ontology (GO) annotations (Ashburner et al. 2000). We found significantly similar peptides in the Swissprot-Uniprot database for 9991 out of total 20,221 transcripts (49.4%), and for 9421 transcripts (46.6%) we retrieved at least one GO term (see File S1). Note that the Trinotate pipeline also retrieves annotations from other sources (e.g., KEGG or PFAM), but since we obtained by far the most annotations from GO terms, which we consider to be most informative, we restricted our enrichment analysis to these annotations. The R package GOseq (Young et al. 2010) was used to detect over- and underrepresented GO terms by using a FDR adjusted *P*-value <0.05. By using information theoretic similarity concepts, we estimated similarities between sets of GO terms (similarity calculation based on Schlicker et al. 2006; implemented in R package GOsim, Frohlich et al. 2007). For this analysis, we removed GO terms whose direct "parent" GO term was also included in the same dataset to avoid pseudo replication.

NEUTRAL EXPECTATION OF DIFFERENCES IN GENE EXPRESSION

Nonnative populations are likely to become different from the source population due to founder effects and subsequent genetic drift. To evaluate the extent to which gene expression differences between populations are the result of neutral processes versus directional selection, a null model of gene expression differences is needed. We employed a comparison of $F_{ST} - M_{ST}$ values, where F_{ST} is the differentiation index derived from putatively neutral molecular marker loci, and M_{ST} is the proportion of total variance in gene expression explained by the variance between populations (Whitehead and Crawford 2006; Hughes et al. 2015). M_{ST}, as well as the related and more commonly used index Q_{ST} (used for quantitative phenotypic traits), are derived from the variances within and between populations, and are directly comparable to F_{ST} values (Whitlock 2008; Leinonen et al. 2013). The conventional interpretation is that traits or gene expressions with Q_{ST} or M_{ST} values higher than F_{ST} values are putative signatures of natural (directional) selection, while values lower than F_{ST} are signatures of stabilizing selection and Q_{ST} or M_{ST} equal to F_{ST} indicates neutral evolution of traits or gene expressions (Whitlock 2008; Leinonen et al. 2013).

F_{ST} values were recalculated from a previously published dataset of 13 microsatellite loci (Michaelides et al. 2015) using Arlequin 3.5.1.3 (Excoffier and Lischer 2010). M_{ST} values were calculated from the expected mean squares of a one-way ANOVA with "introduction" as response variable (see File S2 for details). This calculation was executed for variance-stabilized count data for every transcript, and independently for each lineage and temperature, resulting in four sets of M_{ST} estimates ("Fr-cool," "Fr-warm," "It-cool," and "It-warm"). The rationale for subdividing the dataset is that we are interested in comparing M_{ST} values between the two lineages and that the experimental design did not allow precise estimates of family variation independently of the temperature treatment.

Since these calculations provide only point estimates of F_{ST} and M_{ST} values, and since these estimates are generally associated with large variances, we followed the approach of Whitlock and Guillaume (2009) to derive the expected distribution of M_{ST} values based on the F_{ST} estimates for microsatellite markers and the variance of gene expression within populations (Va_{within}; see File S2). Assuming the approximation of $M_{ST} = F_{ST}$, which should be true for neutral traits, we simulated one million neutral M_{ST} values ($M_{ST\_neutral}$) by sampling the distribution of observed F_{ST} values and the variance in gene expression within each population (Va_{within}) under the Chi-square distribution of Lewontin and Krakauer (1973); (R script adapted from Lind et al. 2011). We evaluated the observed M_{ST} values of the entire transcriptome ($M_{ST\_observed}$) and of the subgroup of genes differentially expressed following introduction (M_{ST_DEG}) against the M_{ST} values under neutral evolution ($M_{ST\_neutral}$). We considered transcripts with associated M_{ST} values that lie above the 97.5% confidence interval of $M_{ST\_neutral}$ to be candidates under directional selection.

DIFFERENTIAL GENE EXPRESSION

To gain a first overview of the broad patterns of variation in gene expression profiles, we applied a principal component analysis to the full dataset by using the R package DESeq2 (Love et al. 2014). After we confirmed that samples derived from a pool of embryos are clustering closely with samples derived from single embryos (Fig. S2), we exclude the pooled samples from further analyses to avoid deflating within-population variances. We used a FDR (false discovery rate) adjusted *P*-value <0.01 as the cutoff for differential expression. First, we interrogated the dataset for differentially expressed genes by fitting a full factorial model that contained "lineage" (Fr – It), "temperature" (cool – warm), and "introduction" (native – nonnative), plus all possible interactions as fixed effects. Second, we divided the data into four subsets partitioning out the effect of "lineage" and "temperature," to obtain more specific insights into gene expression changes that are associated with the introduction of lizards to England. This strategy allowed us to also assess if responses are stronger at low incubation temperature, which we might expect for populations adapting to a cooler environment (see Discussion). This analysis produced four sets of genes that are differentially expressed between native and nonnative populations (DEG_{intro}). Third, we selected the total set of DEG_{intro} for a given lineage and examined to what extent these genes are recruited from a pool of ancestrally temperature-responsive genes. To address the question, we asked if DEG_{intro} are enriched in genes that are differentially expressed in native populations in response to temperature. This set of ancestrally "plastic" genes ($DEG_{ancPlast}$) was obtained fitting a model with "temperature" as sole factor to a dataset containing only native populations. Fourth, we investigated how the extent to which DEG_{intro} respond to temperature has changed following introduction. For native and nonnative populations separately, we fitted a model with "temperature" as sole factor to the set of DEG_{intro} and assessed if the total number and regulation of temperature-responsive genes changed following introduction.

To verify if our results are robust against different strategies of analysis, we also investigated the effect of the introduction by controlling for temperature in the statistical model, instead of analyzing the two incubation temperatures separately (see above). The results of these latter approaches produced very similar findings as the main analyses and are presented in File S3.

TRANSCRIPT CLUSTERING AND DIFFERENTIAL CLUSTER EXPRESSION

To overcome the limitations of differential gene expression analysis at the level of single genes (e.g., the problem of

multiple-testing, general noise in gene expression data; Horgan and Kenny 2011; Conesa et al. 2016), we applied coexpression-based transcriptome clustering (Langfelder and Horvath 2008). This approach reduces the high dimensionality inherent in transcriptomic datasets, and thereby increases the power of detecting modules of genes that exhibit shared gene expression profiles (Meng et al. 2016). We performed coexpression-based clustering of our variance-stabilized transcript count data using the R package WGCNA (Langfelder and Horvath 2008), which constructs a network based on pairwise correlations of transcript expression, and aggregates transcripts that share the same neighbors into modules. We subsequently used the first principal component of each module (eigengene) to summarize its expression in the whole experiment.

Robust pairwise correlations were calculated using biweight midcorrelations, which penalize scores proportionally to their distance from the median (Langfelder and Horvath 2008). We also allowed the two most extreme data points (5%) to be considered as outliers and excluded these from calculations. We constructed a signed network to retain information on the sign of the correlations as well as their strength, and power-transformed it to the lowest exponent that generated a scale-free topology (17), which is expected for gene expression networks (Langfelder and Horvath 2008). We tested a different range of clustering specificities and compared their results to select the parameters that minimized correlation between modules while retaining unique patterns. We opted to apply the most permissive split criterion ("deepSplit" option set to 0), merging modules that diverged at tree height lower than 20% and setting a minimal modules size of 20 transcripts. A heatmap of the eigengene correlations between the resulting modules is shown in Figure S3.

To look systematically for modules that show significant response to any of our explanatory variables, we applied linear-mixed models (LMMs) as implemented in the R package lme4 (Bates et al. 2015) to the eigenvalues of each module. We specified clutch identity as a random factor to account for the expected correlation due to higher genetic similarities within the same clutch. Starting from a full model containing all three terms ("lineage," "temperature," and "introduction") and their interactions, we fitted a model for every combination of those factors. We then compared the resulting model set by using AICc, selecting the models that best fit the data (ΔAICc < 2; Table S1). Only five modules out of total 25 showed multiple models being equally fit, and we selected the model with fewest terms for these five cases (Table S1). To ensure appropriate fit of the models to our data, we examined quantile–quantile plots of sample residuals for the best and null models for each module (data not shown). All model-set comparisons were performed using the R package MuMIn (Barto 2015).

ENRICHMENT ANALYSES

Enrichments or general significance in overlapping sets of transcripts or GO terms was tested by simulating 10,000 permutations of a randomly selected dataset. If the observed number of overlapping transcripts or GO terms was larger than the 97.5% confidence interval of the permutated dataset, the enrichment was considered to be significant.

Results

PATTERNS OF VARIATION IN GENE EXPRESSION

The principal components showed that the eight experimental groups clearly separate into four clusters according to the two main effects, incubation temperature and lineage (Fig. 2A). The signal of introduction, setting native and nonnative populations apart, was substantially smaller. However, within lineages and temperatures, native embryos were significantly more similar to each other than to their nonnative counterparts (see dashed arrows in Fig. 2A). The direction of divergence between native and nonnative populations was different for the two lineages; that is, the principal component values did not fall along the same axis (Fig. 2A). This pattern was consistent with the prediction from putatively neutral microsatellite markers, which revealed that the nonnative populations of French and Italian origin were more dissimilar to each other than were the native populations of French and Italian origin (Fig. 2B).

DIFFERENTIALLY EXPRESSED TRANSCRIPTS

Using a model including all interaction terms, we found that 21.7% (4393) of all transcripts were differentially expressed in response to incubation temperature and 19.8% (3998) showed differences between lineages (Table S2). The number of transcripts that were consistently differentially expressed between native and nonnative populations were fewer, but still substantial (3.9%, 783 differentially expressed transcripts). There were also a significant number of transcripts for which we identified a significant interaction between lineage and introduction (2.4%, 476 differentially expressed transcripts; Table S2).

We partitioned our dataset into four subsets along the major factors (lineage and temperature) to identify transcripts that were differentially expressed (DEG_{intro}) between nonnative and native populations (see below). When comparing the observed M_{ST} values for the four subsets to the $M_{ST\_neutral}$ distribution, we found that substantially more transcripts than expected by chance had M_{ST} values above the 97.5% confidence interval (Table 1; Fig. S4). This effect was particularly pronounced for the French lineage, where there were almost three times as many transcripts with highly divergent than expected by chance. In addition, the vast majority of M_{ST} values associated with the DEG_{intro} identified

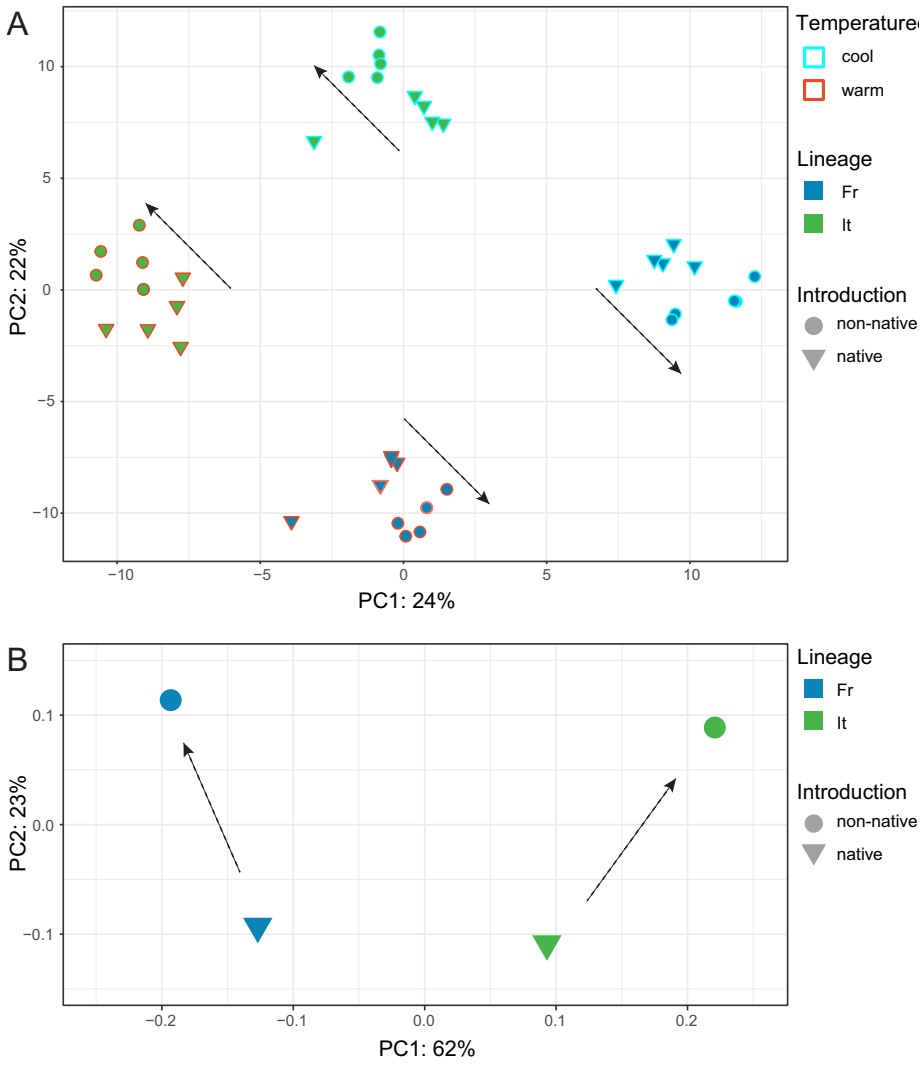

Figure 2. **Principal component analysis of gene expression profiles and genetic divergence at microsatellite loci. (A) Plotted are 40 samples, each representing first and second principal components of regularized logarithm transformed read counts of a single embryo across 20,221 transcripts. The first two principal components explain 24% and 22% of the variation and separate the samples according to lineage and incubation temperature into four distinct clusters. At a given temperature, samples from native lizards show more similar expression profiles than samples from nonnative lizards (dashed arrows mark this trend). (B) Principal components of 13 microsatellites (Michaelides et al. 2015) show the putatively neutral pattern of divergence between native and nonnative populations from Italian and French lineages. Pairwise F_{ST} estimates between populations are: Fr – native and It – native, 0.156; Fr – native and Fr – nonnative 0.125; It – native and It – nonnative, 0.140; Fr – nonnative and It – nonnative, 0.319. The first two principal components explain 62% and 23% of the variation in F_{ST} estimates.**

in the single gene analysis (see below) fall well outside of the 97.5% confidence interval of the $M_{ST\_neutral}$ distribution (Table 1, Fig. 3). Thus, there is evidence that the DEG_{intro} identified in the single gene analysis are candidates for being under directional selection following introduction to England.

Overall, more transcripts were differentially expressed in lizard embryos of French origin and at the warm incubation temperature (Fig. 4). More than 15% of transcripts that differed in their expression between nonnative and native embryos (DEG_{intro}) were consistently up- or downregulated at both temperatures within a lineage (198 (18.7%) versus 79 transcripts (16.0%) for French and Italian origin, respectively; note that all of these transcripts showed the same sign of expression difference at both temperatures; Fig. 4). The overlap of DEG_{intro} between lineages was small, but higher than expected by chance at both cool and warm temperature (15°C: 16 transcripts; 1.9%; neutral expectation: 8, 95% CI [4–13]; 24°C: 20 transcripts; 2.1%; neutral expectation: 10, 95% CI [5–15]). However, only half of these DEG_{intro} shared between lineages showed a consistent direction of expression change. We identified only one transcript that was

Table 1. Comparison of observed M_{ST} values of whole transcriptome and of subset of DEG_{intro} with the neutral expectation derived from F_{ST} values.

Data subset	Mean F_{ST}	Mean $M_{ST\_neutral}$	Mean $M_{ST\_observed}$	Mean M_{ST} of DEG_{intro}	Expected number of genes outside 97.5% CI	Observed number of total transcripts outside 97.5% CI	Fold enrichment	Observed number of DEG_{intro} outside 97.5% CI [percent of all DEG_{intro}]
It/cool	0.137	0.205	0.060	0.808	463–549	599	1.2 ×	257 [91.8%]
It/warm	0.137	0.204	0.074	0.809	462–549	686	1.4 ×	271 [92.5%]
Fr/cool	0.109	0.176	0.119	0.808	463–550	1405	2.8 ×	566 [96.4%]
Fr/warm	0.109	0.174	0.107	0.818	463–548	1509	3.0 ×	652 [97.2%]

The neutral expectation of the number of transcripts with have M_{ST} values higher than the 97.5% confidence interval was estimated using permutation tests. The distributions of the estimated $M_{ST\_neutral}$ and the M_{ST_DEG} are shown in Figure 3, and the distribution of all observed M_{ST} values ($M_{ST\_observed}$) is plotted in Figure S3. DEG_{intro} refers to genes that are differentially expressed between native and nonnative populations.

differentially expressed in all four data subsets, and the direction of the change in nonnative compared to native populations differed between lineages.

Transcripts that were differentially expressed in nonnative versus native populations were significantly (approximately 1.6-fold) enriched for transcripts that showed a temperature-dependence in native populations (i.e., "ancestral plasticity"; Table S3). However, we did not find evidence that transcripts differentially expressed following introduction change their response to temperature, and there was a large overlap of temperature-responsive transcripts between native and nonnative populations (Fr: 124 transcripts; 33%; neutral expectation: 48, 95% CI [37–59]; It: 54 transcripts; 32%; neutral expectation: 21, 95% CI [13–28]). Furthermore, the vast majority (96%) of transcripts that were temperature responsive in the native population ("ancestral plasticity") qualitatively retained their expression profile in respect to temperature following introduction (Table S4).

DIFFERENTIALLY EXPRESSED GENE MODULES

Coexpression-based clustering produced 24 modules of coexpressed genes plus one module that consisted of three genes that showed no significant coexpression (module "gray"; naming of modules by color names is default in the WGCNA software; Fig. 5). The average size of modules was 842 genes, with the largest module containing 3443 genes (module "turquoise"), and the smallest module 30 genes (module "darkgray").

Consistent with the results described above, 18 out of 25 modules showed significant differences in expression at 15°C versus 24°C (Table S1), and the expression pattern of six modules was best explained by temperature alone. While there was no statistical support for a module with lineage as the only explanatory variable, lineage was included as an explanatory factor in the best models for 14 modules. In six of those modules temperature and lineage alone provided the best explanation for the observed expression patterns, in two modules lineage and introduction were selected as best predictors, and in the remaining six modules all three factors best-explained variation in the data. All eight modules that supported "introduction" as a main effect include the interaction term between "introduction" and "lineage" (Table S1), consistent with the lack of convergence in gene expression profiles in nonnative populations revealed above. By comparison, out of the 18 temperature-responsive modules, only two show lineage-specific responses to temperature.

The overall divergence between native and nonnative populations is evident in the graphical representation of the eigenvalues of gene modules (Fig. 5). For example, modules "red," "purple," "salmon," and "tan" have significantly lower expression in French populations introduced to England compared to native French populations, whereas the same modules show higher expression in Italian populations introduced to England compared with native Italian ones. In contrast, the eigenvalues of the two modules "midnightblue" and "lightyellow" are characterized by identical expression profiles across experimental groups except for French native lizard embryos (Fig. 5). A corresponding pattern, but with Italian native lizards exhibiting the divergent expression profile is shown by module "lightgreen." These three modules ("midnightblue," "lightyellow," and "lightgreen") are significantly enriched in genes that are differentially expressed following introduction (percentage of DEG_{intro} in whole dataset: 7.44%; percentage in "midnightblue": 31.80%; percentage in "lightyellow": 37.86%; percentage in "lightgreen": 21.05%).

FUNCTIONAL CHARACTERIZATION OF RELEVANT GENES

Comparing the GO terms associated with genes differentially expressed between native and nonnative populations (DEG_{intro})

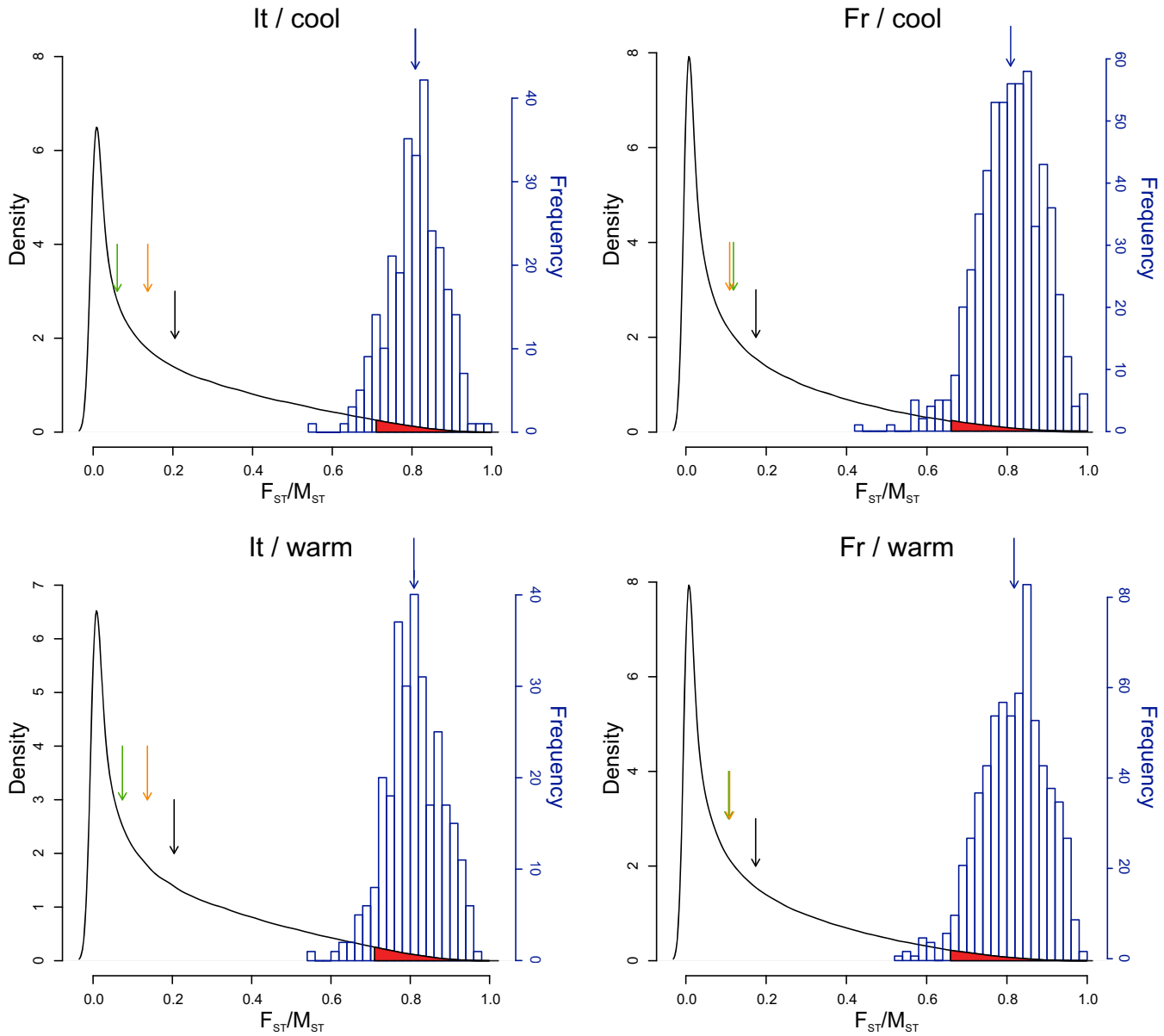

Figure 3. **Neutral expectation of differentiation of gene expression profiles between native and nonnative lizard embryos. Density plots of the simulated distribution of M_{ST} values under neutral evolution ($M_{ST\_neutral}$) are shown (black graphs). The 2.5% tail of the distribution is shaded in red. In addition, histograms of the frequency distributions of the observed M_{ST} values of the differentially expressed genes are plotted in blue (scale on the right). Arrows indicate the average F_{ST} values (orange), average $M_{ST\_neutral}$ (black), average observed M_{ST} values for the entire transcriptome (green), and average observed M_{ST} values for DEG_{intro} (blue).**

against a neutral expectation, we find enrichment of 209, 300, 210, and 244 GO terms for the Fr-cool, Fr-warm, It-cool, and It-warm datasets, respectively. GO terms overlapped within lineages between incubation temperatures (Fr: 38; It: 44; Table S5), and within incubation temperature between lineages (cool: 8; warm: 12). The similarity between the shared group of GO terms between the French and the Italian lineage was significantly higher than expected by chance for the two GO domains "biological process" and "molecular function" (Table 2), suggesting convergence between nonnative populations at the level of gene function. For example, French DEG_{intro} are enriched in the term "purine nucleobase catabolic process" (GO:0006145), while the corresponding Italian group of genes are overrepresented with the term "pyrimidine nucleoside catabolic process" (GO:0046135).

Discussion

Following their introduction to England in the 20th century, wall lizards originating from France and Italy have adapted to the cooler climate experienced in their nonnative range by increasing

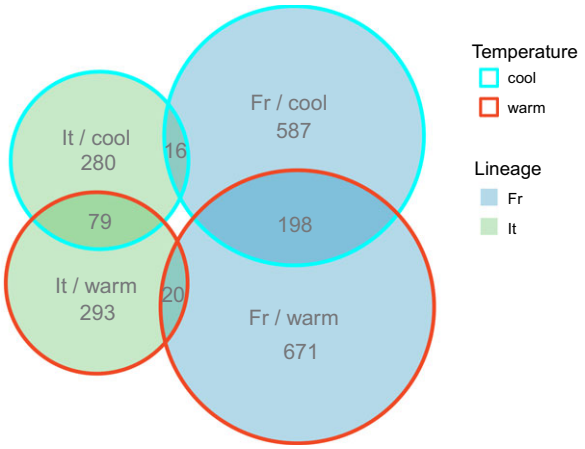

Figure 4. Venn diagram showing overlap of differentially expressed genes in nonnative compared to native lizard embryos (DEG_{intro}). Each circle represents the number of genes that were differentially expressed following introduction in one partition of the data subset according to the two main axes of variation (incubation temperature and geographic origin). The numbers in gray given at the overlap of circles represent the number of genes shared between the individual subsets.

developmental rate (While et al. 2015a). A faster rate of embryonic development shortens incubation duration, which enables lizards to hatch before the onset of autumn despite the low incubation temperatures in England. Our results here suggest this faster developmental rate has been accompanied by adaptive modification of cellular metabolism. However, the genes that have evolved higher or lower expression following introduction showed no, or at best very limited, overlap for lizards of the French and Italian lineages. Nevertheless, we find that these genes share substantial similarity in their putatively assigned gene functions. Our study thus exemplifies that founder effects and other sources of historical contingency can allow convergence of phenotype in the face of divergence of gene expression profiles.

SIGNATURES OF NEUTRALITY AND SELECTION IN TRANSCRIPTOMES

Introduced populations often become genetically more different from each other because of founder effects and drift due to low population size. The wall lizard populations studied here were introduced by humans, likely through the release of tens of individuals, and show a modest reduction in genetic diversity compared to populations in their native range (Michaelides et al. 2016). Indeed, estimates of neutral genetic divergence (F_{ST}) show that nonnative populations of the two lineages are genetically more different to each other than the corresponding comparison of populations from the native range (see also Michaelides et al. 2016), suggesting that genetic drift has played an important role during or following introduction.

The overall divergence in embryonic gene expression between nonnative and native populations (i.e., M_{ST}) followed the pattern predicted by F_{ST}, and hence the majority of variations in transcriptomes among populations are selectively neutral. This result is in line with the limited number of other studies that have compared divergence in transcriptomes to a neutral model based on F_{ST} (e.g., Roberge et al. 2007; Lamy et al. 2011; Hughes et al. 2015). Nevertheless, the analyses revealed more highly divergent transcripts than expected by chance, suggesting that at least part of the transcriptome has been under directional selection since the populations were introduced. This was also supported by the analyses of modules of coexpressed genes, which revealed a small number of modules for which the nonnative and native populations differed substantially from each other. Both types of analyses strongly suggested that the targets of directional selection in gene expression patterns were different for the French and Italian lineages.

The signal of directional selection was particularly strong for the French lineage, a more recent introduction that has retained more neutral genetic diversity (Michaelides et al. 2016). Since populations in western France are genetically more homogenous than in Italy (Michaelides et al. 2015), the more pronounced difference between nonnative and native populations of French compared to Italian origin is unlikely to be caused by sampling bias. Nevertheless, an obvious limitation for inference of selection on transcriptomes, which applies to our study as well, is that a robust rejection of selective neutrality (and environmental maternal effects) requires an experimental design that allows more precise estimates of additive genetic variance (e.g., a quantitative genetic breeding design).

Although few studies have compared divergence in transcriptomes to a neutral model based on sequence data (Leinonen et al. 2013), other studies of genetically distinct populations of animals that inhabit similar environments have found that a (usually very small) part of the transcriptome has converged. This is evidence that the expression of those genes have been under directional selection. For example, Zhao et al. (2015) showed that around 1% of the transcriptomes of two *Drosophila* species exhibit the same changes between pairs of high and low altitude populations. The results from wall lizard embryos are consistent with directional selection on gene expression in populations of both lineages following introduction. However, we found very limited support for convergence of gene expression profiles between lineages in nonnative, cool-adapted, populations. Specifically, while more transcripts with divergent expression between nonnative and native populations were in fact shared between lineages than expected by chance, these transcripts did not show a consistent up- or downregulation in nonnative populations. In addition, the relatively low fold enrichment of highly divergent gene expression in nonnative populations of the Italian lineage suggests that a portion

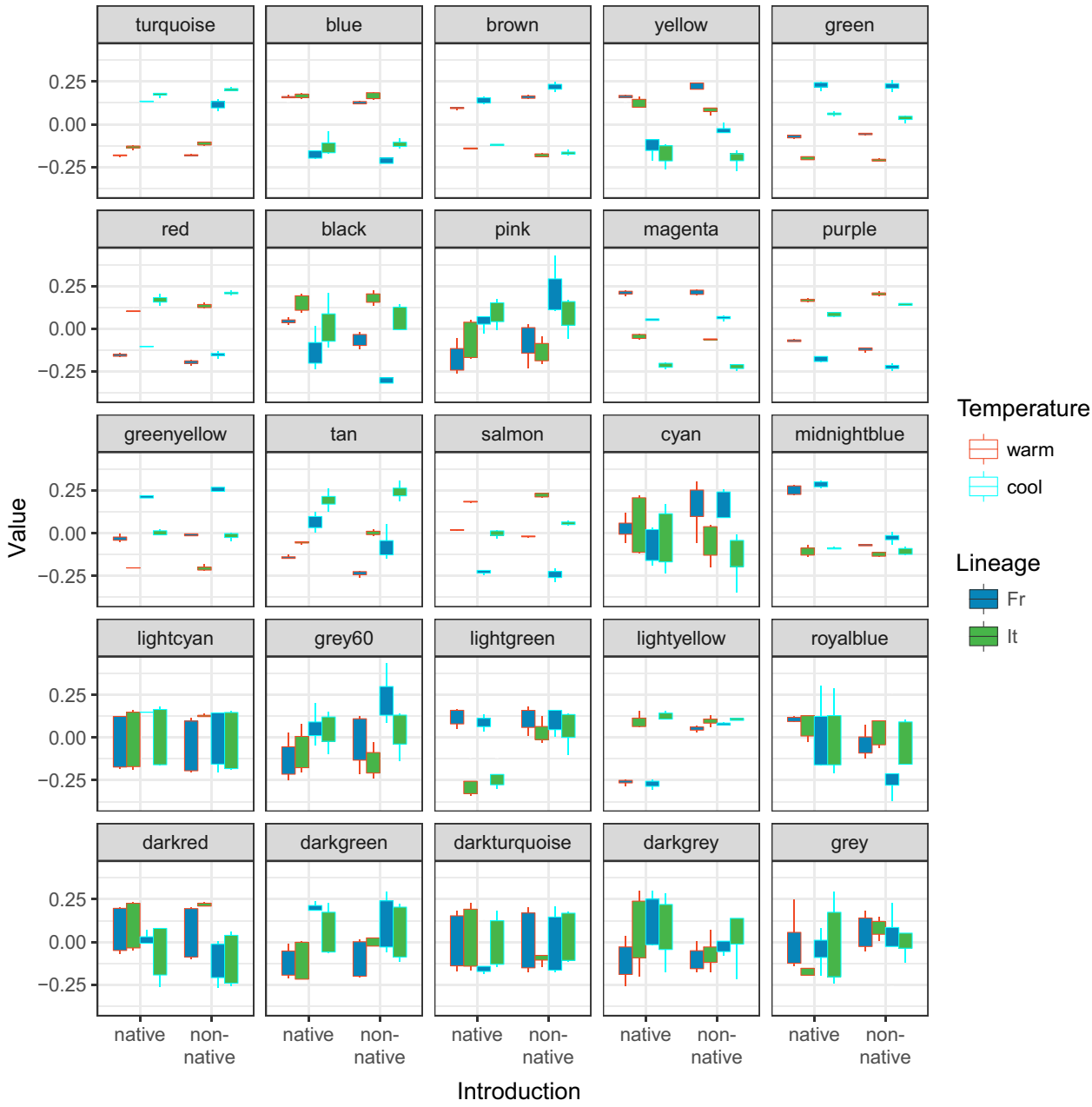

Figure 5. **Eigenvalues of each module of coexpressed genes. The plots show the eigenvalues of the eight experimental groups for each of the 24 differentially expressed modules (plus module "gray"), the eigenvalues of the eight experimental groups are plotted. Panels are ordered according to module size, that is the number of transcripts they contain (largest module, top left; smallest module, bottom right).**

of the putative targets of selection are false positives. This may have limited the overall signal of convergence between lineages. However, given the low number of shared transcripts (fewer than 20 for a given temperature) between lineages, together with the limited signal in the Italian lineage, we conclude that there is no robust evidence for convergence in the expression of particular genes in nonnative populations.

Despite limited evidence for convergence in gene expression, we did find considerable similarity in the putative functionalities associated with genes that were differentially expressed in nonnative populations. For example, genes differentially expressed in embryos from nonnative populations of both lineages were highly enriched for GO terms associated with nucleotide- and glucose-metabolism. Although functional annotations should be interpreted with caution, the enrichment of functional categories suggests that nonnative populations of both lineages exhibit an increase in absolute transcription and replication rate, a higher cellular metabolism and faster cell cycle. Thus, these results are

Table 2. Comparison of similarities between sets of GO terms between French and Italian lineage.

Category	Number of GO terms Fr	number of GO terms It	Observed similarity	95% CI of simulated similarity
BP	10	14	0.2356	0.0645–0.1641
MF	12	13	0.1072	0.0408–0.1071

Both categories BP ("biological process") and MF ("molecular function") show significant similarity between the two lineages. Note that the number of GO terms in each category does not correspond to the total number of enriched GO terms since not all GO terms were associated with an "Information content" that is a prerequisite for calculating similarities (Schlicker et al. 2006). Category CC ("cellular component") had too few GO terms with an associated "Information content," and therefore no similarity was calculated.

consistent with the faster developmental rate of embryos from nonnative populations, a difference that is apparent already at this early stage of development.

The lack of overlap in modules of coexpressed genes under putative directional selection, and at best a very modest overlap of individual genes, implies that there is a very large number of variants that can contribute to thermal adaptation. In nonnative populations, the standing genetic variation available to natural selection will largely be determined by founder effects. The English wall lizard populations studied here are isolated with no gene flow, which restricts the likelihood of convergence of gene expression profiles when populations are adapting to the same conditions. The situation could be different for a more natural range expansion. For example, common wall lizards are abundant even at thermally challenging altitudes across their native range. Because high-altitude lizard populations often show an adaptive increase in developmental rate, there are opportunities to test if populations that adapt independently to cool climate without the bottlenecks and genetic isolation associated with an introduction event show more consistent gene expression profiles. More generally, such comparisons of populations with different demographic histories may be useful to identify how historical contingencies influence the extent of convergent evolution at the molecular level, and thus the repeatability of adaptive evolution at different levels of biological organization.

TEMPERATURE-DEPENDENT GENE EXPRESSION AND ADAPTATION

Despite the highly conserved embryonic stage we based our analysis on, there was a strong effect of temperature on transcription profiles with as many as 20% of all transcripts showing differences in their relative expression at 15°C versus 24°C. There does not appear to be any comparable data for other vertebrate embryos, but this figure is consistent with what has been reported for temperature-dependent gene expression in *Drosophila melanogaster* and *D. simulans* where 10–20% of all genes responded significantly to a temperature difference of 8˚C (Zhao et al. 2015). The existence of temperature-specific gene expression suggests that it should be possible for organisms to adapt to low temperature without necessarily changing their response to high temperature. In contrast to their native counterparts, wall lizard embryos in England are likely to consistently experience temperatures below 20°C (While et al. 2015a). One could therefore expect divergence between nonnative and native populations to be particularly pronounced at very low temperature. This does not appear to be the case, however. Within each lineage, the putatively adaptive gene expression differences that have accumulated in nonnative populations were equal in magnitude across the two incubation temperatures. This result may reflect the strong selection for shorter incubation in nonnative populations since a faster developmental rate at high temperatures can have a disproportionate effect on incubation period even if such temperatures are encountered only rarely (While et al. 2015a). Indeed, cool-adapted populations of ectotherms often develop and grow faster also at high temperatures that should only occasionally be encountered in the wild (Angilletta 2009). In our study, some 16–18% of transcripts that were differentially expressed between the nonnative and native populations (i.e., DEG_{intro}) showed a consistent response at both 15 and 24°C. These genes are perhaps particularly likely candidates for directional selection for faster developmental rate, not the least since they were highly enriched for processes related to nucleotide metabolism and transcription, as described above.

There is a growing interest in how environment-dependent gene expression may change during adaptation to novel environments. On the one hand, maladaptive plasticity in gene expression is expected to quickly become eliminated by natural selection. For example, in guppies, gene expression under putative directional selection in a predator-free environment showed reduced sensitivity to predatory cues (Ghalambor et al. 2015). In the context of adaptation to cool climate, this should involve selective removal of extreme gene expression profiles at low temperature, resulting in an overall weaker temperature-dependence. On the other hand, strong temperature-dependent expression may reflect adaptive plasticity. In this case, selection in more extreme thermal environments may exaggerate the temperature responsiveness of genes that already show some degree of thermal sensitivity (Lande 2009). Thus, both reduced and increased plasticity in gene expression are possible outcomes of adaptation to an extreme environment.

There is some empirical evidence that transcripts that are highly responsive to temperature also figure disproportionally as candidates for climate adaptation. In the estuarine fish

Fundulus heteroclitus, eight out of 11 genes that were upregulated in muscle at cool acclimation temperatures were downregulated in cool-adapted versus warm-adapted populations (Dayan et al. 2015). Our results also provide some support for the prediction that adaptation to cool climate will preferentially involve genes that show native temperature responsiveness (i.e., ancestral plasticity). The putative adaptive gene expression differences in lizard embryos from England were enriched for transcripts that were significantly up- or downregulated with temperature in native populations from both Italy and France. However, there was no evidence for a consistent increase or decrease in the temperature sensitivity of expression of those genes. Thus, it appears as if evolution in nonnative populations did not tinker with the thermal sensitivity of gene expression, but rather adjusted constitutive expressions. Suffice to say that the results suggest that evolutionary adaptation to temperature preferentially involves modification of the same regulatory interactions that also make embryos developmentally responsive to temperature.

AUTHOR CONTRIBUTIONS

N.F., G.M.W., and T.U. conceived the study, designed the experiment, and collected the data. N.F. analyzed all data, except for WGCNA analyses that were performed by A.R. N.F., A.R., and T.U. interpreted results and wrote the manuscript. G.M.W. revised the draft. All authors approved the final version of the manuscript.

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DATA ARCHIVING

Illumina reads were deposited in the NCBI SRA database under the accession number SRP113322 (BioProject PRJNA394646, BioSample SAMN07357074).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Overview of the experimental design and details on the bioinformatical pipeline.
Figure S2. Principal component analysis of full dataset including samples derived from pool of embryos.
Figure S3. Heatmap showing similarity between the 25 modules.
Figure S4. Observed MST values of gene expression profiles between native and non-native lizard embryos.
Table S1. Expression patterns of the 25 gene expression modules.
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Table S3. Comparison of gene sets that show expression changes following introduction (DEGintro) and gene sets that respond to temperature in the lizards from the native range (DEGancPlast).
Table S4. Comparison of temperature-responsive genes within the set of genes that are differentially expressed following introduction.
Table S5. GO terms significantly enriched in genes differentially expressed following introduction.
Supporting Information
Supplementary file 2. Calculation of M_{ST}
Supplementary File 1 Results of 2-factorial analyses of 'French' and 'Italian' datasets