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Multi-species comparisons of snakes identify coordinated signalling networks underlying post-feeding intestinal regeneration

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Several snake species that feed infrequently in nature have evolved the ability to massively upregulate intestinal form and function with each meal. While fasting, these snakes downregulate intestinal form and function, and upon feeding restore intestinal structure and function through major increases in cell growth and proliferation, metabolism and upregulation of digestive function. Previous studies have identified changes in gene expression that underlie this regenerative growth of the python intestine, but the unique features that differentiate this extreme regenerative growth from non-regenerative post-feeding responses exhibited by snakes that feed more frequently remain unclear. Here, we leveraged variation in regenerative capacity across three snake species—two distantly related lineages (*Crotalus* and *Python*) that experience regenerative growth, and one (*Nerodia*) that does not—to infer molecular mechanisms underlying intestinal regeneration using transcriptomic and proteomic approaches. Using a comparative approach, we identify a suite of growth, stress response and DNA damage response signalling pathways with inferred activity specifically in regenerating species, and propose a hypothesis model of interactivity between these pathways that may drive regenerative intestinal growth in snakes.

1. Introduction

Snakes have emerged as a model system in which to study the regulation of intestinal form and function due to the extreme degree of intestinal modulation (5–10-fold) and regenerative growth that some heavy-bodied, infrequently feeding species experience upon feeding after a prolonged fast. At the completion of digestion, these snakes exhibit intestinal atrophy through decreases in cell proliferation and increases in apoptosis of enterocytes, reductions in microvillus length and downregulation of metabolism and digestive function [1–4]. Immediately following the ingestion of a meal, the intestine is rapidly restored, resulting in up to 100% increases in intestinal wet mass, fivefold increases in microvillus length, 44-fold increases in metabolic rate and the upregulation of intestinal function within 24 h [1–4]. This extreme regenerative response has been primarily studied in the Burmese python (*Python molurus bivittatus*) [3,5–13], but has also been identified in other infrequently feeding snake species, including other python species and several rattlesnake and boa species [1,4,14–17]. By contrast, frequently feeding snakes do not regulate intestinal form and function to this degree and instead exhibit relatively narrow

regulation (approx. twofold) similar to that of most vertebrates, including humans [8,16,18]. This extreme regenerative phenotype appears to be highly correlated with feeding ecology rather than phylogeny: distantly related snake species with similar feeding ecologies possess comparable extreme regenerative capacity upon feeding, yet some closely related species with divergent feeding ecologies exhibit divergent phenotypes (extreme versus minimal intestinal remodelling) [9,15]. Notably, while examples of tissue and limb regeneration have been investigated in other vertebrates, and reptiles specifically [19–24], post-feeding regenerative growth in snakes is unique, given that it is not a response to tissue loss or damage through injury (i.e. as in limb or tail regeneration), but instead occurs with every meal ingested following a period of prolonged fasting and in the absence of injury.

Recent studies have revealed the apparent role of cellular growth, metabolic, lipid signalling and stress response pathways during regenerative intestinal growth in the Burmese python [5,6,25], but have not compared these responses to other species. It is therefore unknown what distinguishes the regenerative response from the modest regulatory response associated with feeding, and thus, a number of crucial questions about regenerative mechanisms remain. For example, is regeneration achieved through the activation of unique signalling pathways in infrequently feeding species, or instead through greater-magnitude activity of 'normal' post-feeding cellular responses associated with feeding? Do species with similar regenerative phenotypes achieve these responses through the same mechanisms, or have different species evolved different mechanistic solutions for intestinal regeneration? And can we leverage the natural variation in regenerative phenotypes present across different snake species as a comparative framework for dissecting essential mechanisms underlying intestinal regeneration?

This study begins to address these questions by incorporating an additional regenerating species that possesses the capacity for regenerative growth, the prairie rattlesnake (*Crotalus viridis*), as well as a non-regenerating species, the diamondback watersnake (*Nerodia rhombifer*), with previous and new data from the Burmese python. We use analyses of transcriptomic and proteomic data across fasted and post-feeding time points to comparatively dissect and characterize molecular responses associated with regenerative intestinal growth in these species of snakes. Our results demonstrate that the rattlesnake and python intestinal responses to feeding are characterized by a suite of growth and stress response signalling mechanisms, some of which appear to be unique to these two regenerating species, while others are shared with the non-regenerating watersnake species but are likely to be active at different magnitudes in the two regenerative species. Based on our comparative analyses, we develop a hypothetical model to explain potential signalling networks that may underlie regenerative growth following feeding in the snake intestine.

2. Material and methods

Detailed methods are described in the electronic supplementary material, Methods. In brief, prairie rattlesnakes (*C. viridis*) and diamondback watersnakes (*N. rhombifer*) were sampled under the following conditions: fasted (30 days since last meal), 1 day post-feeding (DPF) and 4 DPF. Intestinal tissue for between three and four individuals per species per time point was extracted, snap frozen in liquid nitrogen and stored at −80°C. Time-series transcriptomic data for *C. viridis* and *N. rhombifer* intestine tissues were generated for this study (available at NCBI: SRP200900) and combined with previously generated data for *P. m. bivittatus* (NCBI: SRP051827). Pairwise exact tests of differential gene expression were performed using DEseq2 v. 1.12.4 [26] in R [27] and inferences of pathway and regulatory molecule activity were generated using Ingenuity Pathway Analysis (IPA) [28]. Label-free quantitative proteomics data were generated for the python and watersnake (https://doi.org/10.5061/dryad.db660b8). Differentially abundant proteins were identified with DEseq2 and used to generate additional regulatory molecule activity inferences in IPA. Relevant scripts and code are available on GitHub (https://github.com/blairperry/3snake-RegenerativeGrowth).

3. Results

(a) Rapid and massive changes in gene expression in regenerating species

Between fasting and 1 DPF, the regenerating python exhibits the largest number of differentially expressed (DE; $p < 0.05$) genes (2559), followed by the regenerating rattlesnake (1439); the number of DE genes in the non-regenerating watersnake is substantially smaller (793; figure 1). The python and rattlesnake shared 767 DE genes between fasted and 1 DPF, 563 of which were uniquely DE in these two regenerating species. Pairwise comparisons of 1 DPF and 4 DPF revealed a considerably larger response in the python (1595 DE genes) compared to that observed in both the rattlesnake (376) and watersnake (194) during this interval (figure 1*c*). Across all three species, many genes with significant up- or downregulation between fasting and 1 DPF showed no differential expression between 1 DPF and 4 DPF (figure 1*d*). A smaller number of genes showed a change in the direction of differential expression, continued differential expression in the same direction (i.e. upregulated fasted versus 1 DPF and upregulated 1 DPF versus 4 DPF) or delayed regulation (i.e. not DE in fasted versus 1 DPF, but DE in 1 DPF versus 4 DPF; figure 2*a*).

(b) Conserved regulatory molecule and pathway activity in regenerating species

To infer patterns of canonical pathway and regulatory molecule activity following feeding based on our transcriptomic data, we first performed core analysis in IPA [28] using all DE genes for each of the three species and identified pathways and upstream regulatory molecules (URMs) that showed one of two patterns predicted to be informative for dissecting mechanisms of regenerative growth: (i) those with significant inferences of regulatory activity in only the two regenerating species ($p < 0.05$), and (ii) those with significant inferences of activity in all three species ($p < 0.05$). IPA analyses of all DE genes between fasted and 1 DPF inferred significant regulatory activity of pathways associated with cellular growth, proliferation and metabolism signalling in all three species, including the PI3 K/AKT signalling, ERK/MAPK signalling, PDGF signalling, insulin receptor signalling and JAK/Stat signalling pathways (electronic supplementary material, figure S1A). Additionally, multiple pathways associated with cellular stress responses were inferred to regulate DE genes between fasted and 1 DPF,

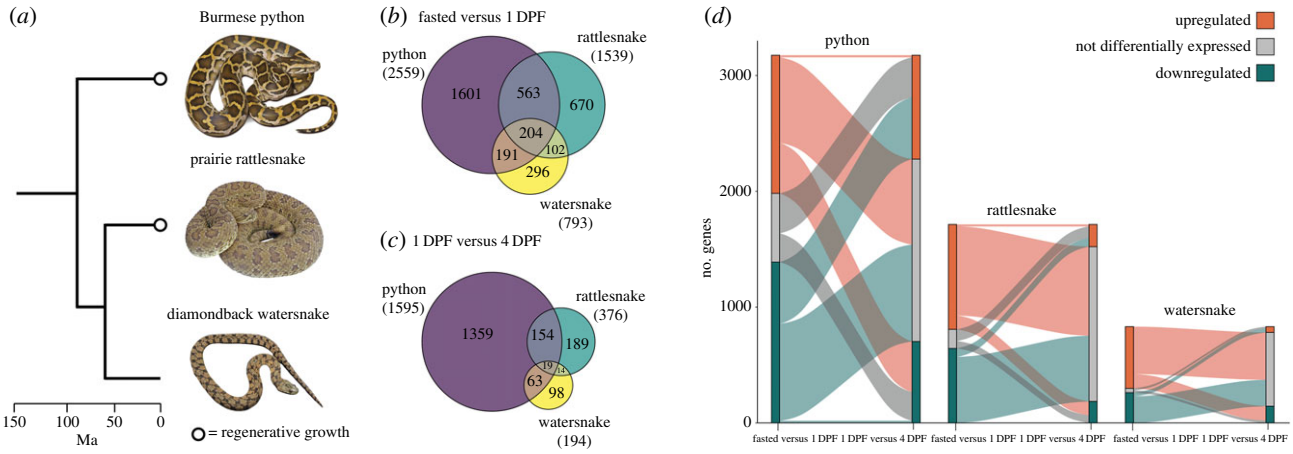

Figure 1. Divergent species that experience post-feeding regenerative growth exhibit similar gene expression responses. (*a*) The Burmese python and prairie rattlesnake both exhibit regenerative organ growth after feeding, despite being separate by roughly 90 million years of divergence. (*b*,*c*) Venn diagrams of DE (*d*,*e*) genes in the Burmese python, prairie rattlesnake and diamondback watersnake in pairwise comparisons between (*b*) fasted and 1 DPF, and (*c*) 1 DPF and 4 DPF. (*d*) Alluvial plots summarizing the number of upregulated ($p < 0.05$), downregulated ($p < 0.05$) and not DE ($p > 0.05$) genes for fasted versus 1 DPF and 1 DPF versus 4 DPF pairwise comparisons in the Burmese python, prairie rattlesnake and diamondback watersnake. Ribbon width represents the number of genes exhibiting a specified pattern of expression across the two pairwise comparisons (i.e. upregulated in fasted versus 1 DPF and downregulated in 1 DPF versus 4 DPF). Genes that were not DE in both pairwise comparisons are not shown.

including the NRF2-mediated oxidative stress response pathway and pathways associated with endoplasmic reticulum (ER) stress and the unfolded protein response (electronic supplementary material, figure S1A). Fewer pathways were inferred to drive gene activity in the regenerating python and rattlesnake alone in this analysis (electronic supplementary material, figure S1A), several of which are associated with DNA damage repair and tumour suppression. URMs associated with growth, metabolism and stress response signalling, including nuclear factor erythroid 2-like 2 (NFE2L2), insulin receptor (INSR), sterol regulatory element binding transcription factor 1 and 2 (SREBF1/2), several peroxisome proliferator-activated receptor (PPAR) molecules and x-box binding protein 1 (XBP1) were inferred to be significantly activated in all three species between fasted and 1 DPF, while few URMs were significant only in the python and rattlesnake (electronic supplementary material, figure S1B). Several pathways with inferred activity between fasted and 1 DPF, including the protein ubiquitination pathway, aldosterone signalling in epithelial cells and ER stress pathway, were also inferred to be actively regulating genes in all three species during the later 1 DPF versus 4 DPF interval (electronic supplementary material, figure S1C). Additionally, multiple URMs including XBP1, activating transcription factor 4 (ATF4) and NFE2L2 were inferred to be inhibited or downregulated during this later time point compared to their inferred activation between fasted and 1 DPF (electronic supplementary material, figure S1D).

To further dissect regulatory mechanisms that may explain unique patterns of gene regulation in the two regenerating species, we performed separate IPA core analyses on targeted subsets of DE genes that were (i) DE only in the python and rattlesnake and (ii) DE in all three species. The resulting IPA inferences of pathway and regulatory molecule were categorized based on patterns of overlap between the analyses of these two gene sets: regulatory mechanisms inferred only from analyses of DE genes shared between all three species were considered 'feeding' mechanisms, those inferred only from analyses of DE genes shared between the python and rattlesnake were considered 'regeneration unique' and mechanisms inferred from analyses of both gene sets were considered to be 'shared' between the feeding and regenerative response (figure 2). 'Feeding' pathways (figure 2*a*, 'feeding') included many of the same growth and stress response pathways inferred in the above analyses based on all DE genes (electronic supplementary material, figure S1). 'Shared' pathways inferred from analyses of both gene sets indicate that some pathways may respond with greater magnitude and/or breadth (in terms of the number of DE genes being regulated) in regenerating species (figure 2*a*, 'shared'), including the NRF2-mediated Oxidative Stress Pathway, which was previously implicated in regenerative growth in studies of the Burmese python [6]. 'Regeneration unique' pathways included many pathways associated with DNA damage repair and tumour suppression, as well as several growth and metabolism pathways including the insulin receptor and insulin-like growth factor 1 (IGF-1) signalling pathways, ERK5 signalling and JAK/Stat signalling (figure 2*a*, 'regen unique'). Inferences of 'shared' URMs (figure 2*b*, 'shared') suggest that the two regenerating species respond to feeding by differentially expressing additional sets of genes potentially regulated by NFE2L2, XBP1, INSR, which are major regulators within the NRF2-mediated oxidative stress response, unfolded protein response and insulin receptor signalling pathways, respectively. This indicates that although these URMs show activity in all three species, they are potentially regulating a larger number of DE genes in species that show regenerative post-feeding responses (pythons and rattlesnakes), which are not DE in the non-regenerating watersnake. These URMs may therefore contribute to regeneration-specific signalling beyond a baseline level general feeding response signalling. 'Regeneration unique' URMs included fibroblast growth factor 21 (FGF21), a known regulator of growth and metabolism [29], and matrix metallopeptidase 3 (MMP3), which is involved in the breakdown of the extracellular matrix during tissue remodelling and growth and has specifically been implicated in limb regeneration in newts [24] (figure 2*b*).

To assess the potential interaction among inferred canonical pathways, networks of pathways were constructed based

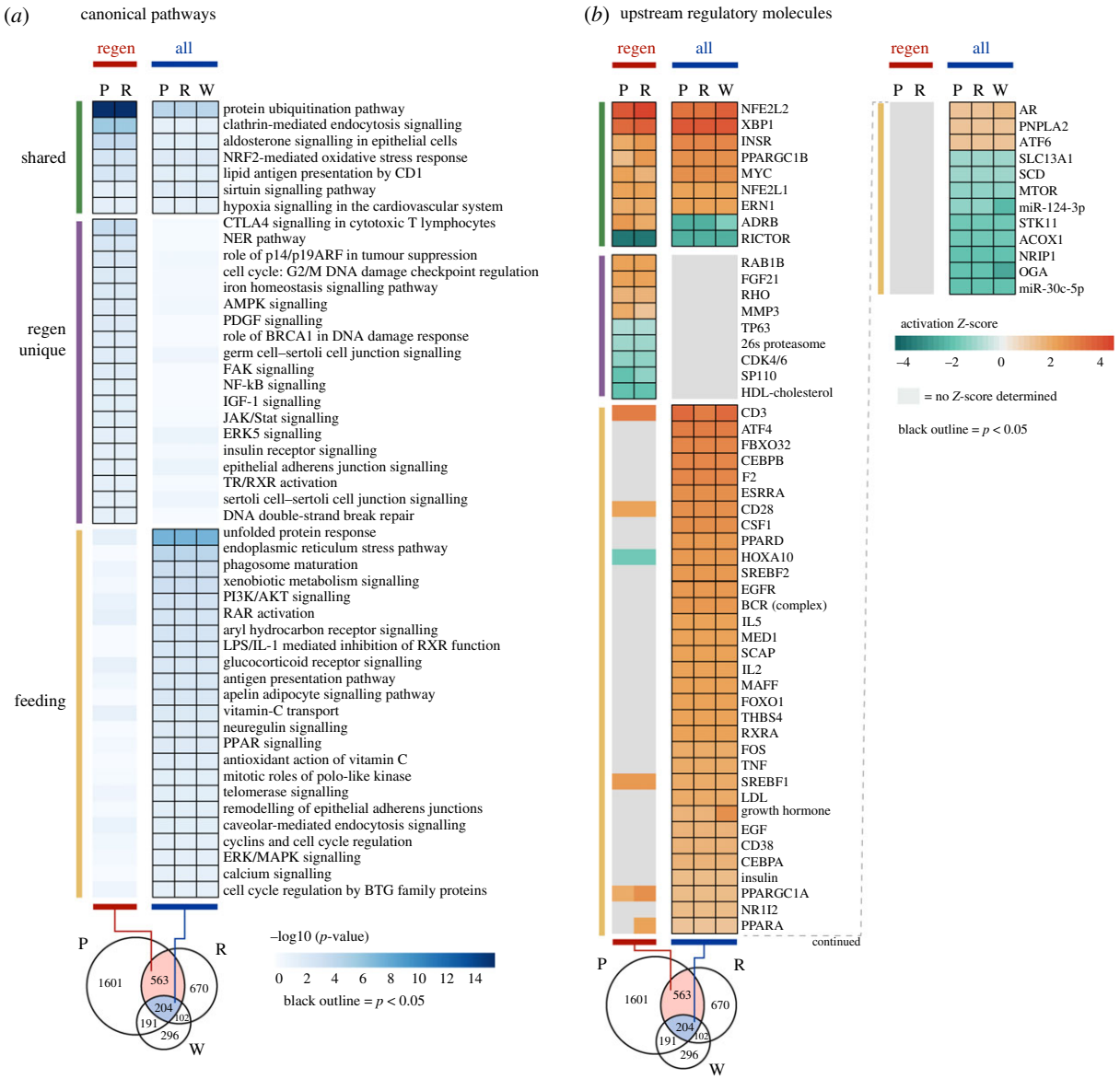

Figure 2. Canonical pathway and URM activation inferences based on comparisons of fasted versus 1 DPF RNAseq data. (*a*) Canonical pathways enrichment of DE genes between fasted and 1 DPF based on genes shared uniquely between the two regenerating species ('regen', left column) and genes shared between all three species ('all', right column). Black outlines denote $p < 0.05$. (*b*) Predicted URM activity based on regen and all gene sets. Cells with a black outline indicate significant enrichment and predicted activity ($p < 0.05$ and $|z| > 1$).

on the overlap of genes underlying inferred pathway activity (figure 3). In these networks, a connection between two pathways indicates that at least 50% of the genes underlying the inferred activity of one pathway were also underlying the inference of the other pathway. The feeding response network, generated from 'feeding' and 'shared' pathways described above, features a large interconnected group of pathways associated with cellular growth, metabolism and homeostasis (figure 3*a*). The NRF2-mediated oxidative stress response pathway and hypoxia signalling in the cardiovascular system pathway overlap with pathways within this growth-related cluster, suggesting the potential integration of growth and oxidative stress response signalling during the feeding response. Other stress response pathways did not show direct overlap with this group; these include the unfolded protein response and endoplasmic reticulum stress pathway, which are connected with the growth-related group via the protein ubiquitination pathway, aldosterone signalling in epithelial cells pathway and glucocorticoid receptor signalling pathway.

The regenerative response network, generated from 'regeneration unique' and 'shared' pathways described above, also exhibited an interconnected group of growth-related pathways (figure 3*b*), although the pathways within this cluster were distinct from growth-related pathways in the feeding response network (figure 3*a*). In this regenerative response network, NRF2-mediated oxidative stress response was again directly interconnected to this growth-related group, but here via the JAK/Stat signalling pathway. This network also features a group of cell junction signalling pathways and a group of DNA damage repair pathways, one of which (Role of BRCA1 in DNA Damage Response) connects directly with the growth-related group via AMPK signalling, with other DNA damage response pathways forming a separate cluster of interconnected pathways (figure 3*b*).

Based on interconnected pathways inferred from DE genes unique to the python and rattlesnake and known biological interactions and consequences of these pathways, we developed a hypothesis network for how growth and stress response mechanisms drive regenerative growth in snakes

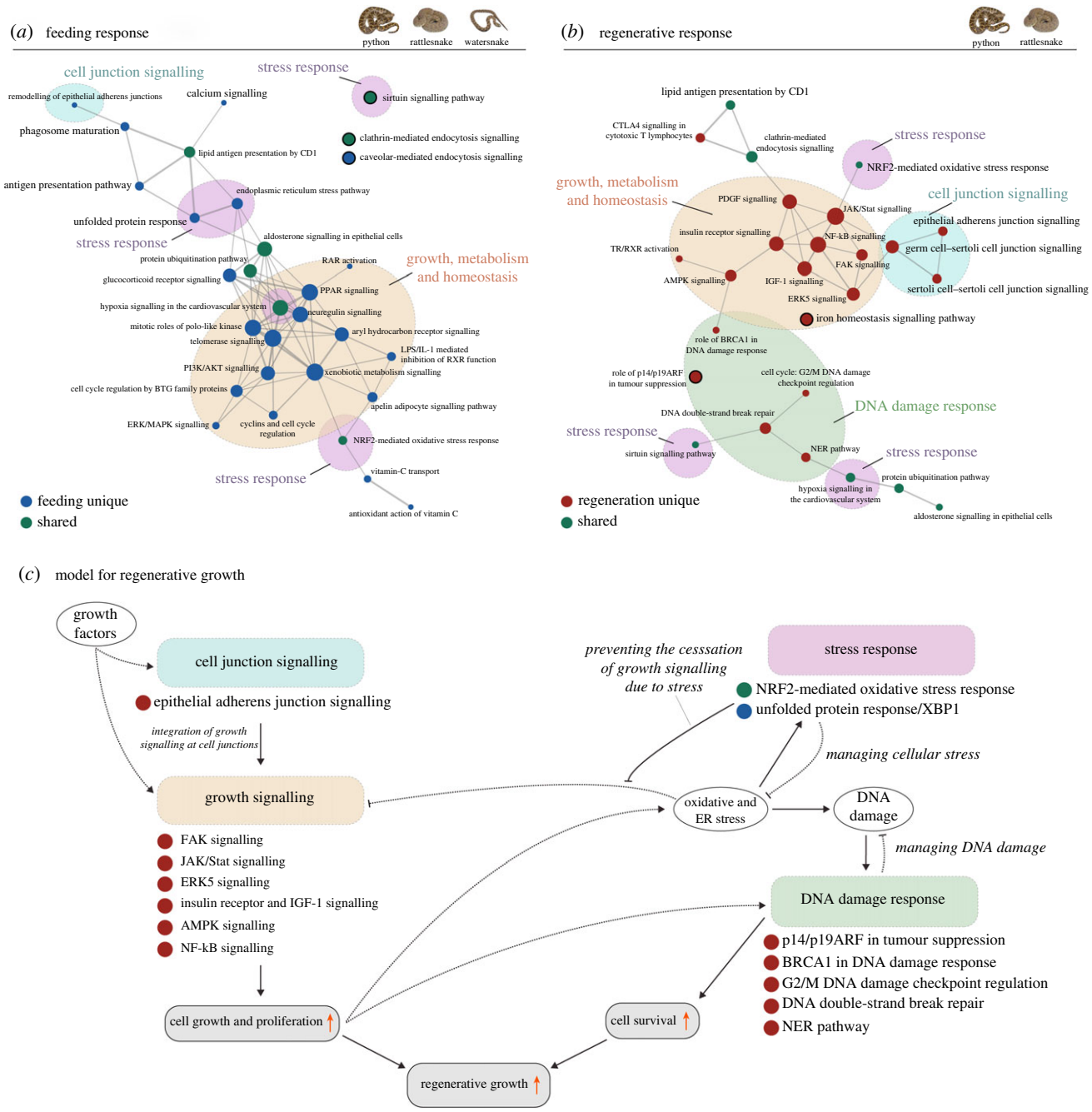

Figure 3. Overlapping canonical pathway predictions characterizing regenerative and feeding responses and a hypothesis model for regenerative growth in snakes. Networks showing the overlap in genes underlying canonical pathways with predicted activity from analyses of (*a*) DE genes shared between all three species and (*b*) DE genes shared between the python and rattlesnake but not the watersnake. A connection between two pathways indicates that at least 50% of the genes underlying the significant prediction of activity in one of the pathways also underlie the prediction of the other pathway, whereas pathways that are not connected to any others (circles with grey outlines) do not share greater than 50% of the genes underlying their prediction with any other pathway. Dotted circles represent manual annotation of pathways with similar functions. (*c*) A hypothesis model for how the integration of growth and stress response signalling drive regenerative growth in snakes.

(figure 3*c*). Key features of this model are the stimulation of regenerative growth through growth factor signalling via cell junction signalling as well as cell surface receptor signalling (e.g. insulin receptor), and the interaction and coordination of multiple growth pathways with stress response and DNA damage response pathways (figure 3*c*).

(c) Proteomic changes underlying regenerative growth

For comparison with inferences based on RNAseq data, we generated quantitative shotgun proteomic data for an overlapping set of samples. We successfully quantified intestinal protein abundance for 857 and 637 proteins for the python and watersnake, respectively. In both the python and watersnake, the number of proteins showing significant changes in abundance between time points (FDR < 0.1) was greatest between fasted and 4 DPF (figure 4*a*). Of the 68 differentially abundant proteins in the watersnake, 53 were successfully matched to an orthologous python protein ID and were used in downstream characterization and analysis. The 12 differentially abundant proteins between fasted and 4 DPF in the python and watersnake were enriched for GO terms relating to cell–cell adhesion and oxidation–reduction processes ($p < 0.05$; electronic supplementary material, figure

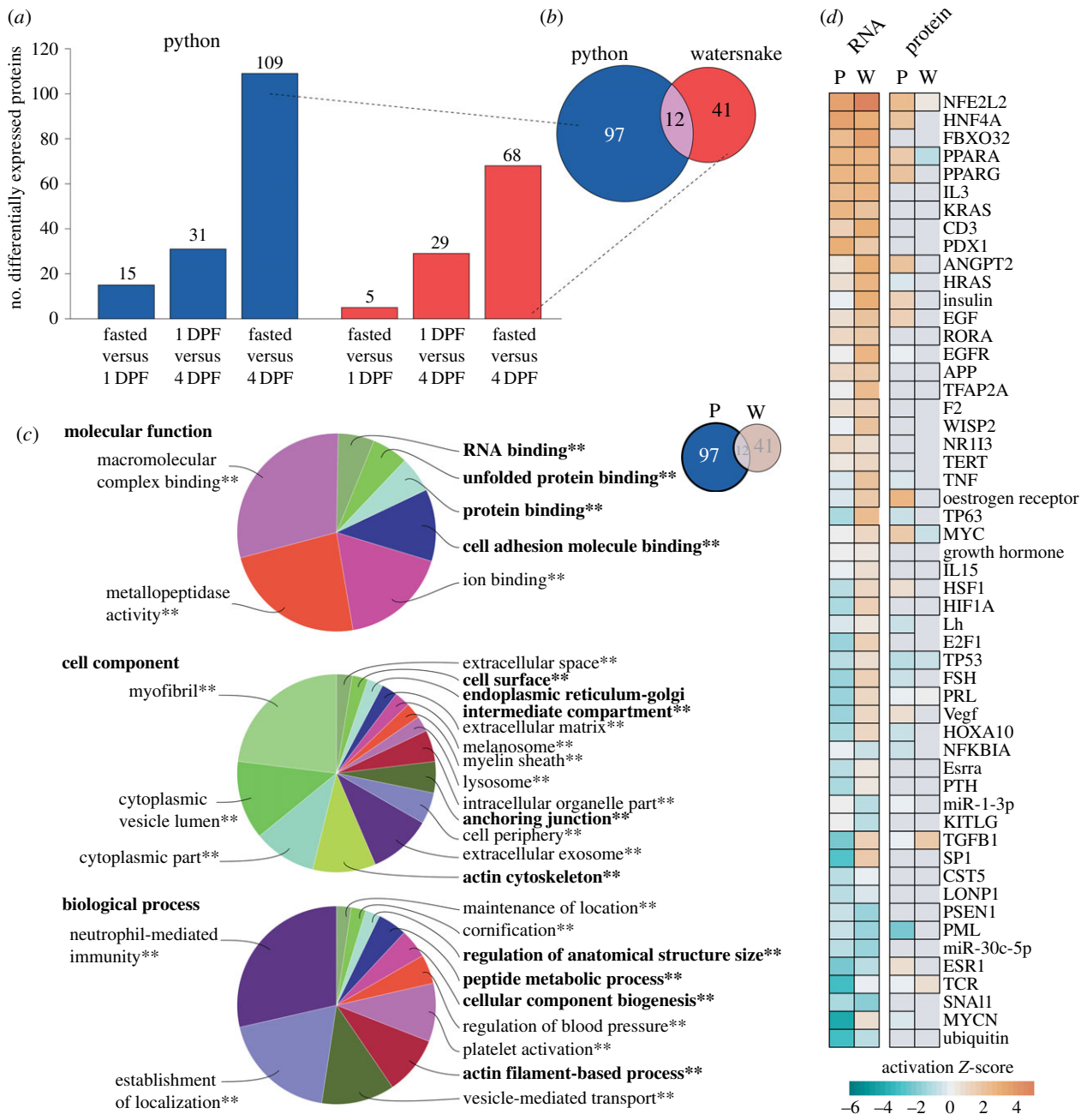

Figure 4. Proteomic comparison of python and watersnake intestine following feeding. (*a*) Numbers of proteins that exhibited significant changes in abundance in pairwise comparisons. (*b*) Venn diagram of proteins showing significant changes in abundance between fasted and 4 DPF in the python and watersnake. (*c*) GO term characterization of proteins with significant changes in abundance between fasted and 4 DPF in the python only. Asterisks denote significant enrichment of a category ($p < 0.05$), and terms with likely involvement in regeneration phenotypes are bolded. (*d*) URM activity inferred from significant changes in protein abundance between fasted and 4 DPF ($p < 0.1$), and significant DE genes between fasted and 1 DPF ($p < 0.05$). Only URMs with significantly inferred activity in the python from both protein and RNA data are shown.

S3). GO term analysis of the 97 proteins differentially abundant only in the python revealed several significantly overrepresented terms relevant to regenerative growth, including RNA and unfolded protein binding, actin cytoskeleton regulation, and regulation of anatomical structure size and cellular component biogenesis (figure 4*c*). At each of the three sampled time points, a weak but significant positive correlation was found between RNA expression and protein abundance in the python and watersnake when excluding data points with low RNA expression or low protein abundance ($p < 0.05$; electronic supplementary material, figure S8). In both species, the correlation between RNA and protein abundance was weakest at the fasted time points and strongest at 1 DPF (electronic supplementary material, figure S8).

IPA core analysis based on differentially abundant proteins between fasted and 4 DPF was compared with pathway and URM activity predictions inferred from patterns of gene expression. Relatively few canonical pathways were inferred to have significant activity based on protein data alone (electronic supplementary material, figure S4A), probably due to the small size of the input datasets. However, several pathways that were inferred to have significant regulatory activity in the python based on gene expression data were also inferred to be significant based on differential protein abundance, including the growth and metabolism-related VEGF signalling pathway. The NRF2-mediated oxidative stress response pathway was inferred to be significantly active in both protein and RNA-derived analysis in

the python and watersnake (electronic supplementary material, figure S4A). URM analysis showed more consistency between analyses based on protein and gene expression datasets (figure 4*d*). Many URMs inferred from gene expression to be activated between fasted and 1 DPF in the python and watersnake showed similar activation patterns based on differential protein abundance, including URMs associated with key growth and stress response pathways such as NFE2L2, KRAS, PPARA, PPARG and EGF (figure 4*d*).

4. Discussion

Interest in leveraging snakes to study the mechanisms underlying extremes of vertebrate organ regenerative growth, including intestinal regeneration, has steadily increased since the discovery of their extreme post-feeding regenerative capacities over 20 years ago [9]. Recent molecular studies that have made progress in understanding the signalling mechanism underlying these responses have focused on the Burmese python [5,6], but have lacked a cross-species comparative context that might differentiate post-feeding regeneration responses from general feeding responses. Here, we provide the first multi-species comparison of post-feeding organ regenerative responses in snakes by analysing the response of two species that do, and a third that does not, regenerate upon feeding. Our results indicate that the regenerating python and rattlesnake exhibit significant differential expression of thousands of genes following feeding, including a large number of shared genes that do not respond in the non-regenerating watersnake. Responsive genes in the two regenerating species show greater overlap with one another than they do with the non-regenerating watersnake, indicating that some mechanisms of regenerative growth responses are shared between these two regenerating species despite approximately 90 million years of divergence [30–32].

(a) Inferences of growth pathway activity between regenerating and non-regenerating snake species

To investigate signalling mechanisms that may differentiate regeneration versus feeding responses, we separately inferred pathway and regulatory molecule activity for DE genes shared between all three species (i.e. those likely to be associated with a general feeding response) and genes DE only in the two regenerating species (i.e. those uniquely associated with the regenerative response). In these targeted analyses, we found evidence for largely non-overlapping sets of canonical pathways and regulatory molecules regulating the core DE genes of the general feeding response in all three species versus the DE genes shared between the python and rattlesnake, suggesting that regenerative growth in these two species involves unique regulatory activity otherwise not active in regulating the feeding response. Our inferences suggest that the general feeding response largely comprises a distinct set of pathways associated with cellular growth, metabolism and homeostasis (figure 2, 'feeding'), including PI3 K/AKT signalling, which was previously suggested as a potential central regulator of regenerative growth in the Burmese python [6]. In addition to growth signalling pathways, multiple stress-response pathways were inferred to be involved in the general feeding response, including the unfolded protein response and endoplasmic reticulum stress pathways.

The regenerative response involves a distinct set of growth and metabolism pathways (figure 2, 'regen unique'), including known regulators of vertebrate growth, tissue repair and regeneration such as the IGF-1 signalling and insulin receptor signalling pathways [33,34]. Insulin receptor signalling and associated downstream pathways have been implicated in reptile longevity, growth and stress response [35–37], and have undergone rapid evolution in snakes [38]. Insulin receptor signalling also interacts with stress response pathways that have been implicated by this and previous studies in the regenerative growth response [6]. Previous studies of the Burmese python have demonstrated that the concentration of circulating insulin, one of the main initiators of the insulin receptor signalling cascade, increases sixfold with 24 h of feeding [12]. Unique activity of insulin receptor signalling is therefore a promising candidate for a high-level driver of regenerative growth in snakes.

Our analyses also identified pathways and URMs that were inferred in analyses of distinct DE gene sets associated with both the feeding and regenerative responses (figure 2, 'shared'), suggesting that more broad and/or higher magnitude stimulation of these signalling pathways that otherwise exhibit a baseline level of activity during the feeding response may also contribute to the regenerative response. Notably, this group of overlapping regulatory mechanisms included the NRF2-mediated oxidative stress pathway, which was previously suggested to be involved in regenerative growth in the Burmese python [6]. The NRF2 pathway overlaps with distinct growth pathways in both the regenerative and feeding response networks, suggesting the potential for direct integration of growth and stress signalling responses during both feeding and regenerative responses. NFE2L2, the primary regulatory molecule within the NRF2 pathway, was also inferred as a major regulator in both the regenerative and feeding responses. Additionally, XBP1 and INSR, major regulatory molecules within the unfolded protein response and insulin receptor signalling pathways, respectively, were inferred as URMs in analyses of distinct DE gene sets associated with both the regenerative and feeding responses, indicating a potentially expanded regulatory role of these URMs during regenerative growth.

Broadly, our results highlight shared patterns of signalling activity between divergent regenerating species and raise questions about the number of times this regenerative response may have evolved in snakes and the degree to which aspects of the regenerative response may be driven by shared ancestral regulatory programmes versus convergent evolution of regulatory programmes in divergent snake lineages. While convergent evolution of complex signalling programmes may seem unlikely, large-scale metabolic adaptation and convergent evolution has been demonstrated previously in snakes, and thus cannot be readily discounted as an explanation for the phylogenetic dispersion of regenerative growth phenotypes and the regulatory pathways that underlie these phenotypes [39,40].

(b) Activation of stress response signalling during regeneration

Oxidative and other cellular stresses are known to impair tissue repair and regeneration in vertebrates [33,41,42], and links between regulation of stress responses and regeneration are beginning to emerge in the literature [43–45]. In rats, the

transition from an oxygen-poor prenatal environment to an oxygen-rich post-natal environment corresponds with a cessation of regenerative capacity in heart tissue due to induced DNA damage inflicted by increased oxidative stress [45]. Additionally, one of the most well-studied vertebrate systems of tissue regeneration is the zebrafish, which inhabits a hypoxic aquatic environment and thus experiences a lesser degree of oxidative stress during regenerative growth [43–45]. A previous study focused on post-feeding organ regenerative response in the Burmese python identified the NRF2-mediated oxidative stress response pathway as having the greatest upregulation in activity of all inferred pathways in the intestine, kidney and liver [6]. Given the rapid increases in metabolism (up to 44-fold [3]) and cell proliferation following feeding in regenerating snake species and previous evidence for a role of stress responses in python organ regeneration, it is logical that a coordinated and highly activated armada of stress response pathways may play a role in the extreme regenerative growth observed in some snakes.

The NRF2-mediated oxidative stress response pathway was inferred to be a regulator in both the general feeding and regenerative responses. Thus, the NRF2 pathway is likely associated with feeding regardless of regeneration phenotype, but may play a more broad and highly stimulated role during regeneration in these two species. Mitigation of oxidative stress by NRF2 has been shown to play a vital role in liver tissue repair in mice by preventing insulin and insulin growth factor 1 (IGF-1) resistance that occurs via the phosphorylation of insulin receptor substrates by serine/threonine kinases that are activated by oxidative stress [33,46,47]. In NRF2-deficient mice, insulin resistance prevents the insulin signalling pathway from properly activating PI3 K/AKT and MAPK signalling pathways, two major pathways of growth and anti-apoptotic signalling, thus impairing tissue growth and repair [33]. Our results, together with the emerging role of NRF2 in regeneration, suggest that the action of NRF2 may play an important role in facilitating regenerative growth by permitting activity of growth mechanisms that otherwise negatively respond to oxidative stress.

The unfolded protein response (UPR), which senses and mitigates ER stress, was an inferred regulator of the general feeding response and is likely to be involved in mitigating ER stress associated with the high degree of cell turnover, exposure to metabolites and toxins, and general secretory nature of digesting intestine tissue [48]. While the entire UPR pathway was not inferred to be a regulator of the regeneration, XBP1, a major regulatory molecule within the IRE1-XBP1 signalling cascade of the UPR [49], was inferred to be an active regulator in both feeding and regenerative responses. XBP1 has been identified as an important factor in preventing tumour formation during regeneration of intestinal epithelial tissue following injury in mice [50], and the broad activation of XBP1 signalling may serve a similar role during regeneration in the python and rattlesnake, although further study would be necessary to confirm the role of this regulatory cascade in the regenerative response.

Our analyses suggest that pathways associated with DNA damage responses are uniquely involved in the regenerative response in the python and rattlesnake. This apparent involvement of a DNA damage response is likely to play a role in facilitating the high degree of cell proliferation required for rapid tissue growth. The involvement of these DNA damage response mechanisms, and particularly those associated with tumour suppression, is intriguing given that snakes, and reptiles in general, exhibit lower incidences of cancer than mammals [51]. Future studies into the specific means by which snakes activate DNA damage responses during regenerative growth may provide new insight into tumour suppression mechanisms in vertebrates.

(c) Insight into the regulation of regeneration from proteomic analyses

Our integrated analysis of transcriptomic and proteomic data provides complementary support for a number of key inferences regarding mechanisms and activation of signalling networks. Core analyses in IPA based on shifts in protein abundance between fasted and 4 DPF produced broadly similar inferences of URM signalling as did analyses of DE genes between fasted and 1 DPF, including consistent activity of stress and growth URMs such as NFE2L2, KRAS, EGF and others. The lag time between transcriptomic and proteomic responses together with the rapid response time of regenerative phenotypes also suggests that other means of regulation, such as post-translational modification of proteins, are probably also important in directing signalling that underlies the regenerative response. Future work to explore the role of post-translational modifications in the early phases of regenerative growth in snakes would provide an important dimension to our understanding of signalling that initiates regeneration.

(d) A model for the regulation of regenerative growth in snakes

We generated a model for signalling underlying regenerative intestinal growth in snakes based on inferences of regulatory mechanisms from this study and documented interactions among these mechanisms in other vertebrates (figure 3*c*). In this model, growth signalling pathways are activated by circulating signal molecules, such as insulin or other growth factors, with some of these signals potentially integrated via cell junction signalling in intestinal epithelial cells [52]. As growth signalling promotes cellular growth and proliferation, the buildup of reactive oxygen species and ER stress activate stress response pathways including NRF2-mediated oxidative stress response and the UPR [8,33], which in turn act to mitigate stress and prevent the cessation of growth signalling [33,53]. In response to initial and/or constitutive increases in cellular stress, DNA damage response pathways are also activated to ensure proper replication of cells and promote cell survival during proliferation [45,54]. Although preliminary, this model provides a hypothesis that can be further tested with additional analyses and experiments, and as such, presents a valuable step towards understanding how extreme bouts of regeneration might be accomplished in vertebrates.

5. Conclusion

Major advances in genomics have enabled the development of new vertebrate model systems that have traditionally lacked genomic resources but possess interesting phenotypes. Snakes are an example of such a system, and new genomic

resources now allow for intensive study of their extreme and medically relevant phenotypes, including regenerative growth following feeding [55–57]. By studying multiple species of snakes that do and do not experience regenerative growth upon feeding, we were able to begin to identify signalling mechanisms that may underlie extreme intestinal regeneration in snakes and distinguish these from mechanisms that are instead associated with a feeding response. Our findings highlight the value of employing a comparative approach to dissect a complex physiological response, and suggest that a combination of mechanisms uniquely activated in regenerating species and mechanisms shared with a typical feeding response, but regulating a greater number or distinct set of genes, may drive regenerative intestinal growth in snakes. We developed a hypothesis for how growth and stress response pathways might coordinate extreme intestinal regenerative growth while managing cellular stress and DNA damage associated with the extreme nature of this growth (e.g. 100% increases in mass in 24 h in pythons [9]). Our inference suggests that extreme regenerative growth in snake requires the coordination of stress response, DNA damage response and pro-survival signalling in addition to growth signalling. Testing and validating the precise role of these pathways and interactions among them is a priority for future studies and may enable further insight into regenerative signalling mechanisms with therapeutic potential for treating human conditions ranging from digestive diseases to cancer. From an evolutionary perspective, our findings raise interesting questions regarding the evolution of the regenerative response among snakes and pose further questions about how this phenotype may have influenced (or been driven by) major features of snake ecology. Considering the diversity of snakes, our analyses also raise the question of how broadly the three study species characterize the dichotomy between those that undergo regeneration upon feeding and those that do not, and future studies incorporating a greater diversity of species will be valuable for testing the generalizability of our conclusions across different snake lineages.

Data accessibility. Time-series transcriptomic data for *C. viridis* and *N. rhombifer* intestine tissues are available from the NCBI Short Read Archive at NCBI: SRP200900, as are previously generated data for *P. m. bivittatus* (NCBI: SRP051827). Label-free quantitative proteomics data are available from the Dryad Digital Repository at: https://doi.org/10.5061/dryad.db660b8 [58]. Relevant scripts and code are available from GitHub (https://github.com/blairperry/3snake-RegenerativeGrowth).

Competing interests. We declare we have no competing interests.

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