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Novel ecological and climatic conditions drive rapid adaptation in invasive Florida Burmese pythons

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Abstract

Invasive species provide powerful in situ experimental systems for studying evolution in response to selective pressures in novel habitats. While research has shown that phenotypic evolution can occur rapidly in nature, few examples exist of genome-wide adaptation on short “ecological” timescales. Burmese pythons (*Python molurus bivittatus*) have become a successful and impactful invasive species in Florida over the last 30 years despite major freeze events that caused high python mortality. We sampled Florida Burmese pythons before and after a major freeze event in 2010 and found evidence for directional selection in genomic regions enriched for genes associated with thermosensation, behaviour and physiology. Several of these genes are linked to regenerative organ growth, an adaptive response that modulates organ size and function with feeding and fasting in pythons. Independent histological and functional genomic data sets provide additional layers of support for a contemporary shift in invasive Burmese python physiology. In the Florida population, a shift towards maintaining an active digestive system may be driven by the fitness benefits of maintaining higher metabolic rates and body temperature during freeze events. Our results suggest that a synergistic interaction between ecological and climatic selection pressures has driven adaptation in Florida Burmese pythons, demonstrating the often-overlooked potential of rapid adaptation to influence the success of invasive species.

KEYWORDS

genomic scans, invasive species, natural selection, regenerative organ growth

1 | INTRODUCTION

The most striking examples of evolution involve rapid phenotypic adaptation in natural populations (Grant & Grant, 2002; Losos, Warheitt, & Schoener, 1997), but few studies have linked genomic change to phenotypic evolution occurring over a small number of generations (though see Campbell-Staton et al., 2017; Epstein et al.,

2016; Reid et al., 2016). Invasive species are valuable models for understanding such links because they are often subjected to strong selective pressures due to the novelty of environmental conditions they face in non-native environments (Reznick & Ghalambor, 2001; Schoener, 2011).

Among the most widely known and impactful invasive species in the United States is the Burmese python (*Python molurus bivittatus*)

(Engeman, Jacobson, Avery, & Meshaka, 2011; Schleip & O'Shea, 2010; Willson, Dorcas, & Snow, 2011). The Burmese python is a large constricting snake native to Southeast Asia (Barker & Barker, 2008) that has received substantial attention due to their recent and highly successful invasive colonization of South Florida (Engeman et al., 2011; Willson et al., 2011). Burmese pythons were first discovered in Florida in the early 1980s (Meshaka, Loftus, & Steiner, 2000) and were considered established by the mid-1990s (Collins, Freeman, & Snow, 2008; Snow, Brien, Cherkiss, Wilkins, & Mazzotti, 2007). This population is thought to have originated from the release of pet pythons, including a catastrophic release event resulting from the destruction of an animal import facility during Hurricane Andrew in 1992 (Willson et al., 2011). The ecological impact resulting from predation on endangered species by pythons within Florida's Everglades National Park (ENP) is extensive, and the economic impact is estimated to be at least \$83,892 per snake per year (Smith, Sementelli, Meshaka, & Engeman, 2007). These snakes prey upon many bird and mammal species, including species listed under the US Endangered Species Act (Dove, Snow, Rochford, & Mazzotti, 2011; Reed, 2005; Snow et al., 2007), and have been implicated in recent severe declines in small mammal populations (Dorcas et al., 2012; McCleery et al., 2015).

Several lines of evidence suggest that invasive Florida Burmese pythons may be under substantial selection pressures. First, invasive Burmese pythons reside at the margin of climatically suitable habitat within the United States (Jacobson et al., 2012; Pyron, Burbrink, & Guiher, 2008) and several studies have found high cold-induced mortality in Burmese pythons relocated from native Southeast Asia to more temperate areas (Avery et al., 2010; Dorcas, Willson, & Gibbons, 2011; Jacobson et al., 2012). Moreover, acute climatic events, including rapid shifts in temperature, also periodically impact South Florida. For example, 50%–90% mortality was documented in South Florida Burmese python populations during a freeze event in January 2010 (Mazzotti et al., 2011). Collectively, this suggests that the more temperate environment in Florida (compared to tropical Southeast Asia) imposes strong selection pressures on the invasive Burmese python population.

In addition to being ill-suited to the subtropical climates of Florida, the invasive Burmese python population has experienced a fundamental shift in prey ecology. The ecology and physiology of Burmese pythons have been strongly shaped by the monsoonal ecosystems of their native Southeast Asia, where they experience major seasonal shifts in prey availability. Indeed, Burmese pythons represent an important and unique model system for studying extreme physiological regulation (Secor, 2008; Secor & Diamond, 1995, 1998). These snakes have adapted to enduring long periods of fasting (based on their native ecology) by massively upregulating and downregulating their metabolism and their organ size and function between meals to conserve energy during long fasts associated with their native ecology (Secor, 2008; Secor & Diamond, 1995, 1998). For example, the python heart, small intestine, liver and kidneys can increase 40%–100% in mass, and their metabolism can increase up to 40-fold, all within 48 hr of feeding (Secor, 2008; Secor

& Diamond, 1995, 1998). Accordingly, Burmese pythons are presumably poorly adapted to the year-round prey availability typical in South Florida. However, the establishment and expansion of invasive Florida Burmese pythons have coincided with dramatic reductions in small mammal populations (Dorcas et al., 2012; McCleery et al., 2015), indicating a potential ecological shift due to more consistent prey availability in comparison with monsoonal Southeast Asia. The expansion of this population in an ecosystem so different from its native range therefore raises the question of whether rapid evolution and adaptation may have played a role in the success of this invasive species.

Given the success and rapid proliferation of the invasive Burmese python population, especially in the face of strong ecological selection pressures, we were interested to test for evidence of rapid evolution (i.e., allele frequency fluctuations) and selection-driven adaptation. Further, we were motivated to determine whether putatively selected genomic loci are associated with physiological traits linked to the novel climatic and ecological pressures present in Florida. To address these aims, we collected and analysed multiple complementary data sets, including ecological, genomic, transcriptomic and morphological data, and integrated the results of genomic scans, differential expression analysis and histological analyses to test for corroborative evidence of rapid adaptation in the invasive Florida Burmese python population.

2 | MATERIALS AND METHODS

The sections below outline the general details of our methodology. A more detailed version of our full methods is provided as Supporting Information.

2.1 | Overview of sample collection

Ninety-seven Burmese python (*Python molurus bivittatus*) samples were collected from South Florida as part of ongoing conservation efforts by state and federal agencies under approved collecting and IACUC protocols. These samples were obtained during two general time periods: (a) $N = 48$ samples from 19 May 2003 to 17 June 2009 and (b) $N = 49$ samples from 30 October 2012 to 6 December 2013 (Supporting information Figure S1), with most sampling separated by only seven years. These two time periods are temporally separated by an extreme freeze event that occurred in January 2010, and we therefore refer to them as pre-freeze and post-freeze, respectively. Supporting information Table S1 contains complete information for all samples used in this study.

2.2 | Evaluating shifts in feeding ecology and climate in Burmese pythons from Florida

The establishment and proliferation of the invasive Burmese python population have corresponded with large declines in small mammal populations (Dorcas et al., 2012; McCleery et al., 2015), which form

a consistent source of prey in the Florida ecosystem. To understand whether more consistent prey availability has led to more frequent feeding in invasive Burmese pythons, we analysed five years of data collected from Burmese pythons that were captured, euthanized and necropsied as part of control efforts in South Florida. Specifically, we quantified the proportion of snakes with a meal item in their gut (i.e., snakes actively digesting) between 2003 and 2008.

We also used ecological niche modelling (ENM) to reconstruct the climatic niche of the Burmese python based on climatic variables associated with its native range and to project the suitable invasive range in the United States (Elith et al., 2006). We selected 90 geo-referenced localities throughout the species native range, extracted climatic niche data from 11 bioclimatic variables from the WORLDCLIM DATA set v 1.4 (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) and constructed climatic niche models using MAXENT v. 3.3.3k (Phillips, Anderson, & Schapire, 2006), following our previously published approach (Card et al., 2016; Jezkova et al., 2015; Schield et al., 2015), with 500 maximum iterations, a convergence threshold of 0.00001, a regularization multiplier of 1 and 10,000 background points. We first ran five model replicates using climatic layers spanning the entire world. We also ran a second set of 10 models, where we constrained climatic layers to the areas of interest (i.e., southern half of Asia and southeastern United States). The two sets of models were very similar and therefore we only present the average model from the first set of models. We visualized this model in ARCGIS v. 10.3 using three logistic probability thresholds: (a) minimum training presence threshold (i.e., the lowest logistic probability inferred in the native range); (b) equal training sensitivity and specificity logistic threshold; and (c) 10th percentile training presence logistic threshold (90% of samples in the native range have a logistic probability equal or higher than this threshold).

2.3 | RADseq library generation, sequencing and data processing

We used a modified version of the Peterson, Weber, Kay, Fisher, & Hoekstra (2012) protocol to prepare double-digest RADseq libraries for the 48 pre-freeze and 49 post-freeze samples. RADseq sampling targeted approximately 20,000 loci from throughout the genome per individual, which was estimated based on an *in silico* digestion and size selection of the Burmese python genome (Castoe et al., 2013). Genomic DNA was digested simultaneously with rare (*Sbf*I; 8 bp) and common (*Sau*3AI; 4 bp) cutting restriction enzymes. The final pooled library was sequenced using 100-bp paired-end reads on an Illumina HiSeq 2000 lane. Raw Illumina reads were filtered to remove PCR clones (using an 8-bp unique molecular identifier), demultiplexed using STACKS v. 1.35 (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011; Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013) and were quality-trimmed using TRIMMOMATIC v. 0.33 (Bolger, Lohse, & Usadel, 2014). We used the RADcap analysis pipeline (Hoffberg et al., 2016) to map reads and infer genotypes based on the Genomic Analysis Toolkit (GATK) best-practices guidelines (DePristo et al., 2011; McKenna et al., 2010; Van der

Auwer et al., 2013). We used BCFtools (Li, 2011; Li et al., 2009) and VCFtools (Danecek et al., 2011) to exclude variants around indels and filter variants based on genotype quality score, read depth and minor allele frequency (see Supplementary Materials & Methods for more details).

2.4 | Analyses of population structure

Given the invasive Burmese python population was established from pet-trade snakes originating from various native-range regions and populations (Hunter et al., 2018), we used our inferred sample genotypes at RAD locus variable sites to estimate how many source populations comprise the invasive population with no *a priori* information about potential source population. We used the LEA package v. 1.0 (Frichot, François, & O'Meara, 2015; Frichot, Mathieu, Trouillon, Bouchard, & François, 2014) in the R statistical environment (v. 3.3.; R Core Team, 2018) to estimate the number of ancestral populations, commonly referred to as *K*, which in this case should correspond to the number of source populations given the relatively recent introduction of pythons to Florida. LEA uses an alternative algorithm for estimating population structure from the popular program Structure (Pritchard, Stephens, & Donnelly, 2000), but is much faster and more accurate than Structure in the presence of inbreeding (Frichot et al., 2014). It also implements a cross-entropy criterion (Alexander & Lange, 2011; Frichot et al., 2014) based on replicates (*N* = 10 replicates with our data set) to estimate the number of population clusters in the data.

2.5 | Inferring and visualizing the between-time site frequency spectrum

The two-dimensional site frequency spectrum (2D-SFS) offers an intuitive way to visualize the density in minor allele frequencies and how they shift between pre-freeze and post-freeze populations. We used *δaδi* (Gutenkunst, Hernandez, Williamson, & Bustamante, 2009) to calculate and visualize the folded 2D-SFS after projecting down to a sample size of 45 for each population. We created two 2D-SFS matrices by inverting the placement of each population time point site frequency spectrum on the *x*- or *y*-axis, effectively creating two transposed 2D-SFS, which we used to calculate linear Poisson residuals between the time points and visualize the change in the 2D-SFS between pre-freeze and post-freeze populations.

2.6 | Multivariate scans for signatures of selection

We used a custom Python script to calculate six metrics to evaluate the degree of allele frequency fluctuation between the pre-freeze and post-freeze populations: (a) the absolute value of allele frequency change $|\Delta AF|$, (b) population allelic differentiation (F_{ST}) based upon Weir & Cockerham (1984), (c) the absolute differentiation in genetic diversity (D_{XY}), (d) the fluctuation in nucleotide diversity (pre-freeze – post-freeze; $\Delta \Pi$), (e) the difference in heterozygosity between populations (pre-freeze – post-freeze; ΔHet) and (f) the fluctuation

in Tajima's D statistic (pre-freeze – post-freeze; ΔT_{ajD} ; (Tajima, 1989). Combining information from two or more summary statistics provides increased power to detect loci under natural selection (Evangelou & Ioannidis, 2013; François, Martins, Caye, & Schoville, 2016; Grossman et al., 2010; Lotterhos et al., 2017; Ma et al., 2015; Randhawa, Khatkar, Thomson, & Raadsma, 2014, 2015; Utsunomiya et al., 2013), and thus, we employed a multivariate outlier approach to identify genetic loci with strong signatures of directional selection based on the six univariate statistics. We used MINOTAUR (Verity et al., 2017) to estimate the Mahalanobis multivariate distance (Mahalanobis, 1936) based on the six metrics described above. The top 2.5% of Mahalanobis distance measures were taken to indicate variants putatively under selection, and this threshold reflected a natural break in the Mahalanobis multivariate distribution and in associated bivariate plots between pairwise univariate statistics (Supporting information Figure S6). We also used ApproxWF (Ferrer-Admetlla, Leuenberger, Jensen, & Wegmann, 2016), which uses a Bayesian model-based approach to explicitly account for demography, to estimate selection coefficients (s) based on the fluctuation in allele frequencies between the pre- and post-freeze populations. These independent analyses identify regions of the genome putatively under selection based either on how much of an outlier a region is based on a composite measure of population genetic statistics (Mahalanobis distance) or whether a Bayesian highest posterior distribution of selection coefficients is significantly different from 0 (no selection; the ApproxWF analysis). We used permutation tests to test the null hypothesis that the allele frequency estimates are not significantly different between the two populations. We also conducted forward-time, Wright–Fisher simulations of neutral genetic drift, matching the sample sizes, locus length and other important factors from our empirical data, to more directly address whether fluctuations in population genetic statistics were beyond what would be expected under genetic drift (see Supplementary Materials & Methods for full details). Higher densities of loci in the empirical data set versus the simulated data sets at more extreme values of population genetic statistics provide evidence for directional selection, versus neutral genetic drift, producing patterns of high allelic fluctuation between population time points.

2.7 | Using synteny with the *Boa constrictor* genome to identify genomic regions in the python

The Burmese python reference genome suffers from relatively low contiguity, and some genome scan outliers were on small python genome scaffolds or were located near the ends of scaffolds. To enable more meaningful analyses of the genomic context of genome scan outliers, we took advantage of the highly contiguous *Boa constrictor* reference genome (Bradnam et al., 2013) and the fact that snake genomes have well-conserved genome size, karyotype, and regional synteny (Alföldi et al., 2011; Matsubara et al., 2006; Singh, 1972) to infer linkage of python scaffolds. We used Satsuma (Grabherr et al., 2010) to map each entire Burmese python genome scaffold to their syntenic location in the *Boa constrictor* genome, and this synteny

information was used to link, order and orient adjacent python scaffolds (for regions of interest) based on their alignment to the highly contiguous *Boa constrictor* genome.

2.8 | Estimating gene expression for Florida Burmese pythons

Given findings from the selection scans (see Results and Discussion), we were interested in comparing patterns of small intestine gene expression from invasive pythons in Florida with previous estimates of expression patterns from controlled experiments involving commercial trade pythons (Andrew et al., 2015, 2017; Castoe et al., 2013). These experiments leveraged replicate sampling of captive Burmese pythons taken at the following controlled time points: fasted (30 days since last meal), 1-day post-feeding (1DPF), 4DPF and 10DPF. We downloaded the raw read data from these previous studies from the NCBI SRA database. These data were combined with newly generated small intestine RNAseq data from seven pythons from the invasive Florida population collected in January of 2016 (see Supporting information Table S1 for details of sampling). Due to regulatory constraints, we were unable to carry out a well-controlled experiment akin to that presented in previous studies (Andrew et al., 2015, 2017; Castoe et al., 2013). However, we were able to leverage the known and well-defined, cyclical pattern of digestive physiology and gene expression following feeding to ensure that snakes were strategically fasted prior to sacrifice. Burmese pythons reach their peak digestive physiological state at 1–2 days post-feeding, and by four days, they are starting to revert to a fasted state. Therefore, snakes that contained no meal item in the gut and that were in captivity without access to food for at least eight days were used for this experiment. We found that these expectations were upheld, as overall gene expression in these seven pythons closely resembled a fasted state in animals from previous well-controlled experiments (Supporting information Figure S11), and we feel that this design is justified for roughly deciphering the digestive tract physiology in fasted modern Florida Burmese pythons.

We normalized raw expression count data using the TMM method (Robinson & Oshlack, 2010) in edgeR (McCarthy, Chen, & Smyth, 2012; Robinson, McCarthy, & Smyth, 2010) and tested for significant changes in gene expression between the fasted invasive python sampling and each of these experimental time points using pairwise exact tests with independent hypothesis weighting (IHW) to minimize the false discovery rate (Ignatiadis, Klaus, Zaugg, & Huber, 2016). Genes identified as significantly differentially expressed (IHW FDR < 0.1) in pairwise comparisons between fasted Florida and fasted experimental animals were analysed using Core Analysis in Ingenuity Pathway Analysis (IPA; Qiagen) to infer differential activity of canonical pathways and upstream regulatory interactions. Annotated genes located on scaffolds that contained putative targets of selection were analysed for GO term (Ashburner et al., 2000; The Gene Ontology Consortium, 2017) and KEGG Pathway (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017; Kanehisa & Goto, 2000; Kanehisa, Sato, Kawashima, Furumichi, &

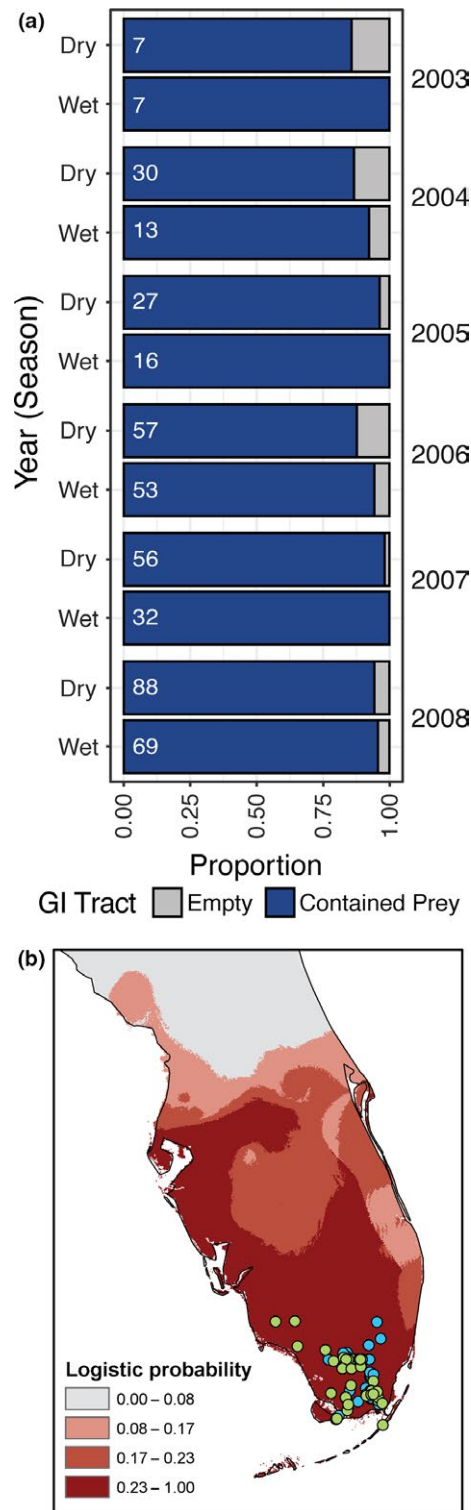


FIGURE 1 Evidence of novel ecological conditions for invasive Burmese pythons in South Florida. (a) Temporal analyses of the proportion of captured pythons containing a food item. White numbers within the bars indicate sample sizes. (b) A map of the sampling used for this work from pre-freeze ($N = 48$; green points) and post-freeze ($N = 49$; blue points) populations and habitat suitability estimates based on the ecological niche modelling of native-range Burmese pythons

Tanabe, 2016) enrichment using the Web-based Gene Set Analysis Toolkit (WebGestalt 2017; Zhang, Kirov, & Snoddy, 2005) and using ClueGO v. 2.2.6 (Bindea et al., 2009) implemented in Cytoscape v. 3.3.0 (Shannon et al., 2003), with ontologies/pathways from GO, KEGG and WikiPathways (Pico et al., 2008; Slenter et al., 2018). As a complement to our ontology- and pathway-based enrichment analyses, we also evaluated the impact of genes on vertebrate phenotype using mouse knockout phenotype enrichment tests conducted using the Mammalian Phenotype Enrichment Analysis (MamPhEA; Weng & Liao, 2010), with manual *post hoc* clustering of similar phenotypes, which were visualized using a wordcloud constructed using the WORDCLOUD2 v. 0.2.0 package in R. We used the GenometriCorr R package (Favorov et al., 2012) to test for spatial autocorrelation between differentially expressed transcripts and genome scan outliers using the Jaccard index of overlap (Jaccard, 1908).

2.9 | Histological analyses of organ morphology

Burmese pythons experience extreme and rapid changes in the morphology of digestive organs when transitioning between a dormant fasted state and an actively digesting state, and we were interested in comparing the morphological state of samples from fasted invasive pythons from Florida to that of experimental animals in carefully controlled fasted and fed states. Cross sections from the anterior third of the small intestine from the seven invasive python samples from Florida taken in January of 2016 were embedded in paraffin, cross-sectioned ($6 \mu\text{m}$) and stained with haematoxylin and eosin on glass slides. Existing paraffin blocks from 3 replicate animals each from controlled fasted, 3DPF and 10DPF time points were also obtained, cross-sectioned and stained in the same manner. We took replicate measures of cell sizes for all four organs for the three experimental time points and for fasted samples from the invasive Florida population were compared using an ANOVA with *post hoc* Tukey's honest significant difference tests of pairwise comparisons. We also visualized and measured the state of intestinal microvilli using transmission electron microscopy, as intestinal microvillus regression is incredibly rare in vertebrates, yet an extreme and reliable hallmark of the downregulation of intestinal physiology and function in Burmese pythons (see Figure 5c; Lignot, Helmstetter, & Secor, 2005).

3 | RESULTS AND DISCUSSION

3.1 | Climatic and feeding data indicate ecological shifts in invasive Burmese pythons

Burmese python physiology is highly adapted to monsoonal Southeast Asian ecosystems with major seasonal shifts in prey availability that lead to these snakes enduring long periods of fasting (Secor, 2008; Secor & Diamond, 1995, 1998). In Florida, however, invasive pythons have constant access to

prey and thus feed year-round (Dorcas et al., 2012). Our analyses, based on five years (2003–2008) of necropsy data from the Florida population of Burmese pythons (Florida Burmese pythons, hereafter), indicated that an annual average of 94% of captured snakes contained a meal (97% in wet season and 91% in dry season; Figure 1a). Though no directly comparable data exist for Burmese pythons in their native range, similar data collected for *Morelia spilota*—an Australian pythonid snake species with similar feeding ecology—found that 73.9% of samples contained prey items in the digestive tract during the summer, which is the monsoonal season where snakes feed most (Slip & Shine, 1988). We used a binomial test to test whether the observed proportion of actively digesting Burmese pythons in Florida year-round (94.3%; $N = 455$ samples) is significantly greater than expected based on what we would expect in Southeast Asia, using data from *Morelia* as a proxy. The result of this test was significant ($p < 0.01$), which indicates that Florida Burmese pythons are feeding year-round at an overall rate that far exceeds what we expect from native-range populations. This shift in feeding rates represents a major ecological shift from the “feast-famine” feeding patterns associated with their native range.

We also found that invasive Florida Burmese pythons experience climatic conditions that are distinct from their native range. Our ecological niche models agreed with previous estimates (Pyrón et al., 2008) that this population persists at the margin of the predicted climatic suitability of this species (Figure 1b). Further supporting this inference, Burmese pythons exhibit high mortality (50% or higher) when relocated to more temperate U.S. locations (Avery et al., 2010; Dorcas et al., 2011) and during freeze events in South Florida (Mazzotti et al., 2011). We hypothesized that these novel ecological factors—more extreme cold climatic events and consistent prey availability—have acted as strong selective catalysts to drive the evolution of Florida Burmese pythons.

3.2 | Genomic evidence for rapid evolution driven by natural selection in Florida Burmese pythons

To test for evidence of evolution and selection on genetic variation through time, we generated genomic data (using ddRADseq; Peterson et al., 2012) from Florida Burmese pythons collected before and after a January 2010 freeze event that occurred in South Florida, which is known to have caused high python mortality (50%–90%; see Supporting information Figure S1 for temporal ranges of sampling; Mazzotti et al., 2011). Our filtered variant data set contained 1,021 variants across 23,041 nuclear loci sampled from 97 Florida Burmese pythons (48 sampled before and 49 after the freeze event; Figure 1b; Supporting information Figure S1, Table S1) and indicates that the Florida population was likely derived from 2 to 3 distinct source populations (Figure 2a). These findings align with importation records indicating two main native-range sources (Engeman et al., 2011) and expand on previous microsatellite analyses (Collins et al., 2008) to provide genomic evidence for a panmictic invasive

population. Among all samples, we found that 3.5% of sequenced RAD loci contained one or more variants (i.e., multiple alleles) in the Florida population.

To test whether selection on standing genetic variation is leading to temporal fluctuations in allele frequencies, we compared genomic variation in our RAD loci between the pre- and post-freeze populations. Evidence for rapid genomic evolution and adaptation in the Florida Burmese python population through time were evident in the two-dimensional allele frequency spectrum and from genomewide population genetic diversity statistics (Figure 2b; Supporting information Figure S2). The allelic fluctuation between empirical pre- versus post-freeze Florida population samples was also significantly different than random population assignments of samples, indicating evolution across our temporal samples (Supporting information Figure S3). Using forward-time simulations across a range of plausible demographic scenarios, we found that the largest empirical allele frequency changes are unlikely to have occurred due to neutral genetic drift alone (Supporting information Figures S4 and S5). Collectively, these results provide strong support for rapid evolution of the Florida Burmese python population and the role of selection at a subset of genomic regions.

To further test for evidence of locus-specific signatures of selection, we used multiple genomic-scan approaches to survey our genomewide variant data set. We identified evidence of temporal genetic differentiation driven by selection at several loci by summarizing six population genetic statistics using a multivariate composite measure (Mahalanobis distance) that identified multivariate outliers (Figure 3a; Supporting information Figures S6 and S7; Lotterhos et al., 2017; Verity et al., 2017). We also conducted an independent estimation of locus-specific selection coefficients (s) based on temporal allele frequency changes using a Bayesian approach that explicitly accounts for demography (Ferrer-Admetlla et al., 2016). Selected variants based on Bayesian HPD not overlapping 0 (i.e., no selection) were found in many of the same genomic regions that contained outlier Mahalanobis distances, indicating that these regions are evolving under directional selection (Figure 3b). These two approaches together implicated 12 candidate genomic regions as likely influenced by directional selection between pre- and post-freeze event pythons. To guard against our results being strongly driven by a small number of extreme outlying samples (e.g., samples with highly differentiated genotypes), we compared allele frequency fluctuations between the two time points for the full data set and a subsampled data set. Specifically, we ran a PCA on sample genotypes to identify the eight samples with the most outlying or divergent genotypes (Supporting information Figure S8A). Half of these samples were from the first time point while the other half were from the second time point. Excluding these outliers, we re-calculated allele frequency fluctuations and found remarkably little difference between these estimates and estimates from the full data set (Supporting information Figure S8B), suggesting our inferences about changes in genotypes

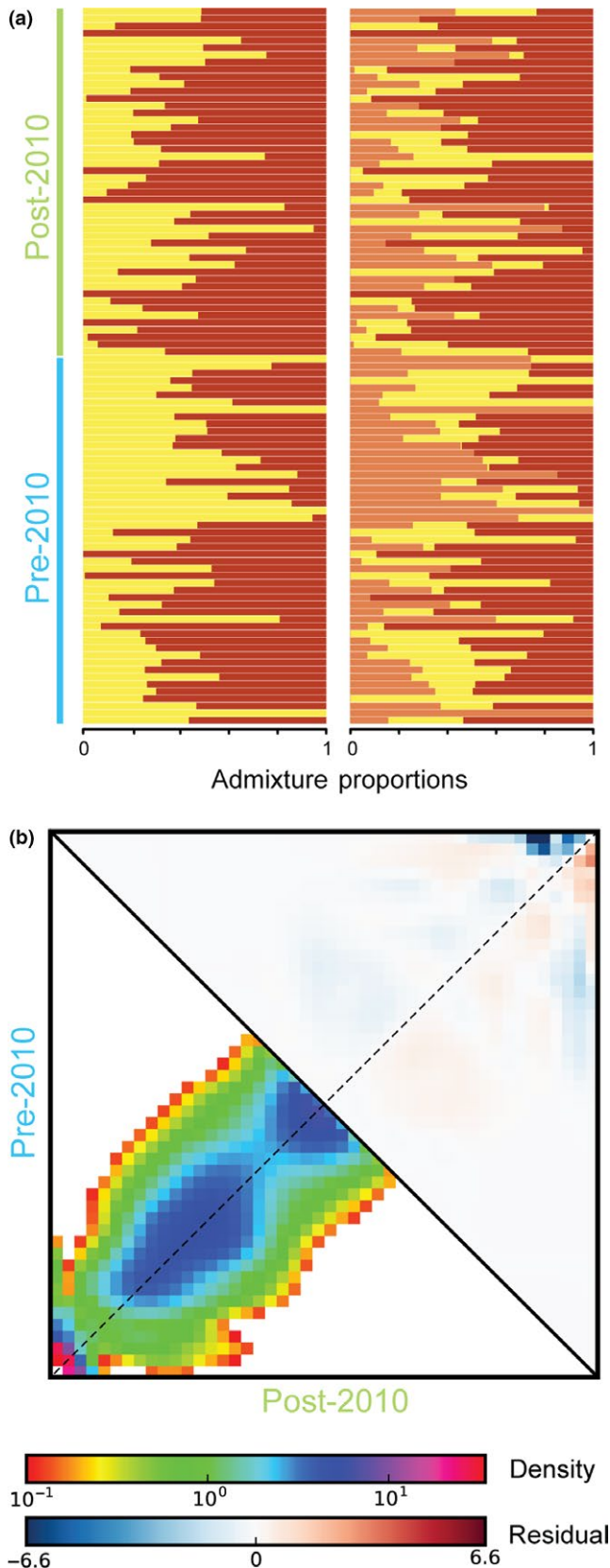


FIGURE 2 Genomic evidence for mixed ancestry and genetic evolution in the invasive Burmese python population. (a) Structure plot showing the admixture proportions for $K = 2$ and $K = 3$ source populations. (b) Allele frequency shifts in the Florida population illustrated by the 2D site allele frequency spectrum (below solid diagonal) and the residual change in allele spectrum density between the two time points (above solid diagonal). Each axis represents the distribution of minor allele frequencies for variant loci at the time point, which was projected down to a sample size of 45

evolution driven by directional selection at a subset of genomic regions.

3.3 | Rapid adaptive evolution targeted genes related to ecological shifts

Given strong evidence for selection-driven evolution, we were motivated to identify the potential functional targets of selection. We used the Burmese python genome annotation to identify genes that are genetically linked to the 12 candidate genomic regions inferred to be under selection (Supporting information Figure S9; Castoe et al., 2013). We used alignments to the more contiguous *Boa constrictor* genome (Bradnam et al., 2013) to identify adjacent syntenic scaffolds in the python (see Supplemental Methods). Seventy-eight genes were identified within the 12 putatively selected genomic regions, and functional annotations for these genes demonstrated striking relevance to physiological features that were *a priori* predicted to be relevant to the novel ecological conditions of Florida. Analyses of associated Mouse Knockout (MKO) phenotypes identified four prominent clusters of phenotypes: sensory perception and responsiveness, thermosensation and hypothermia responsiveness, learning and behaviour, and organ form and function (Figure 3c). Gene ontology (GO) analyses also indicated enrichment for genes linked to cell division, organ growth and development (including calcium signalling), reproduction, immunity and responses to stress, and to neuronal function and behaviour (Figure 3d; Supporting information Figure S10).

Because Burmese pythons are known for their ability to undergo extreme organ growth upon feeding, we cross-referenced genes in these 12 regions with genes relevant to regenerative organ growth (Andrew et al., 2015, 2017) and found several genes in key organ growth regulation pathways. Multiple genes were involved in calcium-mediated signalling, which plays a central role in organ hypertrophy, including *PLEK*, *CHP2* and, importantly, *PPP3R1*, which encodes a regulatory subunit of calcineurin (a key regulator of cardiac hypertrophy), and *PLCE1* (a regulator of processes including cell growth and differentiation). This gene set also included *PITX2*, a regulator of abdominal development, and a long non-coding RNA with homology to *PTEN*—a gene that functions in the mTOR growth pathway that is central to modulating post-feeding organ growth in pythons (Figure 4; Andrew et al., 2015, 2017). Overall, genomic data broadly correspond with

through time are not biased by a small number of extreme samples. Collectively, population genomic data provide evidence for genomewide evolution (i.e., allele frequency change) and for

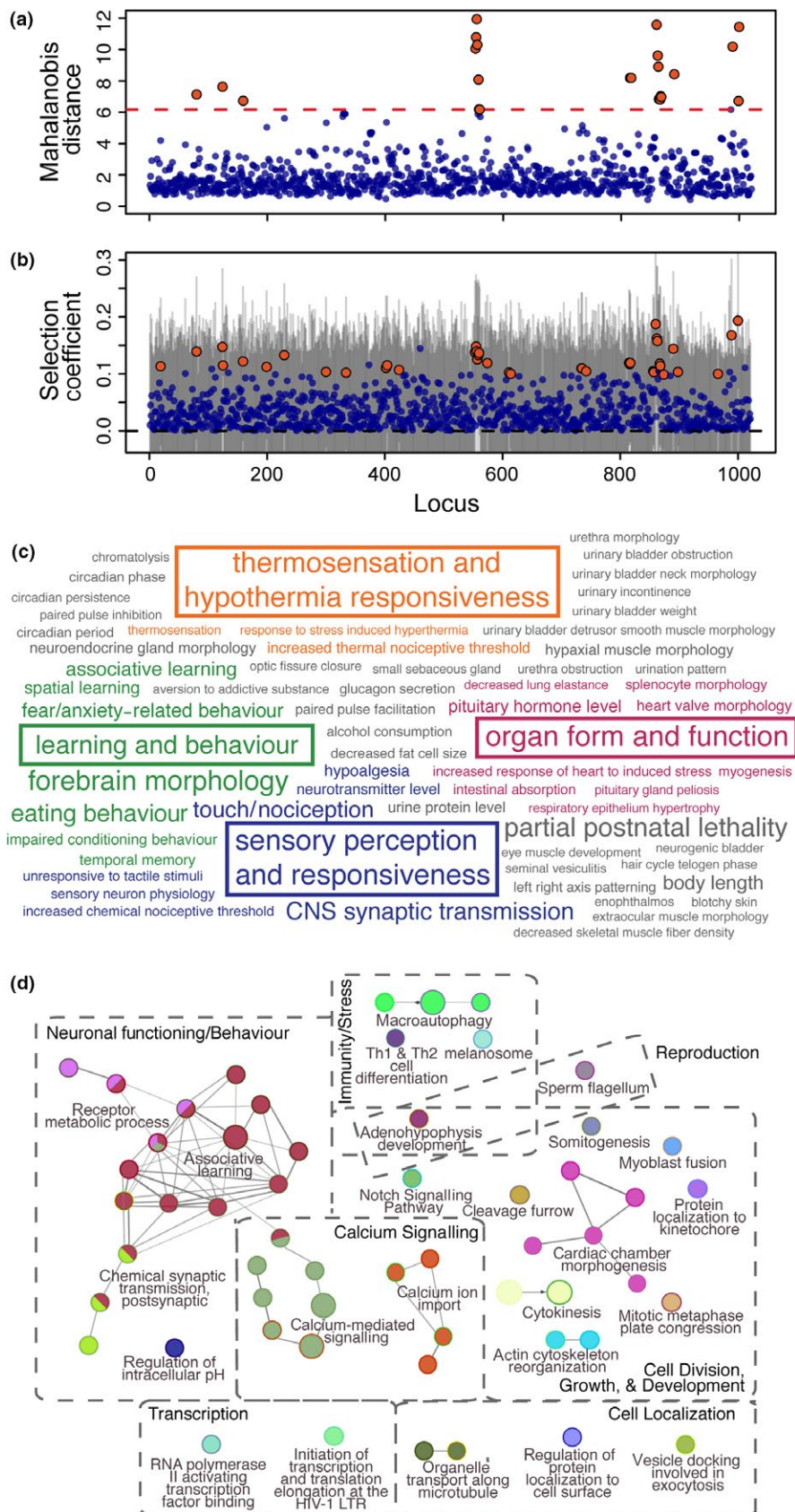


FIGURE 3 Genomewide shifts in population genetic variation indicate selection in genes related to reproduction, behaviour and regenerative organ growth. (a) Manhattan plot of multivariate Mahalanobis distance across variants with points above the 97.5% quantile (red broken line) indicated in red. (b) Manhattan plot of selection coefficients for each genome-wide variant. Grey lines represent the 95% high posterior density for each point (truncated at 0 for visualization). Red points have a 95% high posterior density (HPD) that falls entirely above 0, indicating selection. (c) Word cloud of MKO phenotypes associated with genes in regions with genomic outliers, clustered by colour into broader physiological categories. (d) Enriched GO networks differentiated by colour and clustered into broader physiological categories

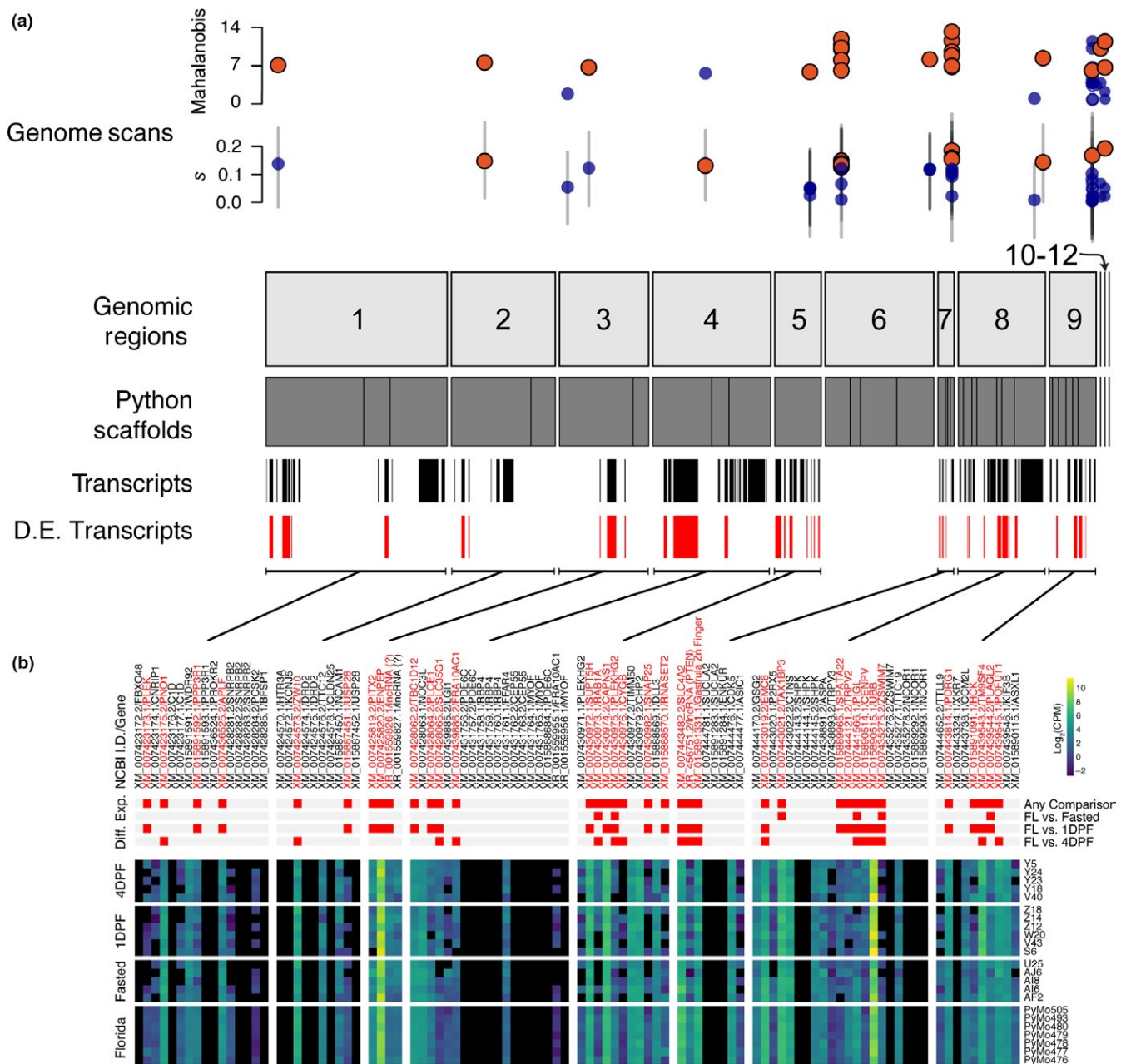


FIGURE 4 Natural selection modulates differential expression and is associated with genes known to play a role in regenerative organ growth. (a) Genome tracks displaying broader context of Mahalanobis distances and selection coefficients in syntenic genomic regions with selected variants, and associated Burmese python genome scaffolds, annotated transcripts and significantly differentially expressed transcripts. Loci with outlier Mahalanobis distances or selection coefficients indicating evidence for selection are shown as orange points and all other loci are indicated in blue (as in Figure 3). Regions were identified from alignments between the Burmese python and *Boa constrictor* reference genomes, and multiple Burmese python scaffolds map to each region (see “Python Scaffolds” track). Region 6 contained no annotated genes. (b) Significant pairwise differential expression comparisons (red in top heatmap and red transcript labels) and normalized expression heatmap (see inset scale of log₂ CPM) for fasted post-freeze Florida Burmese pythons and laboratory pythons in fasted and post-feeding morphological states. CPM, counts per million; D.E., differentially expressed; DPF, days post-fed; FL, invasive Florida Burmese python; lncRNA (?), long non-coding RNA with unknown homology

ecological and climatic data in implicating strong selection on traits related to thermal tolerance as well as feeding ecology/physiology. Furthermore, these multiple lines of evidence that implicate changes in feeding physiology raise the intriguing question of whether Florida Burmese pythons have adapted to alter their dynamic physiology to a more consistently active state based on increased prey availability in South Florida.

3.4 | Histological and functional genomic data implicate phenotypes linked to ecological pressures and correspond with putative genes under selection

We conducted a second set of experiments to identify whether any evidence outside of genomic allele frequency changes might

corroborate (or refute) our inference that rapid adaptation has occurred that may have altered physiological regulation in the invasive Florida Burmese pythons. We tested for evidence that modern Florida Burmese pythons possess a more upregulated fasting physiological state by comparing gene expression and histological data on organ cell sizes between fasted Florida Burmese pythons captured in 2016 and captive bred laboratory descendants of imported pythons while fasting and at various post-feeding time points. Specifically, we tested whether fasted post-freeze Florida Burmese pythons had substantially different cellular and transcriptomic states compared to fasted laboratory pythons—a pattern that is predicted by our inferences from genomic and ecological data. While we acknowledge that this experiment was not ideally controlled (e.g., common-garden design) due to permitting and regulatory constraints, it did allow us to test for phenotypic evidence that supports the hypothesis of a shift in Florida Burmese python physiology. We found that patterns of gene expression in seven fasted post-freeze Florida Burmese pythons resembled fasted laboratory pythons (Supporting information Figure S11), yet were distinct in several key features. Importantly, when comparing fasted post-freeze pythons and laboratory pythons, we found that an excess of differentially expressed genes was located in putatively selected genomic regions based on our analyses

of population genomic data ($p < 0.01$). This pattern suggests that directional selection in these genomic regions may have driven allelic changes that have modified expression of key genes involved with regenerative organ growth. We cannot, however, discount the role of plasticity, either alone or together with genetic adaptation, in driving patterns of differential expression (Card, Schield, & Castoe, 2018; Ghalambor, McKay, Carroll, & Reznick, 2007). Additionally, five of the six genes identified by our analyses of allele frequency changes, and highlighted above as being important in organ growth, showed differentially expressed transcripts between fasted post-freeze and laboratory pythons (IHW FDR < 0.1). We also found that gene expression interpreted in the context of pathways known to mediate regenerative growth in Burmese pythons (Andrew et al., 2015, 2017) indicates that fasted post-freeze pythons exhibit pathway and upstream pathway regulatory molecule states that are intermediate between the fasted and fed states in laboratory pythons (Figure 5a). Lastly, we examined cell sizes from four organs in fasted post-freeze pythons and found that they more closely resemble actively digesting laboratory pythons more so than fasted laboratory pythons (Figure 5b,c). While the transcriptome and histological data alone do not provide definitive proof of adaptation, it is notable that transcriptome and histological results support the independent predictions

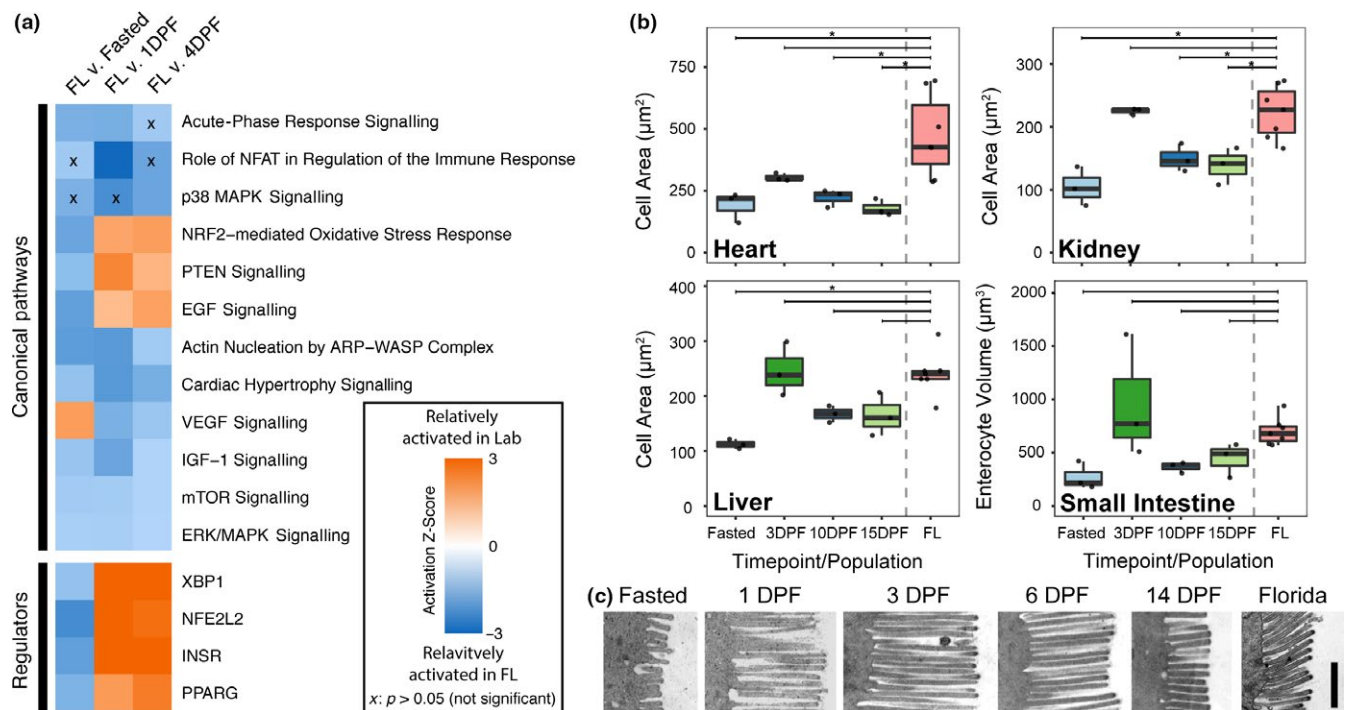


FIGURE 5 Cellular and anatomic evidence for unique, upregulated fasted physiological states in adapted Florida Burmese pythons. (a) Relative activation states for canonical pathways and upstream regulatory molecules previously shown to be important in python regenerative organ growth based on gene expression data. Pairwise comparisons represent relative activation between fasted post-freeze Florida Burmese pythons and laboratory pythons in fasted and post-feeding morphological states. (b) Boxplots showing cell size measurements between laboratory pythons in fasted and post-feeding morphological states and in fasted post-freeze Florida Burmese pythons for four organs. Horizontal bars indicate pairwise comparisons between the measurements from the fasted invasive Florida Burmese pythons and respective treatments from the laboratory pythons, with an asterisk indicating a statistically significant difference (Tukey's HSD p -value ≤ 0.05). (c) Example electron micrographs of proximal intestinal microvilli at several key time points during the normal feeding cycle in laboratory pythons, and in a post-freeze fasted Florida Burmese python. Scale bar = $1 \mu\text{M}$. DPF, days post-fed; FL, invasive Florida Burmese python

from population genomic and ecological data—that post-freeze Florida Burmese pythons may exhibit a unique and more consistently upregulated physiology.

4 | CONCLUSIONS AND SYNTHESIS

Overall, our results provide evidence for rapid evolution by natural selection in invasive Florida Burmese pythons, together with multiple lines of evidence that adaptation may be linked to freeze tolerance and a shift in feeding physiology. Our ecological data provide compelling evidence for an extreme shift in feeding ecology occurring in invasive Burmese pythons since their introduction to Florida, and field mortality estimates together with our ecological niche models indicate Florida Burmese pythons exist at the margins of their thermal tolerance. Our genomic data demonstrate that evolution (allele frequency change through time) has occurred and that a subset of genomic regions exhibit hallmarks of natural selection. Interestingly, these regions are enriched for genes related to thermal tolerance, behaviour and physiological phenotypes. Finally, independent gene expression and histological data provide an intriguing added layer of support for a shift in Florida Burmese python feeding physiology, which implicates many of the same key genes identified by the population genomic data. These results collectively support the hypothesis that new ecological pressures in Florida, such as a more temperate climate and more consistent prey availability, have driven adaptation by favouring the maintenance of a physiologically active state and enhanced thermoregulatory responsiveness.

A compelling question remains of whether behavioural changes, thermal tolerance and shifts in digestion physiology are linked, and future *in situ* and common-garden experiments that extend previous studies (e.g., Andrew et al., 2015, 2017; Avery et al., 2010; Dorcas et al., 2011; Jacobson et al., 2012; Lignot et al., 2005) would be valuable to test for these phenotypic differences and discern connections between these putative adaptations. Moreover, the relative contributions of longer term, consistent selection pressures versus acute, strong natural selection (e.g., rare freeze events) remain unclear from our analyses. It is possible that rapid adaptation in invasive Florida Burmese pythons may be the result of synergistic interactions between consistent ecological pressures, such as shifts in food availability, and acute climatic pressures associated with periodic freeze events. Fasting laboratory Burmese pythons have among the lowest vertebrate basal metabolic rates, yet upon feeding experience extreme organ growth that coincides with the highest increase in metabolic rate in vertebrates (40-fold; Secor, 2008; Secor & Diamond, 1995, 1998). Indeed, positron emission tomography (PET) scans of fasted versus fed laboratory pythons highlight massive upregulation of tissue metabolism in pythons that corresponds with upregulated organ systems (Secor, 2008; Secor & Diamond, 1995, 1998). In ectotherms, metabolism is known to correlate with body temperature (Guderley, 2004; Seebacher,

2005, 2009; Seebacher, Elsey, & Trosclair, 2003), which suggest physiologically upregulated pythons may maintain higher body temperature. Collectively, these data suggest that physiologically upregulated, hypermetabolic pythons—either due to having recently fed or due to heritable variation in their degree of post-feeding downregulation—may be resistant to freezing and may explain how the high mortality 2010 freeze event could have catalysed adaptive evolution. *In situ* adaptation of Burmese pythons to the South Florida environment has broad ecosystem-scale ramifications for persistence and expansion of this impactful invasive species. This and other examples (Phillips, Brown, Webb, & Shine, 2006) also demonstrate the surprising evolutionary potential of invasive species, and the importance of accounting for adaptation in predicting the outcomes of biological invasions.

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DATA AVAILABILITY

New raw Illumina sequencing data have been accessioned at the NCBI SRA (Accession nos SRP103526 and SRP158404). See Supporting information Table S1 for individual sample accession information. Data analysis scripts and intermediate and final data sets have been accessioned using figshare (<https://doi.org/10.6084/m9.figshare.6979940>).

AUTHOR CONTRIBUTIONS

D.C.C. and T.A.C. designed the experiment. D.C.C., B.W.P., R.H.A., M.R.R., F.J.M., K.M.H., M.E.H. and T.A.C. were involved in sample acquisition. D.C.C., B.W.P., R.H.A., D.R.S., A.S.Y., A.L.A., T.J., G.I.M.P., N.R.H., M.R.W. and T.A.C. contributed to data analysis and interpretation. D.C.C. and T.A.C. wrote the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

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

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