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Transcriptomic bases of a polyphenism

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Abstract

Polyphenism—in which multiple distinct phenotypes are produced from a single genotype owing to differing environmental conditions—is commonplace, but its molecular bases are poorly understood. Here, we examine the transcriptomic bases of a polyphenism in Mexican spadefoot toads (*Spea multiplicata*). Depending on their environment, their tadpoles develop into either a default "omnivore" morph or a novel "carnivore" morph. We compared patterns of gene expression among sibships that exhibited high versus low production of carnivores when reared in conditions that induce the carnivore morph versus those that do not. We found that production of the novel carnivore morph actually involved changes in fewer genes than did the maintenance of the default omnivore morph in the inducing environment. However, only body samples showed this pattern; head samples showed the opposite pattern. We also found that changes to lipid metabolism (especially cholesterol biosynthesis) and peroxisome contents and function might be crucial for establishing and maintaining differences between the morphs. Thus, our findings suggest that carnivore phenotype might have originally evolved following the breakdown of robustness mechanisms that maintain the default omnivore phenotype, and that the carnivore morph is developmentally regulated by lipid metabolism and peroxisomal form, function, and/or signaling. This study also serves as a springboard for further exploration into the nature and causes of plasticity in an emerging model system.

KEYWORDS
developmental plasticity, gene expression, phenotypic plasticity, phenotypic variation, spadefoot toad

1 | INTRODUCTION

Among the most spectacular forms of phenotypic plasticity is "polyphenism," in which multiple, discrete phenotypes arise from a single genotype as a result of experiencing differing environmental conditions (sensu Mayr, 1963, p. 670; Michener, 1961). Examples include environmentally influenced sexes (Bull, 1983); different leaf forms on the same plant ("heterophylly"; Wells & Pigliucci, 2000); castes in social insects (Wilson, 1971); seasonal forms (Shapiro, 1976); alternative reproductive forms in organisms ranging from viruses (Ptashne, 2004) to plants (Barnett et al., 2018) to animals (Moczek, 2005; Pienaar & Greeff, 2003); certain predator-induced forms (Agrawal et al., 1999); and alternative resource-use forms found in many organisms (reviewed in Pfennig & Pfennig, 2012). Polyphenisms have long fascinated evolutionary biologists because they are thought to represent a key phase in major, lineage-specific innovations (Mayr, 1963; Nijhout, 2003; Shapiro, 1976; West-Eberhard, 1989, 2003). Thus, clarifying the causes of polyphenism is crucial for understanding the origins of novel phenotypes. Yet, relatively little is known of polyphenism's underlying molecular mechanisms (Lafuente & Beldade, 2019).

Polyphenism can arise when an environmental stimulus breaks down the buffering mechanisms that typically enable robust

development of a particular phenotype, resulting in an altered developmental outcome (Moczek, 2007; West-Eberhard, 2003). The production of environmentally induced phenotypes through such a process has been observed in a variety of taxa (Gangaraju et al., 2011; Queitsch et al., 2002; Rohner et al., 2013; Rutherford & Lindquist, 1998; Sollars et al., 2003). For example, perturbation of the buffering activity of heat shock protein 90 (Hsp90) pharmacologically (i.e., geldanamycin exposure) or environmentally (i.e., temperature increase) results in the production of various distinct leaf and eye morphologies in *Arabidopsis thaliana* and *Drosophila melanogaster*, respectively (Queitsch et al., 2002; Rutherford & Lindquist, 1998). Once exposed, these alternative developmental pathways can be refined by selection into a coordinated developmental switch (Nijhout, 2003). Such developmental switches have been observed in various taxa (e.g., Moczek & Nijhout, 2002; Projecto-Garcia et al., 2017; Ragsdale et al., 2013). For instance, development of alternative discrete mouth forms in the nematode *Pristionchus pacificus* is regulated by the relative activity of a set of environmentally responsive enzymes (Bui et al., 2018; Ragsdale et al., 2013). In short, both a failure of robustness mechanisms and active developmental changes underpin polyphenism. However, the extent to which these two mechanisms influence any particular example of polyphenism is unclear.

Spadefoot toads (*Spea multiplicata*) are especially well-suited for tackling these issues because of their striking larval polyphenism (Pfennig, 1990) that is amenable to laboratory induction and manipulation. Normally, tadpoles of *S. multiplicata* produce an "omnivore morph" body form and eat mostly detritus. This morph is characterized by a long, coiled gut, smooth keratinized mouthparts, many keratinized denticle rows, and small jaw muscles. However, if a young omnivore eats large animal prey (fairy shrimp or other tadpoles), it may develop, via phenotypic plasticity, into a unique "carnivore morph," which specializes on these large prey (Levis et al., 2015; Paull et al., 2012). This morph is characterized by a short gut, pointed mouthparts, few denticle rows, and large jaw muscles (Pfennig, 1992b) and is an evolutionary novelty restricted to the genus *Spea* (Ledón-Rettig et al., 2008). Frequencies of carnivore morph production often vary by sibship and population, with the proportion of carnivores ranging from nearly 0% to nearly 100% for a given lineage (e.g., Levis & Pfennig, 2019; Levis et al., 2017; Pfennig, 1990, 1992a), which points to a genetic basis of plasticity that can undergo evolutionary modification.

The evolution of this polyphenism may have had important evolutionary and ecological consequences (Levis et al., 2018). For example, production of the carnivore morph has allowed *Spea* to access a new, predatory niche. This invasion of a new niche, in which carnivores obtain a high-nutrition meat diet that is in turn associated with accelerated larval development (Pfennig, 2000), presumably explains why *Spea* are among the few amphibians that can breed in highly ephemeral rain-filled pools. Consequently, *Spea* have been able invade a new biome (the desert) that is normally not available to other amphibians. This type of intraspecific variation and niche-width expansion may be an important driver of phenotypic evolution among diverse taxa. Indeed, numerous vertebrate and invertebrate taxa have evolved analogous environmentally induced trophic phenotypes, including protozoans (Kopp & Tollrian, 2003), rotifers (Gilbert, 1973), nematodes (Susoy et al., 2015), many fish (Robinson & Wilson, 1994), and salamanders (Collins & Cheek, 1983). Thus, understanding the causes of carnivore plasticity may provide key insights into an ecologically relevant, and taxonomically widespread, form of phenotypic variation.

Carnivore production is mediated by environmental cues, such as diet (Pfennig, 1990) and competitive environment (Frankino & Pfennig, 2001; Levis et al., 2017), and potentially by internal cues, such as thyroid hormone (Pfennig, 1992b). Previous work identified genes that showed variation in morph-specific expression among populations and species (Levis et al., 2017). However, this earlier study compared molecular phenotypes following environmental induction, and did not necessarily inform the developmental basis of phenotypic variation. Investigating development *before* phenotypic changes are apparent is needed to uncover the nature and mode of origin of the switches and other processes controlling this polyphenism.

In this study, we sought to characterize the transcriptional bases of polyphenism in *Spea*. To that end, we undertook two analyses. First, we identified regulators of plasticity (i.e., genes affecting the expression of other genes involved in development of alternative phenotypes; "switch-influencing" or "switch" genes) and determined if they were associated with a failure of robustness or an active developmental change. Second, we identified important genes and processes for the maintenance of these alternative phenotypes.

2 | METHODS

2.1 | General approach

Based on alternative dietary and competitive conditions that reflect those seen in nature, we investigated the transcriptional bases of the carnivore phenotype. Two days after exposure to carnivore-inducing cues (Figure 1b,c), but before emergence of morphological variation, we sampled tadpoles from numerous sibships. By allowing the siblings of these sampled tadpoles to develop an additional 6 days in carnivore-inducing conditions and measuring their trophic morphology, we were able to identify two groups of tadpoles that ultimately differed in the extent to which their morphology was carnivore-like. Each group was composed of three sibships, and one group (designated "high plasticity" or "HP") had significantly more carnivore-like tadpoles than the other group (designated "low plasticity" or "LP") consisting of the sibships with the least carnivore-like tadpoles. We measured gene expression of the head and body of tadpoles from these two groups that were sampled *prior* to the appearance of morphological differences. We did so because we were interested in the developmental changes *preceding*, and potentially controlling, downstream morphological changes. We first identified genes whose expression differed between the HP and LP groups irrespective of

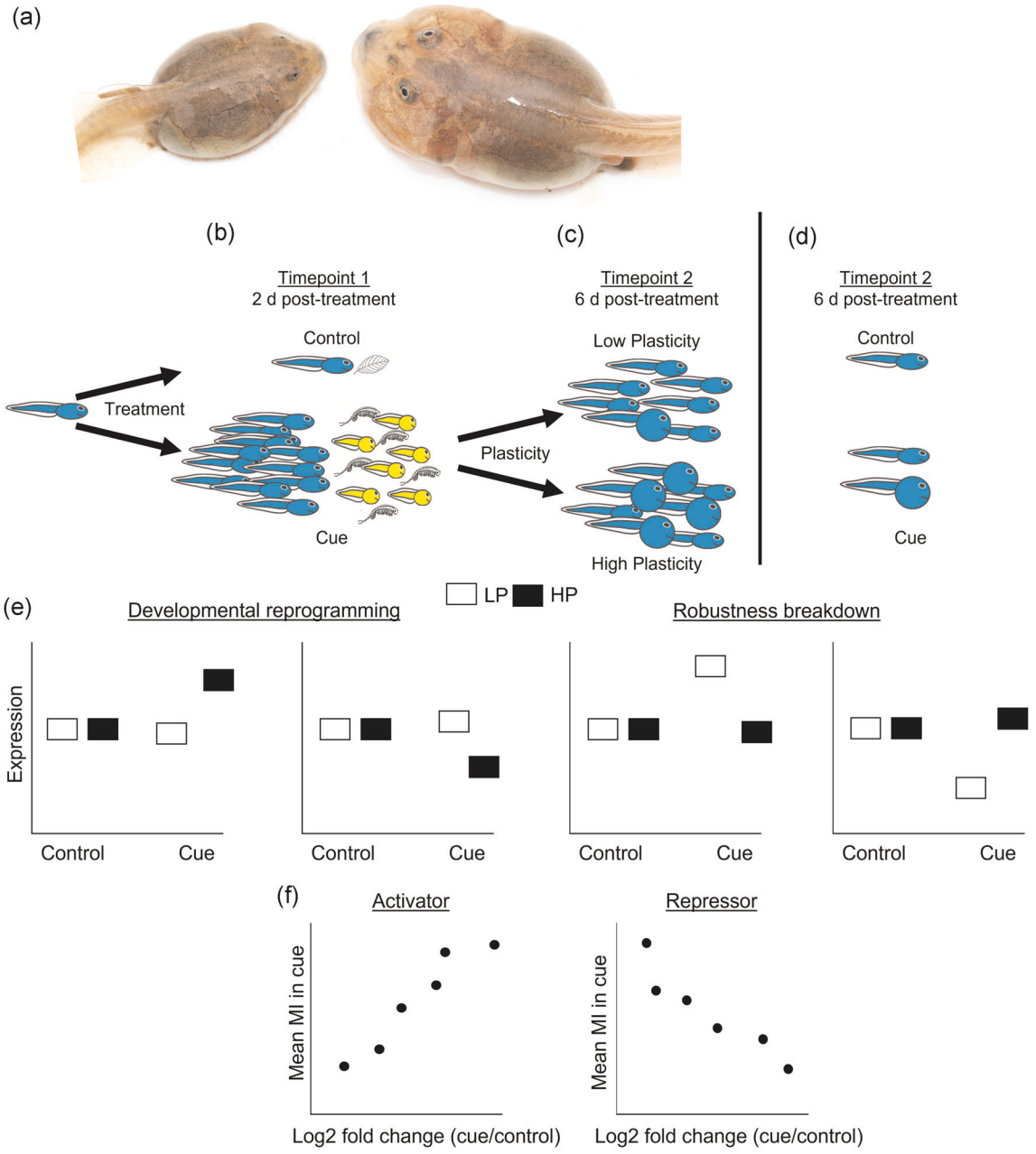

FIGURE 1 (a) Spadefoot toad (*Spea multiplicata*) tadpoles typically develop into an omnivore morph that feed on detritus (left), but can, depending on environmental conditions such as competition and diet, develop into a carnivore morph (right). We collected head and body samples from tadpoles (b) two days after exposure to alternative treatments (Timepoint 1). Treatment conditions were either (top; Control) reared as singletons on a detritus diet or (bottom; Cue) reared at high density and fed detritus, fairy shrimp, and *Scaphiopus couchii* (yellow) tadpoles. c) Sibships were classified as "High Plasticity" (e.g., top) or "Low Plasticity" (e.g., bottom) based on how carnivore-like (depicted by roundness in this example) their tadpoles were after six days of exposure to the cue treatment. (d) Liver and brain tissue were collected from control omnivores, cue omnivores, and cue carnivores 6 days after exposure to their respective treatments. (e) Diagrammatic representations of expression patterns for developmental reprogramming and robustness breakdown switch genes. (f) Diagrammatic representations of relationship between molecular and morphological plasticity for activator and repressor switch genes. MI denotes morphological index; our measure of plasticity in this study (see main text for details) [Color figure can be viewed at wileyonlinelibrary.com]

environmental conditions. We then identified candidate switch-influencing genes using two approaches (see below) and determined if these genes were associated with the breakdown of buffering mechanisms (genes where the LP group expression changed in the cue relative to the control and was significantly different from the HP expression level in the cue) or activation of developmental reprogramming (genes where the HP group expression changed in the cue relative to the control and was significantly different from the LP expression level in the cue). Next, because a gene's position in regulatory networks, rather than just its level of expression, could affect downstream developmental programs, we identified genes whose membership in co-expression networks differed between HP and LP groups. Finally, we identified genes and processes important for the functioning of alternative morphs by comparing liver and brain gene expression of carnivores and omnivores (Figure 1d).

2.2 | Experimental design and sample collection

Adult Mexican spadefoot toads (*S. multiplicata*) were collected on July 10, 2018 from a newly formed (< 1 d old), rain-filled pond near Portal, Arizona (lat. 31.9142, long. −109.0836) and returned to the nearby Southwestern Research Station. We collected 12 calling *S. multiplicata* males and 12 females that were extracted from amplexus with other males. Each toad was washed with water (to remove any sperm) and placed alone in a labeled plastic bag filled with air. We then transported all toads to the Southwestern Research Station, and, within 4 h of collection, we randomly paired each female to a male. Each such pair was placed in a separate tank filled with 6 L of dechlorinated well water and keep in a dark room. After approximately 8 h, upon visual confirmation that oviposition had occurred, we removed the adults from the tanks and began aerating the eggs. Two days after their eggs hatched, for each sibship (ranging in size from ~450–1000 tadpoles), tadpoles were divided into two treatments. Twelve tadpoles per sibship were randomly chosen to be reared individually and fed 10 mg of fish food daily; fish food mimics the detritus on which omnivores feed in form and nutrition (Pfennig et al., 1991). In this "control" treatment, tadpoles were not exposed to cues that elicit carnivore production. Keeping each sibship separate, the remaining tadpoles were divided into five boxes of 80 tadpoles (400 tadpoles per sibship) and fed fish food, live fairy shrimp, and live *Scaphiopus couchii* tadpoles. These numbers of tadpoles and replicates were chosen to balance the need for high densities to elicit a developmental response and the need to include multiple sibships to capture variation in responses (the number of tadpoles per sibship ranged from ~450 to ~1000) and because of physical constraints on space for rearing boxes. This was the "cue" treatment, because competition, shrimp consumption, and tadpole consumption contribute to the development of carnivores (Frankino & Pfennig, 2001; Levis et al., 2015; Pfennig, 1990). Note that we were not trying to identify the relationship between gene expression and exposure to a *particular* cue; we were instead interested in flooding tadpoles with well-known carnivore-inducing cues to identify sibships that differed in morph production. We opted to manipulate the tadpole's rearing environment because its relationship with morph development has received considerable research attention and because direct manipulation of any particular developmental processes (e.g., hormone treatment) may pre-suppose the mechanisms required for plasticity and bias our findings toward those mechanisms. In essence, since this is the initial attempt at uncovering the molecular basis of spadefoot plasticity, we wanted to have an ecologically informed, but mechanistically agnostic, experimental design that can inform subsequent work on particular pathways or processes. Tadpoles remained in their treatments for two days (i.e., they were 4 days old) before we randomly sampled five control tadpoles and five cue tadpoles per sibship, euthanized them with a 0.8% aqueous solution of tricaine methanesulfonate (MS-222), and placed them in a microcentrifuge tube filled with RNAlater. Samples remained at room temperature for 24 h to allow RNAlater penetration and were then frozen at −20°C until they were shipped to the University of North Carolina overnight on dry ice. They were then kept at −80°C until further processing.

The remaining tadpoles were allowed to develop for an additional six days until the different morphs were observable. When tadpoles were 10 days old, we randomly sampled per sibship: five control omnivores, five cue omnivores (i.e., individuals exposed to the inducing cue, but remained as omnivores), and five cue carnivores (i.e., individuals exposed to the inducing cue, and developed into carnivores). We processed these tadpoles as above. The remaining tadpoles in the cue treatment were euthanized and preserved in 95% ethanol for morphological measurements.

To assess sibship-level morphology, and consequently plasticity, we measured the ethanol-preserved tadpoles using previously published methods (Pfennig et al., 2007). Specifically, we measured the width of each tadpole's orbitohyoideus muscles (OH; carnivores have a larger OH for their body size) and scored its mouthparts (MP) on an ordinal scale that ranged from 1 (*most omnivorous*) to 5 (*most carnivorous*). After averaging left and right OH values and correcting the mean for body size, (by regressing log OH on log snout-vent length), we combined these two metrics into a single morphological index ("MI") by taking the first principal component from a principal component analysis. Larger values of MI correspond to more carnivore-like morphology with larger OH muscles and more serrated and notched mouthparts. Since singleton omnivores (i.e., control tadpoles) from our sample population do not tend to differ in their trophic morphology (pers. obs.; Kelly et al., 2019), we used the average MI of each sibship's tadpoles in the cue treatment as a proxy for that sibship's degree of plasticity. This metric tells us how each sibship responds, on average, to being reared in a carnivore-inducing environment. Measurements were taken blindly with respect to an individual's morph, treatment group, and the sibship's ultimate designation as HP or LP. In fact, these measurements determined HP and LP designations.

By performing a simple analysis of variance and Tukey's honestly significant difference (in JMP Pro 15), we identified the three sibships with the highest MI (HP) and the three sibships with the lowest

MI (LP) for use in gene expression analyses. We restricted our focus to these extreme samples to identify those differences with the largest effect on morph development.

2.3 | Sample processing

Immediately before RNA extraction, for 4 days old samples, each tadpole's head was separated from its body and the tail was removed from the body using a fine scalpel. For 10 days old samples, each tadpole's liver and brain were removed using forceps. In all cases, the tadpole was submerged in RNAlater during dissection. Thus, we had four sample types: body, head, liver, and brain. Body and head samples were from 4 days old tadpoles reared in either control or cue conditions. Liver and brain samples were from 10 days old control omnivores, cue omnivores, and cue carnivores. Samples were homogenized in TRIzol reagent with Dounce all-glass tissue grinders. We extracted RNA from three individuals per sibship per sample type (head, body, liver, brain) using Invitrogen PureLink extraction columns with TRIzol reagent. We obtained RNA from 36 head (9 replicates per plasticity-treatment combination), 36 body (9 replicates per plasticity-treatment combination), 54 liver (18 replicates per morph-treatment combination), and 54 brain (18 replicates per morph-treatment combination) samples for a total of 180 samples. For body, head, and liver samples, 3′ RNA-seq libraries were generated using the Lexogen Quantseq FWD kit and sequenced on an Illumina NextSeq 500 using a single end 75 bp kit with an actual read length of 86 bp. Library preparation and sequencing were performed at the Cornell University Institute of Biotechnology. RNA from brain tissue, because of the small volume, was processed by Qiagen Genomic Services using the low input QIAseq UPX 3' sequencing kit and protocol. This generated qualitatively similar data to that produced for other tissues (except that reads were 150 bp), but this procedure was optimized for low inputs of starting material. Thus, all samples of a given tissue or body region were processed in the same way. One limitation of our 4 days old samples is that we do not have tissue-specific patterns which means that expression patterns in different tissues might, in effect, cancel each other out and be undetected by us. Similarly, but likely to a lesser extent, our liver and brain samples are composed of multiple cell types that differ in function. Our results, therefore, may represent only major differences that surpass any inter-tissue or inter-cell type variation in expression.

2.4 | Measurement of gene expression

To measure gene expression, we followed the general procedure described by Seidl et al. (2019) for 3' RNA-seq data. We began by trimming the 3' RNA-seq reads to remove adapter and poly-A contamination using BBDuk (B. Bushnell, https://sourceforge.net/projects/bbmap) with the parameters: ktrim=r k = 23 mink=11 hdist=1 tpe tbo qtrim=r trimq=6 ftl=12 maq=6. Individual reads were then mapped to the *S. multiplicata* genome (Seidl et al., 2019) using STAR aligner (Dobin et al., 2013) with default parameters for the Lexogen Quantseq kit. We used bedtools genomecov (Quinlan & Hall, 2010) to generate bed coverage files at all nucleotide positions and performed peak discovery by using the "findpeaks" function in the R package "pracma" (Borchers, 2019) with the following parameters: nups=1, ndowns=1, zero="0," peakpat=NULL, minpeakheight=500,minpeakdistance=3000, threshold=0, sortstr=FALSE.

We defined the peak as the base with the maximum coverage in each window. We then extracted coverage of each peak ±25 bp for each individual using the bedtools coverage tool and took the average coverage over the 50 bp window surrounding the peak (rounded to the nearest whole number) as our measure of gene expression. Because there is currently insufficient data on 3'UTR location for *S. multiplicata* genes (Seidl et al., 2019), to assign gene identities to our peaks, we used bedtools closest (Quinlan & Hall, 2010) to match our peak to the nearest known *S. multiplicata* genes following Seidl et al. (2019). The best match resulting from a BLASTx of these *S. multiplicata* genes against the Uniprot tetrapod database was used as each gene's identifier. In total, this procedure resulted in expression data for 14,485 protein coding genes.

2.5 | Regulation of plasticity

To identify differentially expressed genes between the HP and LP groups, we used the R package DEseq2 (Love et al., 2014) to evaluate a model containing treatment as a covariate and plasticity level (i.e., HP or LP) as the main explanatory variable. In brief, this package calculates the Wald statistic ($\log_2$ fold change divided by the standard error $\log_2$ fold change) and compares it to a normal distribution to generate a two-tailed *p* value for each gene. The package also performs independent filtering to control for extreme outlier samples, low normalized counts, and genes with no expression, followed by false discovery rate adjustment to account for multiple testing. In short, the program iterates over each gene, fits a negative binomial model based on the groups to be compared (e.g., environment, plasticity level) and then provides output of the comparison after controlling for various factors that could artificially influence results. The model was run separately for head and body samples, and in each case, produced a set of genes whose expression varied between plasticity levels irrespective of treatment. We then input gene IDs into the online gene set enrichment tool g:Profiler (Raudvere et al., 2019; Reimand et al., 2007) to identify the top biological process gene ontology (GO) terms and KEGG pathway terms associated with all differentially expressed genes, HP-biased genes, or LP-biased genes.

While genes whose expression differs between HP and LP groups tell us about key regulators discriminating between these groups, they do not necessarily tell us which genes govern the transition (i.e., the switch) from omnivore morph to carnivore morph. Our next three analyses sought to identify candidates for this role. First, we identified four sets of genes using DESeq2 (Figure 1e).

For all sets, HP and LP groups were significantly different in the cue. The sets were further delineated based on whether HP expression was higher in the cue than in the control, HP expression was lower in the cue than in the control, LP expression was higher in the cue than in the control, or LP expression was lower in the cue than in the control. Essentially, we were testing if the HP group or the LP group changes its expression (up or down) in the cue. We considered cases where the HP group (but not the LP group) changed as being associated with developmental reprogramming. The expression of these genes is changing in the group that exhibits more carnivore-like morphology in the cue environment. In contrast, we considered cases where the LP group changes in the cue, but the HP group does not, as being associated with the breakdown of buffering/robustness mechanisms. That is, we assumed changes that occurred in LP group are at least partly responsible for that group exhibiting more omnivore-like morphology in a carnivore inducing environment.

Our second analysis of plasticity's regulation (Figure 1f) determined the magnitude of expression change ($\log_2$ fold change) across treatments (cue/control) for each gene and found the Spearman correlation between this molecular plasticity and morphological plasticity (i.e., mean MI [determined above]). For this analysis, each sibship was a separate sample such that for every gene we had six measures of $\log_2$ fold change and six measures of MI. Here, we considered genes showing a significant positive relationship between morphological and molecular plasticity as plasticity activators (roughly corresponding to developmental reprogramming) and genes showing a negative relationship as plasticity repressors (roughly corresponding to breakdown of buffering mechanisms). See Figure 1e,f for a schematic of the four regulatory categories—developmental reprogramming, buffering, plasticity activators, plasticity repressors—and how they were identified.

We evaluated biological process GO and KEGG pathway enrichment of the above candidate genes (i.e., developmental reprogramming genes, breakdown of buffering genes, activator genes, and repressor genes) using the g:Profiler online tool (Raudvere et al., 2019; Reimand et al., 2007).

Our third analysis of plasticity's regulation focused on each gene's association with coexpression networks (modules) and how these associations differ between HP and LP groups. To perform this analysis, we followed the tutorial for consensus analysis (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/index.html) using the WGCNA package in R (Langfelder & Horvath, 2008). For this analysis, we focused on HP and LP body samples that were normalized in DESeq2. Modules were detected using the automatic, one-step network construction and consensus analysis pipeline in WGCNA (power = 10, minModuleSize = 30, corType = "bicor," mergeCutHeight = 0.35, TOMType = "signed," maxPOutliers = 0.05). This pipeline identified 24 coexpression networks (modules), determined how associated each gene was with each network (module membership), and determined each gene's correlation with environmental conditions (control to cue).

Using these network association values (that range from −1 to 1), we then compared how similar associations were between HP and LP groups. Specifically, for each group, we ranked the association scores and calculated the rank biased overlap (rbo) between the two lists (Webber et al., 2010) using the "rbo" function in the gespeR package of R (Schmich, 2020; Schmich et al., 2015). Smaller rbo values indicates greater difference between ranked lists and larger values indicate greater similarity. We compared our observed rbo values to rbo values obtained from randomly generating network association scores 1000 times. We retained those genes whose rbo value was significantly smaller than these randomly generated values (i.e., the proportion of rbo values as small or smaller than our observed value was less than 0.05). From these genes, we then retained those whose correlation with the environment had the opposite sign between HP and LP groups. Using this approach, we identified genes whose network membership significantly differed between HP and LP groups and whose response to the environment differed. As with our other analyses, we identified any GO or KEGG terms associated with this class of gene (hereafter: network switch genes) using g:Profiler.

2.6 | Maintenance of plasticity

To determine genes and processes associated with maintenance of plasticity, we used DESeq2 to identify genes whose expression significantly differed between carnivores and omnivores after controlling for sibship and treatment. We focused on liver and brain tissue because our treatments differed in both resource base and social environment (e.g., the differential presence of kin and the intensity of intraspecific competitors)—factors that are known to influence morph determination (Martin & Pfennig, 2009; Pfennig, 1999)—and may have especially impacted the liver and brain. As above, we used g:Profiler to identify biological process GO terms and KEGG pathway terms associated with morph differences in general and then individually for carnivore-biased (i.e., higher expression in carnivores) and omnivore-biased genes.

3 | RESULTS

3.1 | Regulation of plasticity: Differences between HP and LP groups

We first targeted those genes that differ between HP and LP groups using DESeq2. We found 67 and 45 genes that differ between groups for body and head (Table S1) samples, respectively (25 of these genes differed in both tissue types). These genes were relatively evenly split between being HP-biased and LP-biased. No GO or KEGG terms were enriched in any set of genes suggesting a possible lack of systemic pre-conditions being needed for expression of plasticity.

3.2 | Regulation of plasticity: Switch genes

The above analysis identified genes that differ between HP and LP groups and that might set the stage for downstream differences in

morph development. We also sought to identify candidate genes that govern the transition from one morph to another more directly. Here, we focused on expression differences between groups that change with environmental conditions. Our first analysis identified 46 candidate switch genes in the body and 4 candidate switch genes in the head. Of the body genes, 6 were downregulated in the HP group in cue, 33 were upregulated in LP group in the cue, and 7 were downregulated in the LP group in cue. All four of the head genes were downregulated in the HP group in the cue. Thus, we identified 10 developmental reprogramming genes (6 body, 4 head) and 40 breakdown of robustness genes (all body).

Developmental reprogramming genes in the body (i.e., genes where the HP group expression changed in the cue relative to the control and was significantly different from the LP expression level in the cue) were primarily associated with lipid homeostasis, peroxisome form, and function (i.e., peroxisome contents, organization, and metabolic and signaling activity; see Section 4), and sterol metabolism (Table 1A). Genes associated with the breakdown of buffering mechanisms in the body (i.e., genes where the LP group expression changed in the cue relative to the control and was significantly different from the HP expression level in the cue) were not enriched for GO or KEGG terms. Switch genes from the head were likewise not enriched for GO or KEGG terms.

Our second analysis of plasticity's regulation identified 77 and 21 genes in the body and head, respectively, whose expression changes across treatments correlated with the magnitude of sibship-level plasticity. For the body, most of these genes (51) had a repressive relationship such that greater expression plasticity was negatively correlated with morphological plasticity. In the head, by contrast, most of the genes (16) showed an activating relationship such that greater expression plasticity was positively associated with greater morphological plasticity. None of the genes identified here overlapped with those identified above, and there were no shared genes between sample types.

When considering activating and repressing genes together for the body, the GO term "biosynthesis of amino acids" was enriched. When only activators were considered, the GO term "farnesyl diphosphate metabolic process" was enriched. Farnesyl diphosphate is an intermediate compound in the biosynthesis of various molecules, such as sterols. When all head genes were considered, the GO term "citrate cycle (TCA cycle)" was the only one enriched. No other sets of genes yielded significant enrichment.

For our final functional (i.e., GO and KEGG) assessment of plasticity regulators, we formed three sets of switch genes for each sample type (body and head): all genes from both sets of analyses, developmental reprogramming genes plus plasticity activators, and buffering breakdown genes plus plasticity repressors. These observations largely corroborate those presented above. For the body, developmental reprogramming genes plus activator genes were associated with cholesterol/sterol biosynthesis, lipid metabolism, and peroxisome metabolic and signaling activity (Tables 1B and S2). When considering all genes in the head, the citrate cycle (TCA cycle) was enriched. No other set of genes showed enrichment of GO or KEGG terms.

TABLE 1 Top biological process gene ontology (GO) and KEGG pathway terms associated with (A) developmental reprogramming genes in the body and (B) developmental reprogramming genes plus activator genes in the body

Term category	Term name	Term ID	*P*
A. Developmental reprogramming genes (body)			
GO:BP	Lipid homeostasis	GO:0055088	0.0065
GO:BP	Negative regulation of cargo loading into COPII-coated vesicle	GO:1901303	0.0182
GO:BP	Establishment of protein localization to peroxisome	GO:0072663	0.0182
GO:BP	Peroxisomal transport	GO:0043574	0.0182
GO:BP	SREBP-SCAP complex retention in endoplasmic reticulum	GO:0036316	0.0182
GO:BP	Regulation of lipid metabolic process	GO:0019216	0.0182
GO:BP	Protein localization to peroxisome	GO:0072662	0.0182
GO:BP	Peroxisome organization	GO:0007031	0.0182
GO:BP	Protein targeting to peroxisome	GO:0006625	0.0182
GO:BP	Triglyceride metabolic process	GO:0006641	0.0236
KEGG	Fatty acid degradation	KEGG:00071	0.0008
KEGG	Tryptophan metabolism	KEGG:00380	0.0008
KEGG	Beta-Alanine metabolism	KEGG:00410	0.0008
KEGG	Peroxisome	KEGG:04146	0.0020
KEGG	Carbon metabolism	KEGG:01200	0.0035
KEGG	Histidine metabolism	KEGG:00340	0.0271
KEGG	Alpha-Linolenic acid metabolism	KEGG:00592	0.0271
KEGG	Metabolic pathways	KEGG:01100	0.0271
KEGG	Ascorbate and aldarate metabolism	KEGG:00053	0.0271
KEGG	Biosynthesis of unsaturated fatty acids	KEGG:01040	0.0271
B. Developmental reprogramming plus activator genes (body)			
GO:BP	Farnesyl diphosphate metabolic process	GO:0045338	0.0262
GO:BP	Secondary alcohol metabolic process	GO:1902652	0.0286
GO:BP	Response to vitamin E	GO:0033197	0.0286
GO:BP	Sterol metabolic process	GO:0016125	0.0286

(Continues)

TABLE 1 (Continued)

Term category	Term name	Term ID	*P*
GO:BP	Response to light intensity	GO:0009642	0.0286
GO:BP	Cholesterol metabolic process	GO:0008203	0.0286
GO:BP	Secondary alcohol biosynthetic process	GO:1902653	0.0297
GO:BP	Cholesterol biosynthetic process	GO:0006695	0.0297
GO:BP	Sterol biosynthetic process	GO:0016126	0.0332
GO:BP	Regulation of steroid biosynthetic process	GO:0050810	0.0451
GO:BP	Regulation of lipid metabolic process	GO:0019216	0.0482
KEGG	Glyoxylate and dicarboxylate metabolism	KEGG:00630	0.0176
KEGG	Tryptophan metabolism	KEGG:00380	0.0176
KEGG	Valine, leucine and isoleucine degradation	KEGG:00280	0.0176
KEGG	Beta-Alanine metabolism	KEGG:00410	0.0176
KEGG	Fatty acid degradation	KEGG:00071	0.0176
KEGG	Peroxisome	KEGG:04146	0.0343
KEGG	PPAR signaling pathway	KEGG:03320	0.0343

Note: For the full list of significant terms, see Table S2.

3.3 | Regulation of plasticity: Network switch genes

Weighted gene coexpression network (WGCNA) analysis of body samples identified 24 coexpression networks (modules). Using each gene's association with each module for both HP and LP groups, we found 176 genes whose network association and relationship with environment differed between groups (Table S3). Seventy-four of these genes had a positive relationship with environment (positive Pearson correlation from control to cue) in the HP group and the remainder (102) had a positive relationship in the LP group. Recall that we only included genes whose relationship with the environment differed in sign between the two groups. No KEGG terms were enriched, but biological process GO terms that were enriched tended to be related to morphogenesis, skeletal system development, neurogenesis, and regulation of the smoothened signaling pathway (Tables 2 and S4).

3.4 | Maintenance of plasticity

The liver had 3133 genes differentially expressed between morphs. Of these, 1490 genes were carnivore-biased and 1643 genes were omnivore-biased. As expected based on the dietary differences between morphs, various metabolic processes differ in the liver between carnivores and omnivores (Table S5). When considering KEGG terms, carnivore and omnivore livers differ not only in various aspects of metabolism, but also steroid biosynthesis, peroxisome proliferator-activated receptor (PPAR) signaling, and ribosome (Table 3A). This pattern is largely recapitulated in omnivore-biased genes with the addition of the proteasome KEGG term being enriched (Table S5). The carnivore-biased genes had no KEGG enrichment, but they did have numerous metabolic, transcription, translation, and mitochondrial GO processes enriched (Table S5).

The brain had 1348 genes that were differentially expressed between morphs. Of these, 921 genes were omnivore-biased and 427 genes were carnivore-biased. Morphs generally diverged in processes associated with translation, mRNA processing, and various cellular metabolic processes. KEGG terms for morph differences in the brain were associated with several neurological diseases such as Parkinson's disease, Alzheimer disease, and Huntington disease

TABLE 2 Top biological process gene ontology (GO) terms enriched in genes whose network association and relationship with rearing environment differed between high (HP) and low (LP) plasticity groups

Term category	Term name	Term ID	*P*
GO:BP	Embryonic skeletal system development	GO:0048706	0.004
GO:BP	Embryonic organ development	GO:0048568	0.009
GO:BP	Embryonic skeletal system morphogenesis	GO:0048704	0.012
GO:BP	Cerebellar Purkinje cell-granule cell precursor cell signaling involved in regulation of granule cell precursor cell proliferation	GO:0021937	0.012
GO:BP	Positive regulation of smoothened signaling pathway	GO:0045880	0.012
GO:BP	Skeletal system morphogenesis	GO:0048705	0.013
GO:BP	Embryonic organ morphogenesis	GO:0048562	0.017
GO:BP	Positive regulation of cerebellar granule cell precursor proliferation	GO:0021940	0.017
GO:BP	Neuron maturation	GO:0042551	0.017

Note: For the full list of significant terms, see Table S4.

TABLE 3 Significant KEGG pathway terms associated with genes showing a significant difference in expression between carnivores and omnivores in (A) the liver and (B) the brain

Term name	Term ID	*P*
A. Liver genes		
Metabolic pathways	KEGG:01100	1.02E−07
Ribosome	KEGG:03010	2.86E−06
Carbon metabolism	KEGG:01200	3.15E−03
Complement and coagulation cascades	KEGG:04610	6.12E−03
Valine, leucine and isoleucine degradation	KEGG:00280	7.31E−03
Histidine metabolism	KEGG:00340	1.10E−02
Steroid biosynthesis	KEGG:00100	1.17E−02
PPAR signaling pathway	KEGG:03320	2.24E−02
Glyoxylate and dicarboxylate metabolism	KEGG:00630	2.89E−02
B. Brain genes		
Term name		
Ribosome	KEGG:03010	7.55E−13
Parkinson disease	KEGG:05012	3.92E−10
Alzheimer disease	KEGG:05010	1.78E−09
Huntington disease	KEGG:05016	3.50E−09
Oxidative phosphorylation	KEGG:00190	3.50E−09
Nonalcoholic fatty liver disease (NAFLD)	KEGG:04932	2.36E−07
Thermogenesis	KEGG:04714	2.99E−04
Citrate cycle (TCA cycle)	KEGG:00020	7.14E−04
Carbon metabolism	KEGG:01200	1.22E−02

Note: For the full list of significant terms, see Tables S5 and S6.

TABLE 4 Top biological process gene ontology (GO) terms associated with genes showing carnivore-biased expression the brain

Term name	Term ID	*P*
Nervous system development	GO:0007399	9.25E−06
Neurogenesis	GO:0022008	2.43E−04
Generation of neurons	GO:0048699	2.43E−04
Neuron development	GO:0048666	2.43E−04
Neuron differentiation	GO:0030182	3.75E−04
Neuron projection morphogenesis	GO:0048812	6.37E−04
Cell part morphogenesis	GO:0032990	6.37E−04
Cell projection morphogenesis	GO:0048858	7.66E−04
Plasma membrane bounded cell projection morphogenesis	GO:0120039	7.66E−04
Cell morphogenesis involved in neuron differentiation	GO:0048667	1.36E−03
Anatomical structure morphogenesis	GO:0009653	2.02E−03
Cellular component morphogenesis	GO:0032989	2.02E−03
Cell morphogenesis	GO:0000902	2.02E−03
Vesicle-mediated transport in synapse	GO:0099003	5.29E−03
Regulation of anatomical structure morphogenesis	GO:0022603	5.67E−03
Positive regulation of cellular catabolic process	GO:0031331	6.67E−03
Cell morphogenesis involved in differentiation	GO:0000904	9.26E−03
Cell development	GO:0048468	9.43E−03
System development	GO:0048731	1.15E−02
Neuron projection development	GO:0031175	1.22E−02
Regulation of dendrite morphogenesis	GO:0048814	1.22E−02
Regulation of cell morphogenesis	GO:0022604	1.30E−02
Behavior	GO:0007610	1.42E−02
Trans-synaptic signaling	GO:0099537	1.42E−02
synaptic vesicle cycle	GO:0099504	1.61E−02

Note: For the full list of significant terms, see Table S6.

(Table 3B); they were also associated with ribosome and oxidative phosphorylation (Table 3B). Omnivore-biased genes showed a similar pattern, but had additional KEGG terms including spliceosome, pyruvate metabolism, and RNA degradation (Table S6). Carnivore-biased genes, in contrast, had no enriched KEGG terms, but were enriched for GO terms related to nervous system development, behavior, and synaptic signaling (Tables 4 and S6).

4 | DISCUSSION

We sought to identify the developmental basis of a complex, environmentally induced phenotype in spadefoot toad tadpoles. Our findings, together with previous work, lay the foundation for additional efforts to unravel the mechanisms governing development of this evolutionary novelty.

As noted in Section 1, environmentally induced phenotypes arise via developmental reprogramming and/or breakdown of buffering/robustness mechanisms. When comparing expression patterns between HP and LP groups before morph development, we observed the greatest number of differentially expressed genes in the LP lineages. Since these genes are responding to cues that induce the carnivore morph in HP individuals, this observation suggests that (1) those genes differentially expressed in LP individuals are involved in buffering the omnivore phenotype against environmental (dietary and/or social) change, and (2) the carnivore morph may involve, at least in part, a failure to adequately express those genes. However, these genes had diverse functions. Therefore, because these

regulators of plasticity did not appear to be coordinated—in that they did not have any unifying GO or KEGG functions—these results indicate that the dysregulation of buffering may not be an adaptive driver of plasticity, which is consistent with work in other systems (Moczek, 2007; Queitsch et al., 2002; Rohner et al., 2013; Rutherford & Lindquist, 1998; Sollars et al., 2003). In contrast, genes putatively activating carnivore development (i.e., developmental reprogramming genes and plasticity activators; Figure 1e,f; see above for operational definitions of these categories) were associated with lipid metabolism, (chole)sterol metabolism, and peroxisome organization, contents, and activity (Table 1). Since these genes were involved in a relatively unified set of processes, they may be more likely to bring about the coordinated, novel carnivore phenotype. These findings therefore suggest that adaptive plasticity might both come about through the breakdown of robustness mechanisms and by the selective refinement of this plastic response into a system capable of coordinated developmental reprogramming.

Regarding the functional processes implicated in regulating spadefoot tadpole plasticity, it is hardly surprising that we found changes to lipid metabolism were important given the dietary differences between carnivores and omnivores (Paull et al., 2012; Pfennig et al., 2006). One category of lipids that may be especially critical in morph development are sterols; specifically, cholesterol. Cholesterol is a key component of cellular membranes, but more interestingly, it is the precursor to steroid hormones (e.g., thyroid hormone, corticosterone). The thyroid hormone thyroxine has previously been shown to play a possible role in carnivore development (Pfennig, 1992b), and corticosterone has also been implicated in spadefoot morphological development (Ledón-Rettig et al., 2009). Thus, any changes in cholesterol synthesis could have dramatic effects on circulating hormones that are relevant to morph differentiation. Notably, these same changes—lipid metabolism and cholesterol and steroid biosynthesis—have also been implicated in the environmentally-induced developmental acceleration in the spadefoot *Pelobates cultripes* and are part of the evolutionary adaptation (i.e., constitutively rapid development) of another spadefoot (*Scaphiopus couchii*) to a desert environment (Liedtke et al., 2021). These similarities, plus the fact that carnivores develop more rapidly than omnivores (Pfennig, 1992a), suggest a potential link between the morphological polyphenism studied here and plasticity in developmental rate. Further, they highlight the possibility that adaptation to the same desert environment occurred in different spadefoot lineages (*Scaphiopus* vs. *Spea)* through the differential reworking of the same molecular toolkit.

Perhaps the most interesting finding is that the peroxisome might be a key organelle governing phenotypic switching in *Spea*. Peroxisomes are organelles (historically called "microbodies") found in eukaryotic cells that house diverse oxidative reactions and play important, and diverse roles in metabolism, detoxification of reactive oxygen species, and signaling (Kao et al., 2018). In general, the peroxisome is a nexus for lipid metabolism and cellular signaling (Lodhi & Semenkovich, 2014). As described above, changes to lipid processing and signaling (e.g., cholesterol and steroid hormones) are important mechanisms contributing to differences between HP and LP groups. Many of the metabolic changes (e.g., lipid metabolism, fatty acid degradation) and signaling processes underlying these differences (Table 1) may be performed by or within the peroxisome (or are related to peroxisomal function in some way; Cooper, 2000), and this suggests that the peroxisome might be a key organelle regulating phenotypic switching in spadefoots. Indeed, the KEGG term peroxisome proliferator-activated receptor (PPAR) signaling pathway was enriched in those genes implicated in active developmental reprogramming (i.e., a greater number of genes were associated with the term than expected by chance; Tables 1 and S2). PPARs are nuclear receptors whose main function is to induce proliferation of peroxisomes and enact myriad downstream effects ranging from cellular differentiation, development, metabolism, and survival (Feige et al., 2006). Activation of nuclear receptors (e.g., PPARs, thyroid hormone receptor, ecdysone receptor) may be a general feature controlling plastic development (Bui & Ragsdale, 2019). In other systems, PPARs not only regulate the expression of genes involved in fatty acid synthesis, oxidation, and storage, but they also participate in the molecular mechanism of altered metabolic homeostasis (Ferré, 2004). Indeed, PPAR signaling is an important regulator of nutritionally-induced phenotypic change.

Further implicating the peroxisome in spadefoot plasticity are the expression patterns of some key peroxisome-associated genes (i.e., CAT, ACOX1). In particular, CAT was identified as a developmental reprogramming switch gene (Table S7), and thieenzyme this gene encodes is responsible for detoxification of reactive oxygen species within the peroxisome (Lodhi & Semenkovich, 2014). We also found that a gene (ACOX1) involved in peroxisomal fatty acid beta-oxidation (FAO) was a developmental reprogramming switch gene. Peroxisomal FAO regulates whole body metabolism (Moreno-Fernandez et al., 2018), and differences in FAO in peroxisomes might be responsible for some of the physiological and metabolic variation between high and low plasticity groups.

Is such a role for peroxisomes unique to spadefoot plasticity? It is well-known that the number and size of peroxisomes, as well as their protein/enzyme concentration and composition, can be modulated by nutritional factors and environmental stimuli. For example, deficiencies in peroxisomal FAO can prevent adaptive plastic responses—dauer development—in *Caenorhabditis elegans* (Park & Paik, 2017). Similarly, oxidative stress and, by extension the peroxisome, is associated with developmental plasticity (acceleration) in other anurans. In particular, the activity of GPX and CAT (key peroxisome enzymes) is associated with developmental acceleration in the frogs *Rana temporaria* and *Pelobates cultripes* under pond desiccating conditions (Burraco et al., 2017; Gomez-Mestre et al., 2013). Likewise, peroxisomal enzyme activity changes during thyroxine-induced metamorphosis of two other frogs, *R. japonica* and *R. nigromaculata* (Kashiwagi, 1995). Peroxisome number and enzyme expression is affected by thyroxine treatment in still other frog species, *R. catesbeina* and *Xenopus laevis* (Dauça et al., 1983). Importantly, carnivore development in *S. multiplicata* tadpoles also arises via developmental acceleration and is modulated, in part, by

thyroxine (Pfennig, 1992a, 1992b). Since peroxisome form and function, lipid metabolism, and cholesterol and steroid biosynthesis are associated with both spadefoot developmental acceleration and ecomorph polyphenism (see above; e.g., Liedtke et al., 2021), and since environmentally dependent developmental acceleration is a common form of plasticity in diverse amphibian species (Denver, 1997), it is possible that the carnivore morph in *Spea* arose, at least partially, through co-option of this already existing machinery.

When we identified candidate switch regulating genes based on their association with coexpression modules rather than differences in expression level between treatment groups, we found that morphogenic processes—skeletal system development and neurogenesis—were enriched (Tables 2, S3, and S4). More narrowly, we found that the smoothened signaling pathway was enriched in these genes. The smoothened pathway regulates hedgehog signaling and affects transcription of downstream genes by activating expression of Gli transcription factors (Arensdorf et al., 2016). While the smoothened receptor itself was not identified as a network switch gene, both its ligand (sonic hedgehog [SHH]) and ultimate target (the transcription factor glioma-associated onocogene [GLI1]) were identified as network switch genes (Table S3). Related to the above discussion of cholesterol, hedgehog signaling seems to transduce to the smoothened receptor by modulating the receptor's cholesterylation and thereby enabling downstream signal transduction and transcription, and ultimately, promoting developmental change (Huang et al., 2016; Xiao et al., 2017). Moreover, thyroid hormone (a steroid hormone derived from cholesterol) induces SHH signaling and enables intestinal modification in the frog *Xenopus laevis* (Hasebe et al., 2012). This might suggest an additional mechanism by which changes in cholesterol synthesis could give rise to the alternative morphs in *S. multiplicata*. Namely, differences in cholesterol levels (which can be modified by resource use) might differentially activate SHH signaling via the smoothened pathway and lead to downstream morphological changes.

Finally, we also compared expression differences between well-developed omnivores and carnivores in an effort to discern what genes might be responsible for maintaining these two alternative morphs. Unsurprisingly, we found that various aspects of metabolism differed between carnivore and omnivore livers (Table 3). However, steroid synthesis and PPAR signaling also differed. These findings tie mechanisms of morph *maintenance* to mechanisms of morph *induction* (see above), suggesting that the same mechanisms leading to morph production are also necessary for morph maintenance. Such a pattern is not necessarily expected: the molecular "switch" shunting development into alternative phenotypes often does so through activation of different gene regulatory networks that are relatively independent of the switch itself (Projecto-Garcia et al., 2017). Direct manipulation of dietary factors and/or pharmacological treatments might be useful to determine the extent to which the switch mechanism and phenotype-specific gene regulatory networks overlap and interact.

Regarding the brain, we found that omnivore-biased genes were associated with several neurodegenerative diseases (Tables 3 and S6). We also found that carnivore-biased genes were associated with nervous system development, synaptic signaling, and behavior (Table 4). Such changes in brain gene expression are common in organisms encountering a dynamic or enriched environment (Abbey-Lee et al., 2018; Chesler & Williams 2004; Rampon et al., 2000) and are generally consistent with previous work on brain gene expression between spadefoot morphs (Ledón-Rettig, 2021). Our results suggest that the individuals that have more to gain from effectively navigating such a dynamic environment—carnivores—may do so by expanding their nervous system (presumably the brain) and increasing their activity to facilitate changes in behavior accompanying a predatory lifestyle (Ledón-Rettig, 2021; Pfennig, 1999; Pfennig & Murphy, 2000). The prevalence of neurological disease terms in omnivore-biased genes further supports differences between morphs in cognitive function and capacity to navigate a complex environment with diverse resources and socially antagonistic interactions. As with the liver, pharmacological treatment may be a fruitful future avenue for parsing these differences.

While the above patterns and processes certainly contribute to spadefoot developmental plasticity, they are likely not the only ones relevant to this plastic response. Additional switch genes may have gone undetected in our study for at least three reasons. First, the timing of sampling and tissues we sampled were chosen because of their presumed relevance to spadefoot plasticity and were necessarily incomplete when considering the entire sequence of morph development. Thus, our limited sampling in time and across tissues may have caused us to miss additional regulators of plasticity. Second, since we cannot be 100% sure that the tadpoles we sampled before morph development would actually go on to develop into a particular morph, these samples likely harbored more noise in gene expression than "pure" (e.g., carnivore-only) lines would have. This too might have obscured additional relevant genes or pathways. Third, our observed changes in development potentially involved restructuring gene regulatory networks, not just changes in gene expression. An important future direction is to identify carnivore and omnivore gene regulatory networks and determine how their topologies differ across environments and across tissues. Thus, our findings, while not the whole story, provide an important foundation for additional work in this system.

In sum, our results provide important insights into the mechanistic basis of a complex, environmentally induced phenotype: the distinctive carnivore morph found in tadpoles of the genus *Spea*. We found that lipid metabolism, (chole)sterol synthesis, and peroxisome organization and activity are important regulators of carnivore plasticity. We also found that, as suspected, metabolic and cognitive differences characterize the alternative morphs. At the same time, this study serves as a springboard for further work exploring the developmental and evolutionary bases of trophic plasticity in this and perhaps similar systems that produce similar alternative environmentally induced phenotypes.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Nicholas A. Levis, Ian M. Ehrenreich, and David W. Pfennig conceived the study, Nicholas A. Levis, Daniel J. McKay, and David W. Pfennig designed the study, Patrick W. Kelly, Emily A. Harmon, and David W. Pfennig performed the experiment, Emily A. Harmon collected morphological data, Nicholas A. Levis performed tissue processing and sample submission, Nicholas A. Levis analyzed the data and wrote the initial manuscript. All authors contributed to the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

Raw sequencing reads have been deposited in the National Center for Biotechnology Information Sequence Read Archive under Bioprojects PRJNA675835 (body and head) and PRJNA675144 (liver and brain).

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