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Captivity induces large and population-dependent brain transcriptomic changes in wild-caught cane toads (*Rhinella marina*)

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Abstract

Gene expression levels are key molecular phenotypes at the interplay between genotype and environment. Mounting evidence suggests that short-term changes in environmental conditions, such as those encountered in captivity, can substantially affect gene expression levels. Yet, the exact magnitude of this effect, how general it is, and whether it results in parallel changes across populations are not well understood. Here, we take advantage of the well-studied cane toad, *Rhinella marina*, to examine the effect of short-term captivity on brain gene expression levels, and determine whether effects of captivity differ between long-colonized and vanguard populations of the cane toad's Australian invasion range. We compared the transcriptomes of wild-caught toads immediately assayed with those from toads captured from the same populations but maintained in captivity for seven months. We found large differences in gene expression levels between captive and wild-caught toads from the same population, with an over-representation of processes related to behaviour and the response to stress. Captivity had a much larger effect on both gene expression levels and gene expression variability in toads from vanguard populations compared to toads from long-colonized areas, potentially indicating an increased plasticity in toads at the leading edge of the invasion. Overall, our findings indicate that short-term captivity can induce large and population-specific transcriptomic changes, which has significant implications for studies comparing phenotypic traits of wild-caught organisms from different populations that have been held in captivity.

KEYWORDS
Bufo marinus, cane toad, captivity, invasive species, population, transcriptomics

1 | INTRODUCTION

Transcriptomics are essential to answer key questions in molecular biology, from functional genomics and phylogenetics, to biomarker discovery. Investigating gene expression changes is especially important to understand gene function and adaptation. Levels of gene expression are intrinsically labile and can be responsive to both internal and external stimuli. Perhaps one of the most frequently encountered environmental influences in the field of experimental research is captivity. Many organisms are routinely bred in controlled conditions. Captive breeding can lead to genetic changes, resulting from small population sizes and/or adaption to environmental conditions encountered in captivity (Frankham, 2008). These genetic changes can have profound fitness consequences and their mitigation is an essential part of conservation programs (Allendorf et al., 2010; Frankham et al., 2002). Captive breeding can also have large consequences for gene expression levels. For example, nine generations of captive breeding were found to induce large gene expression changes (i.e., ~5000 differentially expressed genes; hereafter, DEGs) and shifts in allele frequency in the zebrafish, *Danio rerio*, (Uusi-Heikkilä et al., 2017). Likewise, a single generation of captive breeding induced substantial gene expression changes (i.e., >700 DEGs) in the steelhead trout, *Oncorhynchus mykiss* (Christie et al., 2016).

In the case of wild-caught individuals, quantitative and/or qualitative measurements of individuals from different populations are often performed in the laboratory, following a period in captivity. In such cases, the observed gene expression levels are usually thought to be representative of wild populations. Yet, some studies have reported transcriptomic changes following a short period of captivity. For example, a couple of days of captivity-induced stress led to a reduction in *Vitellogenin* expression in the liver of a tropical anole, *Anolis pulchellus*, (Morales & Sánchez, 1996). In the three-spined stickleback, *Gasterosteus aculeatus*, six out of 13 of the immunity genes assessed in the whole body had differential expression after 11 months in captivity (Hablützel et al., 2016). Similarly, 11 months in captivity induced gene expression changes in ~1800 genes in gill tissue in the freshwater mussel, *Amblema plicata* (Roznere et al., 2021). In the thecosome pteropod, *Limacina retroversa*, large transcriptomic changes (i.e., >9000 DEGs) were observed in whole bodies after only 14 days in captivity (Maas et al., 2017). It appears, therefore, that short-term captivity can cause moderate to large changes in gene expression, and thus has the potential to be an important confounding factor in many studies. Yet, both the exact magnitude of this effect and how general it is, are not well understood.

Moreover, whether the magnitude and direction of the effects of captivity are similar across populations is also unknown. Studies frequently use common garden experiments to disentangle genetic and environmental effects on gene expression levels (e.g., Meier et al., 2014). Yet, if different populations respond dissimilarly to captive conditions, then any transcriptomic change that would classically be interpreted as a sign of genetic adaptation, might in fact reflect a population-dependent effect of captivity.

The cane toad, *Rhinella marina*, is a good model to investigate the effect of captivity on gene expression levels. This species has been intensively studied in Australia because of its highly invasive abilities, and it has become a case study to understand the causes and consequences underlying the rapid evolution often seen in successful invaders (Shine, 2010, 2014). From 101 individuals originally brought to Australia in 1935, the cane toads' Australian invasion range now stretches over 1.2 million km^2 (Urban et al., 2007). Remarkably, toads have quickly changed while dispersing across the Australian continent (Shine, 2010). As a result, toads at the front of the invasion range show distinct dispersal-related traits compared to toads found in long-colonized areas at the core of the invasion range (Urban et al., 2008). Indeed, range-front toads have different morphological and physiological features compared with range-core toads (Hudson et al., 2018; Hudson, McCurry, et al., 2016; Llewelyn et al., 2010; Phillips et al., 2006). Their behaviour is also different (Alford et al., 2009; Gruber et al., 2017a, 2017b; Lindstrom et al., 2013). Toads from across the invasion range also show distinct gene expression levels in their brain, spleen and muscle tissues (Rollins et al., 2015; Selechnik, Richardson, Shine, Brown, et al., 2019; Yagound et al., 2022). This phenotypic divergence is mirrored by minor changes at the genetic level, in particular in genes involved in the response to climatic conditions (Selechnik, Richardson, Shine, DeVore, et al., 2019).

Understanding the rapid evolution of cane toads across their Australian invasion range often requires that wild-caught individuals spend short to long periods of time in captivity, under controlled conditions, before being assessed for various phenotypic traits (e.g., Brown, Kelehear, et al., 2015; Ducatez et al., 2016; Gruber et al., 2017a, 2018; Kolbe et al., 2010; Llewelyn et al., 2010; McCann et al., 2014). Captive breeding is also a frequent practice to evaluate the heritability of phenotypic variation in common garden experiments (e.g., Brown, Phillips, et al., 2015; Gruber et al., 2017b; Hudson et al., 2018; Hudson, Brown, et al., 2016; Kosmala et al., 2018; Sarma et al., 2021; Stuart et al., 2019). However, how captivity induces phenotypic changes in this species remains unclear.

Few studies of cane toads have documented the effects of captivity on behavioural and physiological traits. Gruber et al. (2018) found that captivity had an influence on the behavioural responses of toads subjected to consecutive assays, and that this effect was different between captive-raised and wild-caught toads. Transport-induced captivity has been shown to increase toads' dispersal behaviour (Pettit et al., 2017). Further, it is well established across vertebrates, including anurans, that captivity-induced stress affects corticosterone and testosterone levels, as well as immune function (Graham et al., 2012; Titon et al., 2017, 2018). However, the effect of captivity on gene expression levels in cane toads has not been studied.

Here, we evaluated the effect of short-term captivity on the brain transcriptome of invasive cane toads by comparing the gene expression profile of wild-caught toads with those of toads captured from the same populations and then maintained for 7 months in captivity under controlled conditions. We compared the transcriptomes of wild-caught and captive toads from both range-front (i.e., newly-colonized) and range-core (i.e., long-colonized) areas to test whether

captivity had an effect of similar magnitude for populations located at each end of the Australian invasion range. We predicted that wild-caught and captive toads would have distinct brain transcriptomes, thus indicating an effect of captivity on brain gene expression levels. Further, based on the existing large phenotypic differences between range-front and range-core populations, we predicted that these two populations would display distinct responses to captivity.

2 | MATERIALS AND METHODS

2.1 | Sample collection

All toads used in this study were adult female cane toads collected in 2014 and 2015 from six sites ($n = 4$–12 individuals per location) representing two populations (Figure 1 and Table S1) of the Australian invasion range. Queensland sites corresponded to the range-core population of the invasion range, where toads had been present for 76 to 80 years at the time of collection (Gruber et al., 2017a). Mean annual rainfall at these sites is respectively 1924.2 ± 605.3 (mean ± SD) mm (Gordonvale), 1997.8 ± 546.8 mm (Cairns) and 2956.7 ± 826.7 mm (Daintree), while mean annual maximum temperature is respectively 29.7 ± 0.3°C (Gordonvale), 29.1 ± 0.4°C (Cairns) and 29.4 ± 0.5°C (Daintree) (Australian Government Bureau of Meteorology [www.bom.gov.au/climate/data/]). Sites from Western Australia corresponded to the range-front, where toads had been present for only 4 to 5 years at the time of collection. Mean annual rainfall at these sites is respectively 721.5 ± 253.7 mm (Purnululu), 1012.0 ± 582.5 mm (Durack River) and 571.5 ± 209.2 mm (Halls Creek), while mean annual maximum temperature is respectively 35.1 ± 0.8°C (Purnululu), 35.1 ± 0.8°C (Durack River) and 33.6 ± 0.8°C (Halls Creek). Toads from Gordonvale and Daintree (range-core), and Durack River and Halls Creek (range-front) were humanely euthanised by lethal injection of 150 mg/kg sodium pentobarbital immediately following capture. We extracted whole brain samples and stored them in RNAlater (Qiagen) at 4°C to preserve tissue integrity before storing them at −80°C prior to RNA extraction. Hereafter, we refer to these samples as "wild-caught" ($n = 8$ individuals for both range-core and range-front populations; Table S1). Toads from Cairns (range-core) and Purnululu (range-front) were maintained in captivity between September 2014 and April 2015 (i.e., total 7 months) at Macquarie University, as described in Gruber et al. (2017a). Euthanasia, whole brain extraction and storage was performed as described above. Hereafter, we refer to these samples as "captive" ($n = 12$ individuals for both range-core and range-front populations; Table S1). All procedures were approved by the animal ethics projects 2013/5805, ARA2013/035, 2014/562 and AEX04-2014.

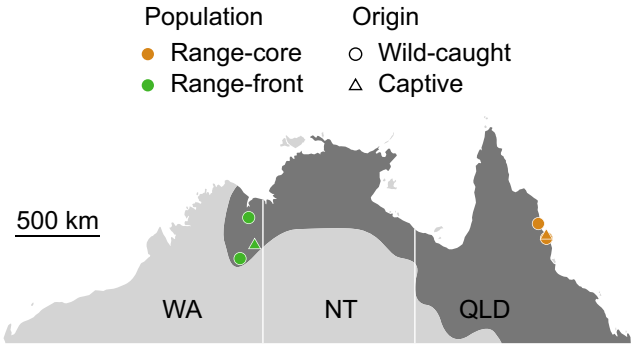

FIGURE 1 Location of samples. NT, Northern Territory; QLD, Queensland; SWA, Western Australia. The shaded area represents the cane toad's Australian invasion range

2.2 | RNA extraction and sequencing

We extracted total RNA from whole brains using Qiagen RNeasy lipid tissue mini kits (Qiagen), according to the manufacturer's protocol. Tissues were homogenized using a Fast Prep-24 Classic homogenizer (MP Biomedicals) and 1 mm Zirconia/Silica beads (Daintree Scientific) for 1 min at 6 m/s. Genomic DNA was digested using a Qiagen RNase-Free DNase set on column during extraction. Extracted RNA was quantified using a Qubit RNA HS assay kit on a Qubit 3.0 Fluorometer (Life Technologies). Library preparation was conducted following the TruSeq mRNA 2 (Illumina) protocol commercially at Macrogen (South Korea). Libraries were sequenced (two lanes of 125 bp paired-end sequencing) on an Illumina HiSeq 2500 platform (Macrogen).

2.3 | Data preprocessing, alignment and gene expression quantification

We checked the quality of the raw data using FASTQC 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). We removed adaptor sequences and we trimmed low quality reads using TRIMMOMATIC 0.38 (Bolger et al., 2014) with the following parameters: ILLUMINACLIP: path/to/TruSeq3-PE.fa:2:30:10:4 HEADCROP:13 AVGQUAL:30 MINLEN:36. We then mapped trimmed reads to the multitissue reference cane toad transcriptome (Richardson et al., 2018) using STAR 2.7.2b (Dobin et al., 2013) in two-pass mode with default parameters. We quantified gene expression from the resultant BAM files using SALMON 1.2.1 (Patro et al., 2017).

There was a similar number of raw reads, post-trimming reads and mapped reads for wild-caught and captive toads (wild-caught toads: respectively 12.2 ± 1.0 million reads, 8.3 ± 0.7 million reads and 3.5 ± 0.3 million reads; captive toads: respectively 11.7 ± 1.8 million reads, 8.2 ± 1.2 million reads and 3.2 ± 0.6 million reads; Table S2).

2.4 | Differential expression analysis

We filtered out genes that had less than 10 counts per million in at least 10 samples using EDGER 3.32.1 (Robinson et al., 2010) with the filterByExpr function in R 4.0.4 (R Core Team, 2021). We normalized

and rlog-transformed counts before computing pairwise correlations for all samples. We then used the resultant correlation matrix to plot a heatmap with pheatmap 1.0.12 (Kolde, 2019) to assess the presence of outliers. This revealed seven outliers, that is, one wild-caught toad and six captive toads (Figure S1). Since the aim of our study was to reveal gene expression differences between captive and wild-caught toads, and since almost all outliers (i.e., individuals with the most different gene expression profile) were captive toads, we decided to exclude all outliers from subsequent analyses and to adopt a more conservative approach. Including outliers would have significantly increased the magnitude of transcriptomic differences between captive and wild-caught toads, for potentially spurious reasons. We performed differential expression analysis with DESEQ2 1.30.1 (Love et al., 2014). To compare the influence of captivity on brain gene expression between range-core and range-front populations, we carried out differential expression analysis for each population separately. We considered any gene with a Benjamini-Hochberg adjusted *p*-value < .05 (Benjamini & Hochberg, 1995) to be significantly differentially expressed between captive and wild-caught toads.

2.5 | Network analysis

We performed gene correlation network analyses for range-core and range-front populations using WGCNA 1.70-3 (Langfelder & Horvath, 2008) to identify clusters of coregulated genes. We selected a soft threshold power using the pickSoftThreshold function according to the authors' recommendations (Zhang & Horvath, 2005). We selected a power of 12 and 10 for range-core and range-front populations, respectively (Figures S2 and S3). We identified gene coexpression modules using the blockwiseModules function. We then tested whether each module was significantly associated with the origin (i.e., captivity vs. wild-caught) of the toads by fitting linear models with Benjamini-Hochberg correction for multiple testing using limma 3.46.0 (Ritchie et al., 2015).

2.6 | Differential variability analysis

We tested whether brain genes differed in their expression variability (hereafter, dispersion) between captive and wild-caught toads for both range-core and range-front populations using MDSEQ 1.0.5 (Ran & Daye, 2017). We normalized gene counts using the trimmed mean of *M*-values (TMM) method (Robinson & Oshlack, 2010) in edgeR. We considered any gene with a Benjamini-Hochberg adjusted *p*-value < .05 to be significantly differentially dispersed between captive and wild-caught toads.

2.7 | Functional analysis

We inferred genes' biological function by performing gene ontology (GO) enrichment analyses using GOSEQ 1.26.0 (Young et al., 2010). We conducted GO enrichment analyses using the probability weighting function (PWF) to adjust for transcript length bias, and the Wallenius approximation to test for over-representation. We adjusted *p*-values with the Benjamini-Hochberg method. We used enrichplot (Yu, 2021) to visualize GO results.

3 | RESULTS

Captivity had a marked effect on brain gene expression in cane toads from populations at both ends of the Australian invasion range. The total number of DEGs between captive and wild-caught cane toads was 951 in range-core populations, and 1972 (i.e., more than twice as many) in range-front populations (Figures 2 and 3). These figures corresponded respectively to 7.3% and 14.9% out of 13,090 and 13,219 total filtered genes. Out of 951 genes showing differential expression between captive and wild-caught toads from range-core populations, 471 (49.5%) genes were upregulated and 480 (50.5%) genes were downregulated (Figure 2a,b). GO enrichment analysis revealed a significant over-representation of GO terms such as "adult locomotory behaviour" and "response to cold" (Figure 2c). There was a larger effect of captivity on brain gene expression in toads from range-front populations, with 1144 out of 1972 (58.0%) genes showing differential expression being upregulated in captive versus wild-caught toads, while 828 (42.0%) genes being downregulated (Figures 3a,b). We found a significant over-representation of GO terms such as "adult locomotory behaviour", "translation" and "cholesterol biosynthetic process" in the GO enrichment analysis (Figure 3c).

By comparison, the total number of DEGs between range-front and range-core populations was only 162 (100 upregulated, 62 downregulated) in captive cane toads, and 49 (21 upregulated, 28 downregulated) in wild-caught cane toads (Figure S4).

GO:0008344 (adult locomotory behaviour) contained respectively 10 and 13 DEGs in range-core and range-front toads, out of which nine were found in both populations (Table S3). Half (50.0% and 46.2%, respectively) of these DEGs were upregulated in captive versus wild-caught toads.

Weighted gene correlation network analysis (WGCNA) identified 37 modules of coregulated genes for range-core populations (Figure S2C), of which eight modules differed significantly between captive and wild-caught toads. Coexpression modules "blue" (1294 genes, $p = .0122$; Figure S5), "black" (715 genes, $p = .0198$; Figure S6), "dark turquoise" (94 genes, $p = .0287$; Figure S7), and "dark olive green" (51 genes, $p = .0393$; Figure S8) contained genes that were on average upregulated in captive versus wild-caught toads. GO enrichment analysis for module "black" showed an over-representation of GO terms such as "response to cold", "response to antibiotic", "response to stress" and "antigen processing and presentation of exogenous protein antigen via MHC class Ib, TAP-dependent" (Figure S6C), thus showing a clear response to environmental conditions induced by captivity. Coexpression modules "dark green" (105 genes, $p = .0079$; Figure S9), "magenta" (433 genes,

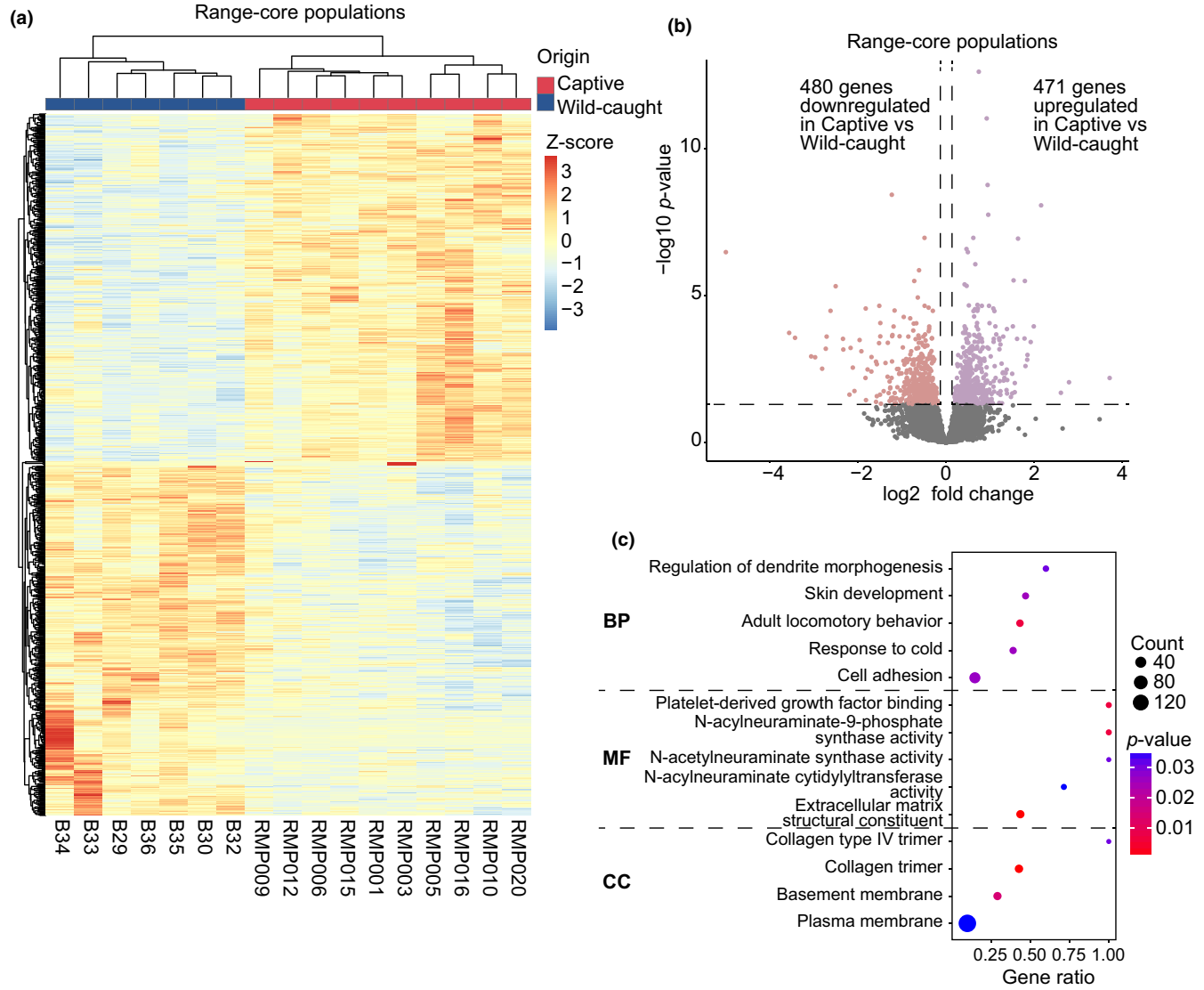

FIGURE 2 Brain gene expression differences between captive and wild-caught cane toads from range-core populations. (a) Heatmap of normalized gene expression values for all differentially expressed genes (DEGs) between captive and wild-caught toads. Columns correspond to individuals. Rows correspond to genes. Colour depicts Z-score normalized gene expression value. (b) Volcano plot of significantly DEGs between captive and wild-caught toads. Nonsignificant genes are represented in grey. (c) GO enrichment analysis of all DEGs between captive and wild-caught toads. The size of each circle is proportional to the number of genes being significantly enriched, while the colour of each circle is proportional to its FDR-corrected *p*-value. Gene ratio corresponds to the proportion of genes being enriched out of the total number of genes in that GO category. BP, biological process; CC, cellular component; MF, molecular function

$p = .0237$; Figure S10), "salmon" (328 genes, $p = .0255$; Figure S11), and "turquoise" (1652 genes, $p = .0267$; Figure S12) contained genes that were on average downregulated in captive versus wild-caught toads. We found a significant over-representation of GO terms such as "learning" (module "magenta"; Figure S10C), lipopolysaccharide and carbohydrate biosynthetic processes (module "salmon"; Figure S11C), and the regulation of transcription in general (module "turquoise"; Figure S12C) in the GO enrichment analyses.

WGCNA identified 14 modules of coregulated genes for range-front populations (Figure S3C), of which two modules differed significantly between captive and wild-caught toads. Coexpression module "turquoise" contained 3618 genes that were on average upregulated in captive versus wild-caught toads ($p = .0150$; Figure S13). GO enrichment analysis showed an over-representation of GO terms like "translation", "protein folding" and "oxidation–reduction process" (Figure S13C). Coexpression module 'brown' contained 1447 genes that were on average downregulated in captive versus wild-caught toads ($p = .0072$; Figure S14). We found a significant over-representation of GO terms related to the general activity of the nervous system (Figure S14C) in the GO enrichment analysis.

When comparing between range-core and range-front populations, we found evidence both for parallel transcriptomic changes due to captivity, and for a larger effect for range-front populations. Out of all DEGs identified between captive and wild-caught toads, 447 were unique to range-core populations, 1468 were unique to range-front populations, and 504 were overlapping between

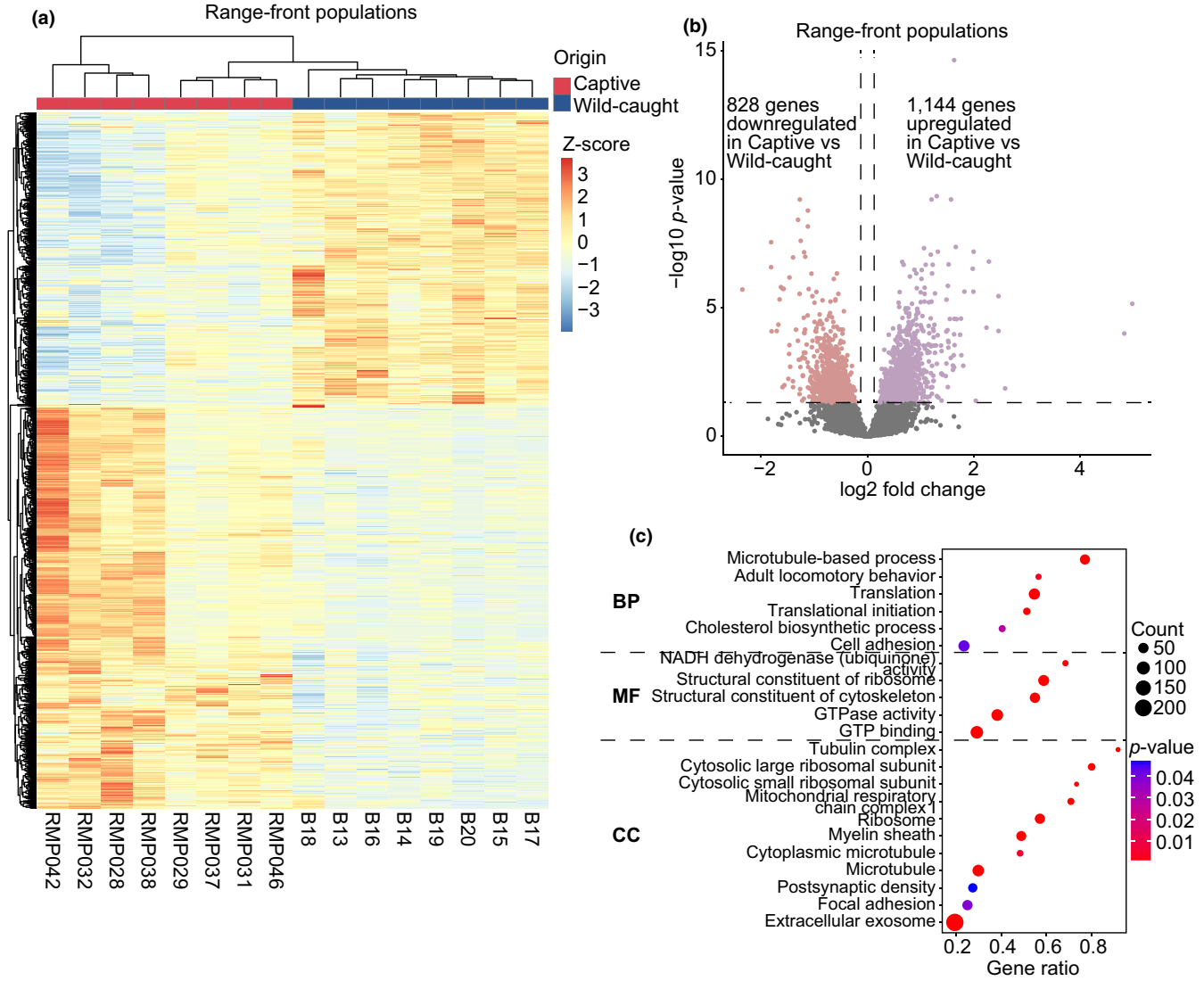

FIGURE 3 Brain gene expression differences between captive and wild-caught cane toads from range-front populations. (a) Heatmap of normalized gene expression values for all differentially expressed genes (DEGs) between captive and wild-caught toads. Columns correspond to individuals. Rows correspond to genes. Colour depicts *Z*-score normalized gene expression value. (b) Volcano plot of significantly DEGs between captive and wild-caught toads. Nonsignificant genes are represented in grey. (c) Gene ontology (GO) enrichment analysis of all DEGs between captive and wild-caught toads. The size of each circle is proportional to the number of genes being significantly enriched, while the colour of each circle is proportional to its false discovery rate (FDR)-corrected *p*-value. Gene ratio corresponds to the proportion of genes being enriched out of the total number of genes in that GO category. BP, biological process; CC, cellular component; MF, molecular function

range-core and range-front populations (Figure 4a). Among these shared 504 DEGs, 501 (99.4%) genes showed parallel changes between captive and wild-caught toads from both populations (Figure 4b). Indeed, 274 (54.4%) genes were upregulated in captive versus wild-caught toads in both populations, while 227 (45.0%) genes were downregulated in captive versus wild-caught toads in both populations. By contrast, two genes (0.4%; Cysteine-rich protein 1 and uncharacterised gene Rm74787t4) were downregulated in captive versus wild-caught toads in range-core populations and upregulated in captive versus wild-caught toads in range-front populations, while one gene (0.2%; uncharacterised gene Rm22763d7790732t1) was upregulated in captive versus wild-caught toads in range-core populations and downregulated in captive versus wild-caught toads in range-front populations. GO enrichment analysis conducted on the 504 DEGs shared between both populations showed an over-representation of GO terms like 'adult locomotory behaviour' (Figure 4d). We found a significant over-representation of GO terms like "lipopolysaccharide biosynthetic process" and "carbohydrate biosynthetic process" (Figure 4c) in the GO enrichment analysis conducted on the 447 DEGs unique to range-core populations. GO enrichment analysis conducted on the 1468 DEGs unique to range-front populations showed an over-representation of GO terms like "translation" and "ergosterol biosynthetic process" (Figure 4e).

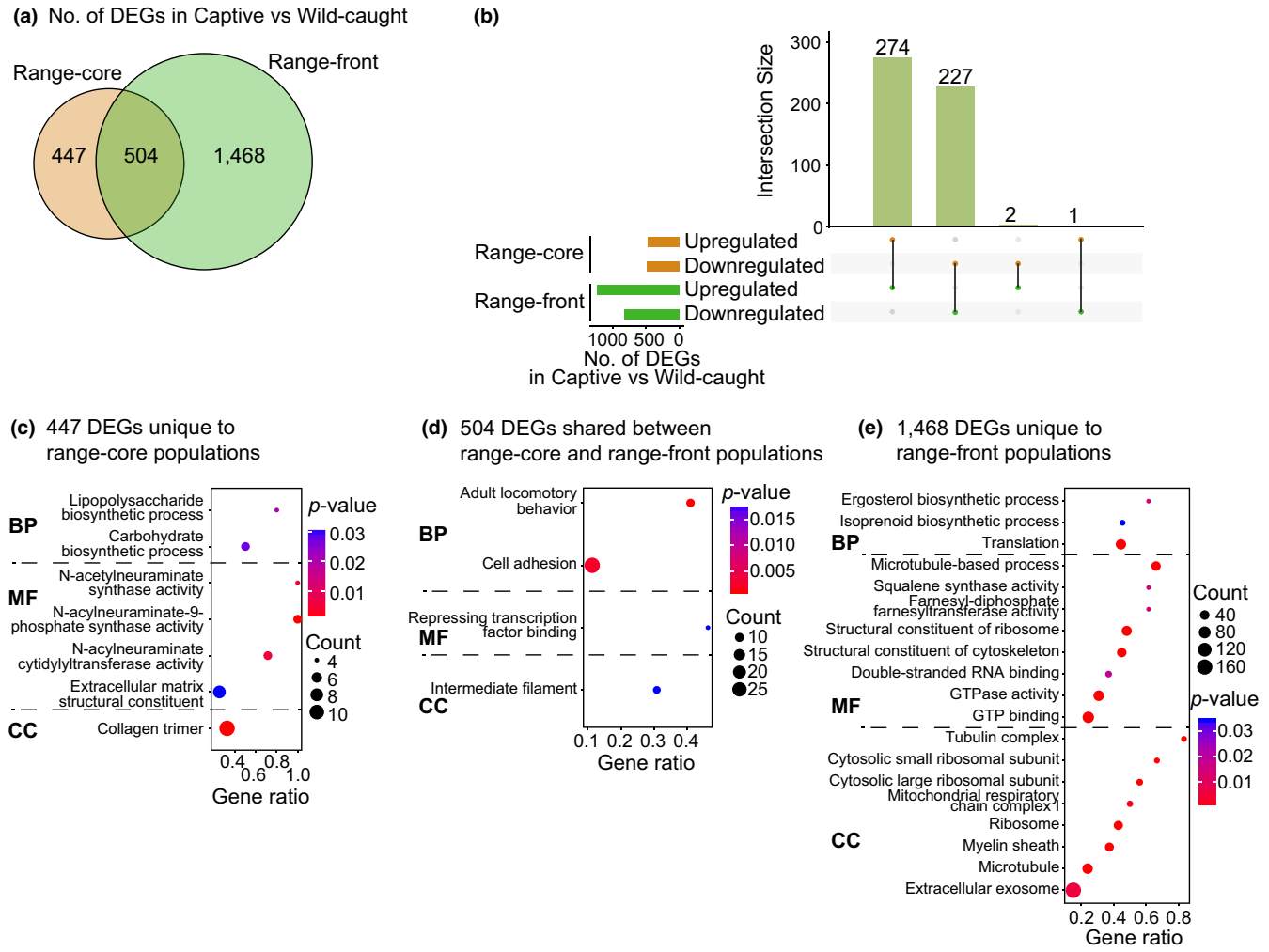

FIGURE 4 Comparison of the brain transcriptomic differences in captive versus wild-caught cane toads between range-core and range-front populations. (a) Venn diagram representing the overlap of differentially expressed genes (DEGs) in captive versus wild-caught toads between range-core and range-front populations. (b) Upset plot representing the overlap of DEGs showing up- or downregulation in captive versus wild-caught toads between range-core and range-front populations. Vertical barplots show the number of genes in any particular combination represented by dots with connecting lines. Genes in this plot correspond to the 504 DEGs overlapping between range-core and range-front populations. (c–e) Gene ontology (GO)enrichment analysis of DEGs between captive and wild-caught toads (c) unique to range-core populations, (d) shared between range-core and range-front populations, (e) unique to range-front populations. The size of each circle is proportional to the number of genes being significantly enriched, while the colour of each circle is proportional to its FDR-corrected *p*-value. Gene ratio corresponds to the proportion of genes being enriched out of the total number of genes in that GO category. BP, biological process; CC, cellular component; MF, molecular function

When looking at the variability in brain gene expression, we found again a greater response to captivity for toads from range-front compared with range-core populations. There were 60 differentially dispersed genes between captive and wild-caught toads from range-core populations, out of which 27 (45.0%) genes were over-dispersed in captive toads, and 33 (55.0%) genes were over-dispersed in wild-caught toads (Figure 5a). By contrast, there were 155 differentially dispersed genes between captive and wild-caught toads from range-front populations, out of which 143 (92.3%) genes were over-dispersed in captive toads, and 12 (7.7%) genes were over-dispersed in wild-caught toads (Figure 5b). GO enrichment analysis failed to find any GO term being significantly over-represented in both populations.

4 | DISCUSSION

We found that cane toads that spent 7 months in captivity had different brain expression levels in ~1000–2000 genes compared to wild-caught toads from the same populations. Previous studies found that captivity-induced stress caused changes in immune genes expression in cane toads (Graham et al., 2012), which was then replicated through an LPS immune challenge (Gardner et al., 2018). Our results generalize these findings at the whole transcriptome level and indicate that short-term captivity can induce large brain transcriptomic changes in wild-caught cane toads. Caution should thus be exercised in studies investigating gene expression in wild animals

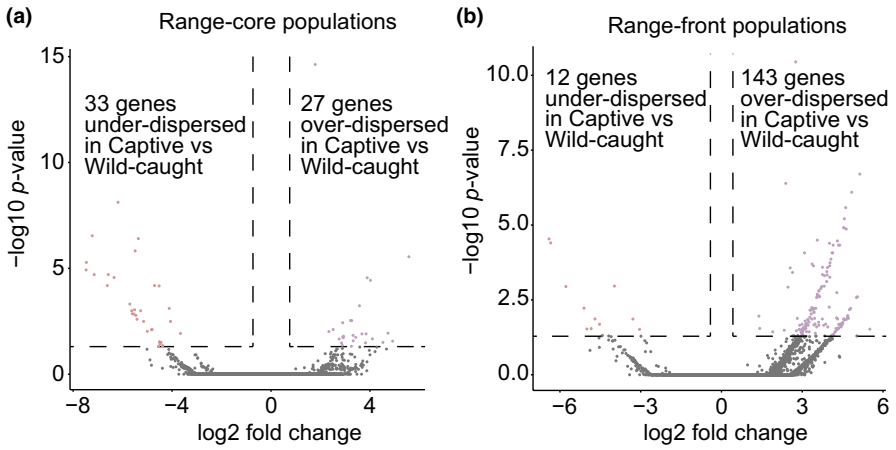

FIGURE 5 Brain gene expression variability between captive and wild-caught cane toads from range-core and range-front populations. (a, b) Volcano plots of significantly differentially dispersed genes between captive and wild-caught toads from (a) range-core populations, and (b) range-front populations. Nonsignificant genes are represented in grey

that have been housed in captivity, as any measurement has the potential to be severely biased.

Our findings add to a growing list of studies reporting changes in gene expression levels following a brief period in captivity across clades as diverse as bivalves, gastropods, fishes, amphibians and reptiles (Hablützel et al., 2016; Maas et al., 2017; Morales & Sánchez, 1996; Roznere et al., 2021). It is, therefore, tempting to infer that captivity-induced transcriptomic change is a general phenomenon, and that its magnitude is relatively large. It is important to note that the influence of captivity goes well beyond gene expression levels, since many other phenotypic traits such as behaviour and physiology can potentially be affected either directly or through cascading effects (du Toit et al., 2012; Gruber et al., 2018).

Interestingly, these short-term effects of a change in environmental conditions on gene expression levels appear to be of greater magnitude than what is often documented for long-term effects. For example, several studies investigating the transcriptomic consequences of domestication have reported relatively minor changes in gene expression levels (Albert et al., 2012; Drew et al., 2012; Fang et al., 2015; Lindberg et al., 2005). Meanwhile, domesticated plants and animals typically have reduced gene expression diversity and genetic diversity compared to their wild progenitors (Liu et al., 2019), which could be a sign of reduced plasticity following artificial selection. Similarly, it is remarkable that, in cane toads, the magnitude of brain gene expression changes following a brief period of captivity (7%–15% of all expressed genes after 7 months; this study) is much higher than observed between Australian wild toads from mesic range-core areas and toads from arid range-front areas (<1% of all expressed genes after ~70 years; this study; Yagound et al., 2022). Stressors in captivity were clearly distinct from those experienced in the wild, as human interaction was substantially increased, constant temperatures were maintained, food sources were regular and water supply was constant (Gruber et al., 2017a). Yet, our results suggest that toads can quickly adjust, at the molecular level, to environmental changes.

A total of 14 genes with brain expression level differences between captive and wild-caught toads from range-core and range-front populations were related to GO:0008344 (adult locomotory behaviour). A further eight genes related to GO:0007612 (learning) were part of the coexpression module "magenta" of the WGCNA and were on average downregulated in captive versus wild-caught toads from range-core populations. Enrichment for these GO terms has been repeatedly found in studies investigating gene expression changes associated with phenotypic differences, for example, between Chinese and western pig breeds (Zhang et al., 2018), sympatric whitefish (*Coregonus clupeaformis*) populations (Hebert et al., 2013), dietary restricted fruit flies (Katewa et al., 2012), or mice selected for increased voluntary wheel-running behaviour (Kavushansky et al., 2018). This suggests a strong influence of captivity on toad behaviour, as has been previously reported (Gruber et al., 2018; Pettit et al., 2017).

Various GO terms significantly enriched between captive and wild-caught toads, including GO:0006950 (response to stress), GO:0009409 (response to cold), GO:0009408 (response to heat), GO:0046677 (response to antibiotics) and GO:0006805 (xenobiotic metabolic process), showed a clear molecular response to captivity-induced stress in toads. Enrichment for GO terms related to the response to stress is often documented in studies investigating the effect of captivity and rearing conditions, for example, in Nile tilapia (*Oreochromis niloticus*) reared under artificially low densities (Ellison et al., 2018), in captive common octopuses (*Octopus vulgaris*) reared under different diet regimes (García-Fernández et al., 2019), in captive spotted seatrouts (*Cynoscion nebulosus*) exposed to acute cold stress (Song & McDowell, 2020), or in captive Australasian snappers (*Chrysophrys auratus*) exposed to a change in thermal regime (Wellenreuther et al., 2019). Key genes related to these GO terms were heat shock protein genes (in particular *HSP90A*), genes that are well known to be upregulated when organisms are exposed to environmental stressors (Santoro, 2000).

The second main finding from our results is the distinct brain transcriptomic response to captivity between toads from long-colonized (range-core) and toads from newly-colonized (range-front) areas. Indeed, the magnitude of the effect of captivity on both gene expression levels and gene expression variability was much larger for range-front toads compared with range-core toads, with twice as many DEGs and more than twice as many differentially dispersed genes between captive and wild-caught toads. Captivity in toads from range-core areas triggered molecular changes related to

behaviour (GO:0008344 and GO:0007612), lipid and carbohydrate metabolism (GO:0009103 and GO:0016051), the immune system (GO:0002481, GO:0002485, GO:0002489 and GO:0002591), transcription (GO:0006351, GO:0006355 and GO:0045893), the response to stimulus (GO:0007186), and the response to stress (GO:0006950, GO:0009409, GO:0009408 and GO:0046677). Changes in expression levels in genes related to transcription have been documented in several studies investigating the transcriptomic consequences of environmental fluctuations, for example, in tambaquis (*Colossoma macropomum*) after a change in thermal conditions (Fé-Gonçalves, Araújo, Santos, & Almeida-Val, 2020), or during winter torpor in Djungarian hamsters (*Phodopus sungorus*) (Haugg et al., 2021). Captivity thus appeared to have affected similar molecular pathways in range-core toads.

By contrast, captivity in toads from range-front areas induced a broad transcriptomic response, with changes in gene expression levels related to translation (GO:0006412, GO:0006413, GO:0032543, GO:0006614, GO:0070126, GO:0070125), protein folding (GO:0006457, GO:0061077), as well as behaviour (GO:0008344), metabolism (GO:0001695, GO:0006695, GO:0009058, GO:0008299, GO:0006696), and the response to stimulus (GO:2000463 GO:0008277). Many studies investigating the effect of captivity-induced stress found an enrichment for GO terms related to translation, for example, heat stress in anole lizards (*Anolis homolechis*) (Akashi et al., 2016), temperature fluctuations in New Zealand silver trevally (*Pseudocaranx georgianus*) (Valenza-Troubat et al., 2022), cold stress in common wall lizards (*Podarcis muralis*) (Feiner et al., 2018), or ammonia stress in magur catfish (*Clarias magur*) (Banerjee et al., 2019), protein folding (e.g., prolonged heat stress in tambaquis [*C. macropomum*]) (Fé-Gonçalves, Araújo, dos Santos, et al., 2020), acute cold and heat stress in spotted seatrouts (*C. nebulosus*) (Song & McDowell, 2020), change in thermal regime in Australasian snappers (*C. auratus*) (Wellenreuther et al., 2019), or cold stress in common wall lizards (*P. muralis*) (Feiner et al., 2018) and metabolism (e.g., diet regime in common octopuses [*O. vulgaris*]) (García-Fernández et al., 2019), acute cold stress in spotted seatrouts (*C. nebulosus*) (Song & McDowell, 2020), water deprivation in desert rodents (*Peromyscus eremicus*) (MacManes, 2017), or irradiation in Mediterranean fruit flies (*Ceratitis capitata*) (Calla et al., 2014). It is, therefore, likely that stressors in captivity played an important role in the molecular response of cane toads from range-front areas. Interestingly, the dispersion analysis revealed an increase in gene expression variability in captive toads, but only from range-front areas. Altogether, these findings suggest that cane toads from newly-colonized areas display increased plasticity compared to toads from long-colonized areas, and support the hypothesis that adaptive plasticity can be important for range expansion (Hendry, 2016). Cane toads display complex patterns of phenotypic plasticity that appear to have changed during their invasion trajectory (Ducatez et al., 2016; Stuart et al., 2019). Differences in plasticity between range-core and range-front toads may be driven by selection pressures related to the environmental heterogeneity experienced along the invasion transect (Lande, 2015; Valladares et al., 2014), potentially reinforced by nonadaptive processes such as spatial sorting (Shine et al., 2011). It would be interesting to investigate gene expression reaction norms to decipher the molecular mechanisms underpinning environmentally-induced phenotypic changes in cane toads, and to better understand the toads' past and future invasion success.

More generally, our findings add to the large list of phenotypic differences documented between range-core and range-front toads, that is usually interpreted as a sign of rapid evolution (Alford et al., 2009; Gruber et al., 2017a, 2017b; Hudson et al., 2018; Hudson, McCurry, et al., 2016; Lindstrom et al., 2013; Llewelyn et al., 2010; Phillips et al., 2006; Rollins et al., 2015; Selechnik, Richardson, Shine, Brown, et al., 2019; Shine, 2010; Urban et al., 2008; Yagound et al., 2022). Transcriptional divergence of key gene regulatory networks is thought to play an important role at the onset of adaptive radiations, before genetic changes can arise, for example, through genetic assimilation (Schneider & Meyer, 2017). Future studies might be able to track whether these differences in gene expression levels are followed by genetic changes across the Australian invasion range. Thus, like many invasive species, cane toads provide a good model to follow evolution in real time (Sax et al., 2007).

The fact that cane toads show population-dependent molecular responses to captivity has an important bearing for future studies. Here, we have clearly shown that it is a poor assumption that the impacts of captivity experienced by one population mirror that of other populations, even if all populations have very little genetic divergence. Therefore, research comparing effects across multiple populations need to account for such biases. Indeed, this effect has the potential to be of greater magnitude than the experimental factor(s) being tested, even in a common garden breeding design. Alleviating this issue might require adding extra controls, such as individuals from each population that spend little-to-no time in captivity, or time series analyses when the tissues studied permits this.

We note that our findings are bound to only two populations, each located at one end of the Australian invasion range. While comparing additional populations would certainly be desirable to draw definitive conclusions about the population-dependent response to captivity, this does not seem currently feasible with Australian cane toads, due to the large homogeneity seen within both range-core and range-front toads (Selechnik, Richardson, Shine, DeVore, et al., 2019).

Notwithstanding the clear difference between range-core and range-front toads in gene expression changes following a brief period in captivity, it is worth noting that both populations also showed parallel transcriptomic changes following captivity. Indeed, ~500 genes showed either up or downregulation in captive versus wild-caught toads from both populations. These overlapping DEGs encompass behaviour-related genes, and probably represent a general molecular response to captivity in cane toads. Parallel transcriptomic changes are still not well understood and have been mostly studied in the context of parallel phenotypic evolution (Arendt & Reznick, 2008). Shared underlying genetic variation (Haldane, 1932), and genetic and developmental constraints (West-Eberhard, 2003)

have been proposed among other factors to explain cases of parallel evolution (Stern, 2013). It is likely that these processes are also at play in cane toads.

GO enrichment analyses are limited to the quality and completeness of the underlying genome annotation. Out of 951 DEGs between captive and wild-caught toads from range-core populations, 824 DEGs (86.6%) had GO annotations, while 70.9% of the remaining 127 DEGs were uncharacterised genes. Likewise, 1618 out of 1972 DEGs (82.0%) between captive and wild-caught toads from range-front populations had GO annotations, while 70.6% of the remaining 250 DEGs were uncharacterised genes. This indicates that we were able to capture most of the functional interpretation of our findings, although some of it was inherently lacking.

Studying gene expression in whole brains may have masked transcriptomic changes occurring in specific brain regions (Nadler et al., 2006). This effect complicates the functional interpretation of our findings, in particular with regards to GO enrichment analyses. More detailed future studies are welcome to confirm our results. Environmental conditions during early development are known to affect adult phenotypic traits in amphibians, including cane toads (Ducatez et al., 2016). We cannot rule out that this effect played a minor role in determining some of the observed phenotypic differences seen between wild-caught and captive toads. Likewise, the large distance and significant environmental differences seen between wild populations (Queensland and Western Australia) and New South Wales where animals were held captive could have added confounding factors to the analysis of the effect of captivity. The fact that temperatures encountered in captivity were closer to those encountered in the wild by range-core toads than to those encountered by range-front toads could have played a role in the greater response to captivity seen for range-front toads (although the enrichment for "response to cold" was found in range-core toads). It is also possible that we missed subtle differences in gene expression levels due to insufficient sequencing depth. Genetic factors are unlikely to have influenced our results, since there is very minimal genetic heterogeneity within each Australian cane toad population (Selechnik, Richardson, Shine, DeVore, et al., 2019).

In conclusion, our findings add to the growing body of research showing that even brief periods of captivity are sufficient to induce relatively large gene expression changes, which have the potential to further many other phenotypic changes. Strikingly, we show here that these effects can be population-specific. Across animal species, it is common practice to bring wild-caught individuals to the laboratory, thus subjecting them to captive environments before measuring phenotypic traits of interests. Researchers should, therefore, be cautious when interpreting such data, as the observed values may be a poor reflection of those present in nature.

AUTHOR CONTRIBUTIONS

Mark F. Richardson, Jodie Gruber, Jack G. Reid and Lee A. Rollins conducted fieldwork. Andrea J. West and Mark F. Richardson conducted laboratory work. Boris Yagound, Andrea J. West, Mark F. Richardson and Lee A. Rollins conducted analyses. Boris Yagound, Andrea J. West, Mark F. Richardson, Jodie Gruber, Jack G. Reid, Martin J. Whiting and Lee A. Rollins contributed to interpretation and writing.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The raw RNA-seq data has been made available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (BioProject PRJNA479937). Scripts used in this study are available at: https://github.com/CaneToadGenomics/Brain-transcriptome-analysis.

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SUPPORTING INFORMATION

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