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Genetic accommodation in the wild: evolution of gene expression plasticity during character displacement

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Abstract

Ecological character displacement is considered crucial in promoting diversification, yet relatively little is known of its underlying mechanisms. We examined whether evolutionary shifts in gene expression plasticity ('genetic accommodation') mediate character displacement in spadefoot toads. Where *Spea bombifrons* and *S. multiplicata* occur separately in allopatry (the ancestral condition), each produces alternative, diet-induced, larval ecomorphs: omnivores, which eat detritus, and carnivores, which specialize on shrimp. By contrast, where these two species occur together in sympatry (the derived condition), selection to minimize competition for detritus has caused *S. bombifrons* to become nearly fixed for producing only carnivores, suggesting that character displacement might have arisen through an extreme form of genetic accommodation ('genetic assimilation') in which plasticity is lost. Here, we asked whether we could infer a signature of this process in regulatory changes of specific genes. In particular, we investigated whether genes that are normally expressed more highly in one morph ('biased' genes) have evolved reduced plasticity in expression levels among *S. bombifrons* from sympatry compared to *S. bombifrons* from allopatry. We reared individuals from sympatry vs. allopatry on detritus or shrimp and measured the reaction norms of nine biased genes. Although different genes displayed different patterns of gene regulatory evolution, the combined gene expression profiles revealed that sympatric individuals had indeed lost the diet-induced gene expression plasticity present in allopatric individuals. Our data therefore provide one of the few examples from natural populations in which genetic accommodation/assimilation can be traced to regulatory changes of specific genes. Such genetic accommodation might mediate character displacement in many systems.

Introduction

Darwin (1859 (2009)) first proposed that the origin of species, and the evolution of trait differences between them, stem ultimately from divergent natural selection that minimizes competitive interactions between initially similar populations. Such trait evolution that arises as an adaptive response to resource competition between species – a process now dubbed 'ecological character displacement' – can thereby explain how new species arise, diversify and coexist (Schluter, 2000; Dayan & Simberloff, 2005; Grant & Grant, 2008; Pfennig & Pfennig, 2012b). Yet, ecological character displacement's underlying proximate mechanisms remain poorly understood (Pfennig & Pfennig, 2012a).

At the mechanistic level, ecological character displacement has traditionally been assumed to arise solely through an evolutionary change in the frequencies of underlying genotypes or alleles (Taper & Case, 1985; Doebeli, 1996; Schluter, 2000; Dayan & Simberloff, 2005; for an empirical example, see Lamichhaney *et al.*, 2016). Indeed, one of the widely cited criteria for demonstrating ecological character displacement is that the phenotypic change must be shown to reflect a *genetic* change (Schluter & McPhail, 1992). However,

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ecological character displacement need not arise through changes in DNA coding sequence. Instead, it might alternatively be mediated by phenotypic plasticity.

To understand how ecological character displacement could arise through phenotypic plasticity, consider that many species can respond adaptively to interspecific competition by facultatively altering resource-use traits (reviewed in Pfennig & Pfennig, 2012b). These induced responses can instigate rapid, widespread and adaptive divergence between competing species (West-Eberhard, 2003; Galloway & Etterson, 2007; Turcotte & Levine, 2016). Ecological character displacement might therefore arise when selection favours the evolution of 'reaction norms' that lessen interspecific competition (where a 'reaction norm' refers to the set of phenotypes expressed by a single genotype under different environmental conditions; sensu Schlichting & Pigliucci, 1998).

The above two proximate mechanisms are not mutually exclusive, however, and they might often act in concert to promote the evolution of ecological character displacement (Pfennig & Pfennig, 2012b). Indeed, character displacement might often evolve from an initial phase in which trait divergence is environmentally induced to a later phase in which this divergence becomes genetically canalized (Wilson, 1992). Such a process might come about if: (1) underlying genetic variation exists in the tendency or manner in which individuals respond to interspecific competition (i.e. if different genotypes exhibit different reaction norms); (2) selection acts on this variation and, by promoting quantitative genetic changes, refines these induced resource-use traits over time (a process known as 'genetic accommodation'; sensu West-Eberhard, 2003); and (3) under recurrent selection to minimize interspecific competition, such environmentally induced resource-use traits eventually evolve to become 'fixed' in the population. In other words, resource-use traits might undergo an extreme form of genetic accommodation known as 'genetic assimilation' (sensu Waddington, 1953) in which ancestral plasticity is lost. This loss of plasticity, and subsequent fixation of the favoured trait through genetic assimilation, can proceed via at least two routes. First, when maintenance or expression of plasticity is costly (Snell-Rood *et al.*, 2010; Murren *et al.*, 2015), selection can actively eliminate it, causing the favoured phenotype to be fixed in the population. Second, plasticity can be lost through mutational degradation or genetic drift (Masel *et al.*, 2007), as might occur when nonfavoured phenotypes are seldom expressed and thereby experience relaxed selection (Kawecki, 1994; Whitlock, 1996; Van Dyken & Wade, 2010). Regardless of how it comes about, genetic assimilation provides a mechanism whereby character displacement could evolve from an initial phase in which trait divergence is environmentally induced to a later phase in which this divergence is genetically fixed. Although this 'plasticity-first' hypothesis for the evolution of character displacement has garnered increasing empirical support (Pfennig & Pfennig, 2012b), little is known of the underlying mechanisms by which plasticity is gained or lost (Sikkink *et al.*, 2014; Ehrenreich & Pfennig, 2016; Levis & Pfennig, 2016).

A plausible mechanism of plasticity-first evolution is via evolutionary changes in the degree of plasticity in gene expression – specifically, in amount of gene product (Gilbert & Epel, 2015). Gene expression is often environment-specific (Aubin-Horth & Renn, 2009; Scoville & Pfrender, 2010; Snell-Rood *et al.*, 2010; Gunter *et al.*, 2013; Morris *et al.*, 2014; McCairns *et al.*, 2016). Moreover, evolutionary shifts in gene expression are increasingly viewed as important in mediating population divergence (Pavey *et al.*, 2010; Pfennig *et al.*, 2010; Thibert-Plante & Hendry, 2011; Morris *et al.*, 2014). If selection is persistent and coarse-grained (where each individual encounters the same selective environment), then formerly induced differences in gene expression might ultimately become fixed.

Spadefoot toads are ideal for exploring these issues. Spadefoots of the genus *Spea* normally produce alternative, diet-induced, larval ecomorphs that utilize different dietary resources: 'omnivores,' which eat mostly detritus (and are the default morph), and 'carnivores,' which are induced by, and specialize on, shrimp and other tadpoles (Pfennig, 1990, 1992; Levis *et al.*, 2015). In addition to differing in diet, these two morphs differ in morphology (carnivores are larger than omnivores in body size and have proportionally larger jaw muscles, a more serrated keratinized beak and a shorter gut); development rate (carnivores develop faster and achieve metamorphosis sooner than omnivores); and behaviour (carnivores are solitary, active swimmers, whereas omnivores are gregarious, sluggish swimmers; Bragg, 1965; Pomeroy, 1981).

In the south-western U.S.A., Plains spadefoot toads, *Spea bombifrons*, and Mexican spadefoot toads, *S. multiplicata*, have undergone ecological character displacement with each other, potentially via genetic accommodation/assimilation (Pfennig & Murphy, 2000, 2002, 2003; Pfennig *et al.*, 2007; Rice *et al.*, 2009). Specifically, in regions where they occur alone (i.e. allopatry), *S. bombifrons* maintains plasticity to produce both omnivores and carnivores. In contrast, in regions where *S. bombifrons* co-occurs with *S. multiplicata* (i.e. sympatry), selection to minimize interspecific competition for detritus has caused *S. bombifrons* to become nearly fixed for producing carnivores only. Indeed, even when reared in the laboratory, *S. bombifrons* tadpoles from sympatric populations are more carnivore-like than conspecifics from allopatric populations from birth (D. Pfennig; unpubl. data). Because these two species have come into secondary contact (Rice *et al.*, 2009), allopatry (where carnivore–omnivore plasticity is present) represents the ancestral condition, whereas

sympatry (where this plasticity has been nearly lost) represents the derived condition.

Two lines of evidence indicate that this shift in morph production and plasticity constitutes ecological character displacement; that is, that competitively mediated selection has caused this divergence. First, controlled experiments in the laboratory reveal that individual tadpoles that are the most similar in resource use to the other species have the lowest fitness when engaged in interspecific competition for food, suggesting that selection would disfavour these individuals in sympatric populations (Pfennig *et al.*, 2007). Second, estimates of selection in the wild reveal that, in contrast to allopatry (where disruptive selection favours both morphs within each species), in sympatry directional selection favours only carnivores in *S. bombifrons* (Pfennig *et al.*, 2007; Martin & Pfennig, 2009, 2010, 2012).

The shift in levels of plasticity from allopatry to sympatry (i.e. ecological character displacement) appears to reflect genetic accommodation. Specifically, experiments have shown that plasticity-mediated shifts in ancestral (allopatric) populations mirror the more highly canalized trait differences observed in derived (sympatric) populations that have undergone character displacement. In these experiments, allopatric *S. bombifrons* that were experimentally exposed to *S. multiplicata* facultatively produced mostly carnivores (Pfennig & Murphy, 2000, 2002), which mirrors ecomorph production among *S. bombifrons* in natural sympatric populations. *Spea bombifrons* produce more carnivores in the presence of *S. multiplicata*, because they are more effective at capturing and consuming shrimp (Pfennig & Murphy, 2000), which induces the carnivore ecomorph. Thus, in sympatric populations of *S. bombifrons*, an environmentally induced ecomorph (the carnivore ecomorph) has been converted into more highly canalized version of this morph through an evolutionary adjustment in regulation of trait expression. In other words, character displacement appears to have arisen through genetic accommodation generally and genetic assimilation specifically (Pfennig & Murphy, 2000, 2002; Pfennig & Martin, 2010).

Among the likely molecular targets of character displacement in this system are genes associated with these two ecomorphs, especially those that are normally expressed more highly in one ecomorph than in the other. Such genes should be particularly prone to experience genetic accommodation, because the more frequently a gene is expressed phenotypically (and, thereby, exposed to selection), the stronger the selection on that gene (Roff, 1996; West-Eberhard, 2003 p. 169). Thus, in a population where a particular ecomorph is recurrently favoured (e.g. the carnivore morph in sympatric populations of *S. bombifrons*), any gene regulating the form or frequency of that ecomorph should undergo genetic accommodation, regardless of whether it is a 'switch' gene (i.e. a gene that determines morphotype) or a 'downstream' gene (i.e. a gene involved in morphotype functionality). Essentially, genetic accommodation of a complex phenotype should occur at numerous loci, as genes encoding diverse functions become finely tuned by selection to produce a phenotype well adapted to local environmental conditions (Hodgins-Davis *et al.*, 2012; Gunter *et al.*, 2013; Pfennig & Ehrenreich, 2014; Schneider *et al.*, 2014).

Leichty *et al.* (2012) identified 25 such biased genes in *Spea* (in these experiments, *S. bombifrons* tadpoles were reared under common conditions on a diet of shrimp only; thus, any differences in gene expression were associated with different trophic morphologies *per se* and not dietary differences). These genes were classified either as 'carnivore-biased' (if they had significantly higher expression in carnivores than in omnivores) or 'omnivore-biased' (if they had significantly higher expression in omnivores than in carnivores). Based on what is known about the functions of these 25 genes (see Leichty *et al.*, 2012), most (if not all) are likely crucial in ecomorph *functionality*. Therefore, these candidate genes can be targeted by selection to ultimately result in genetic accommodation as carnivore–omnivore plasticity undergoes adaptive evolution during character displacement.

Here, we used the spadefoot system to evaluate whether evolutionary shifts in gene expression plasticity accompany – and possibly mediate – the observed character displacement. We specifically sought to test the prediction that morph-biased genes have undergone evolutionary shifts in gene expression plasticity in *S. bombifrons* inhabiting sympatric (derived) populations vs. allopatric (ancestral) populations. As we describe below, our results validated this prediction, suggesting that genetic accommodation of gene expression plasticity might play a general and important role in mediating character displacement.

Materials and methods

Study subjects

We bred six pairs of *S. bombifrons* that had been collected in the wild in the south-western USA and that had been part of an established laboratory colony at the University of North Carolina, Chapel Hill, for 1–2 years. Three pairs of adults had been collected from several populations near Willcox, Arizona. This is a region in which *S. bombifrons* does not co-occur with *S. multiplicata* (representing allopatry), and in which *S. bombifrons* are under selection to produce both omnivores and carnivores (Pfennig *et al.*, 2007). The other three pairs were collected from several populations near Rodeo, New Mexico. This is a region in which *S. bombifrons* does co-occur with *S. multiplicata* (representing sympatry), and in which S. *bombifrons* are under selection to produce only carnivores (Pfennig *et al.*, 2007). Indeed, tadpoles from these sympatric populations have nearly

lost the plasticity to produce both omnivores and carnivores (Pfennig & Martin, 2010). The two collection sites (Willcox and Rodeo) are about 80 km apart. Importantly, these are the main two sites in which the aforementioned studies of ecological character displacement had been conducted.

Breeding was induced by injecting the adults with 70 μL luteinizing hormone releasing hormone agonist (Sigma L2761) and leaving the pairs overnight in nursery tanks. The resulting tadpoles were fed crushed fish food *ad libitum* until they were 8 days old (fish food simulates in form and nutrition the detritus on which *Spea* feed in natural ponds; see Pfennig *et al.*, 2006). At that point in time, a subset of tadpoles was selected from each family and divided haphazardly into one of two diet treatments.

Experimental design

We utilized a common garden design to determine whether *S. bombifrons* from allopatry vs. sympatry (hereafter, different 'selective environments' or 'origins') have evolved differences in the expression of biased genes. To do so, we assigned 10 tadpoles from each family into one of two diet treatments: crushed fish food (hereafter, 'detritus') alone or shrimp and detritus. Tadpoles were reared individually, and detritus-fed tadpoles received 10 mg of detritus every other day and shrimp + detritus-fed tadpoles received 5 mg of detritus every other day as well as 20 live adult brine shrimp twice daily (brine shrimp simulate the fairy shrimp on which wild carnivores feed). After 2 weeks, we selected ~ 3 tadpoles per family per diet treatment (a total of 9 tadpoles per treatment from each selective environment), placed them for 30 s in a 0.1% aqueous solution of tricaine methanesulfonate (MS-222), and immediately flash froze them in liquid nitrogen. These tadpoles were then stored at −80 °C until homogenization (~ 2–3 months). Thus, our samples consisted of 2–4 tadpoles (three on average) per family from six families from two diet treatments ($N = 36$). Based on data from previous, similar experiments, tadpoles likely had an SVL of ~ 13 mm, a mass of ~ 0.45 g, and were approximately Gosner stage 36–37.

RNA extraction and cDNA synthesis

Based on a modified version of the protocol by Leichty *et al.* (2012), we extracted total RNA from whole tadpoles using a combination of TRIzol Reagent and the Ambion PureLink RNA Mini Kit (ref: 12183025). All samples were submerged in 1 mL TRIzol Reagent and homogenized with a rotor stator tissue homogenizer and refrozen at −80 °C until RNA extraction 3 years later. Full details of our RNA extraction protocol are provided in the supplemental material. Following extraction and treatment with DNase, we visually evaluated RNA quality on a denaturing TAE agarose gel according to Masek *et al.* (2005) and determined RNA purity and concentration using a NanoDrop 2000 (Thermo Scientific) (Table S1). For samples of adequate quality and purity, we reverse transcribed 600 ng of total RNA using the Bio-Rad iScript Reverse Transcription Supermix for RT-qPCR (Cat. # 1708841). Reverse transcription reactions consisted of 4 μL iScript RT Supermix, 600 ng of total RNA and enough nuclease-free water to bring the total reaction volume to 20 μL. Reactions ran according to the manufacturer's protocol.

RT-qPCR

We focused on nine biased genes plus one, unbiased, 'control' gene (Table 1). We selected these nine genes from the 25 total biased genes identified by Leichty *et al.* (2012), because these nine genes represented various functional groups. Specifically, these nine genes

Table 1 The nine biased genes (plus a nonbiased control gene) examined in this study, along with the model that best predicted each gene's pattern of expression and that model's effect size.

Gene	Symbol	Bias	Functional group	Best model†	R^2‡
Basic transcription factor 3	*Btf3*	Carnivore	Gene regulation	Diet*Origin	0.264
t-box transcription factor TBX15-like	*Tbx15*	Carnivore	Gene regulation	Diet	0.172
Collagen, type II, alpha 1	*Col2a1*	Carnivore	Structural	Null	0.000
Collagen alpha-1(IX) chain	*Col9a1*	Carnivore	Structural	Null	0.000
Peptidase M20 domain containing	*Pm20d2*	Carnivore	Metabolism	Diet + Origin	0.285
Pancreatic triacylglycerol lipase-like	*Pnlip*	Omnivore	Metabolism	Diet	0.139
Amylase, alpha 2A (pancreatic)	*Amy2a*	Omnivore	Metabolism	Null	0.000
mug1 protein	*Mug1*	Omnivore	Immunity	Diet	0.158
Peptidoglycan recognition protein 1	*Pglyrp1*	Omnivore	Immunity	Null	0.000
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	None (control)	Metabolism	—	—

†See methods for a discussion of the interpretation of the various models; 'Origin' refers to the selective environment of the focal tadpole's parents (allopatry or sympatry).
‡R^2 refers to the marginal effect size (i.e. R^2 of only fixed effects) of the best supported model; calculated using the function 'sem.model.fits' from the package 'piecewiseSEM' in R (sensu Nakagawa & Schielzeth, 2013; Johnson, 2014).

have been implicated in regulatory (*Btf3* and *Tbx15*), metabolic (*Pm20d2*, *Pnlip* and *Amy2a*), structural (*Col2a* and *Col9a*) and immunity (*Mug1* and *Pglyrp1*) functions.

We performed RT-qPCR on these 10 genes using 20 μL reactions of the Bio-Rad iTaq Universal SYBR Green Supermix (Cat. # 172–5121) and its recommended cycle conditions for a standard run on a StepOnePlus thermocycler (Applied Biosystems cat. #: 4376600). Melt curve analysis was also performed for each well to evaluate primer specificity. Reaction components, conditions and primer sequences are provided in Tables S2–S4, respectively. When possible, we used intron-spanning primers to reduce or eliminate the possibility of any nuclear DNA amplification during PCR.

Our plate set-up used a sample maximization design. Specifically, for each gene, we ran five individual tadpoles per treatment on one plate. The remaining four individuals per treatment were run on a second plate (one 'calibrator' individual per treatment from the first plate was also run on the second plate). Interplate calibration (Hellemans *et al.*, 2007) was performed using GenEX version 6 (MultiD Analyses AB). Each tadpole was run in triplicate, and each plate contained no reverse transcription, no template and no SYBR controls in duplicate. For statistical analysis, we omitted readings that the machine called outliers (a replicate that is significantly smaller or larger than the others) or produced large standard deviation warnings (Cq standard deviation > 0.5). We excluded individuals with fewer than two valid technical replicates.

We normalized our qPCR data according to Rieu & Powers (2009). Specifically, we first determined the relative quantity (RQ) of each sample mean from the formula $RQ = \frac{1}{2^{Cq}}$ where Cq is the quantification cycle (i.e. the cycle where the threshold level of fluorescence is met according to instrument defaults). We then normalized RQ values by dividing them by the RQ of a reference gene (glyceraldehyde-3-phosphate dehydrogenase or *GAPDH*). We then took the log base 2 ($\log_2$) of these normalized values to obtain values (termed Cq') that were used for subsequent analysis. Cq' is the relative quantity of gene product normalized to an endogenous control whose expression is invariant across treatments.

Statistical analysis

For each gene, the relationship among diet, selective environment (i.e. origin: sympatry vs. allopatry), and expression level was evaluated using linear mixed-effects models fitted with maximum likelihood in the lme4 package of R (Bates *et al.*, 2014). 'Diet' and 'Origin' (i.e. selective environment) were fixed categorical variables and 'Family' was a random effect. We compared a null model that contained only the random effect to single-factor models that retained the random effect and included either diet or origin as a fixed effect, and to two-factor models (with and without an interaction term). A model was called the 'best model' if it had the lowest AICc value and a likelihood ratio test (using the function 'anova' in R) indicated that it was significantly better than the null model (Table S5). The biological interpretation of each model is discussed below.

If there is no diet-dependent plasticity in gene expression and there has been no evolution of gene expression between selective environments, then the null model is considered the best fit. In contrast, if diet alone is the best model, then detritus- and shrimp-fed tadpoles have different levels of gene expression (i.e. there is diet-dependent plasticity in gene expression), but there have been no evolved changes in plasticity between selective environments. If any of the remaining models is deemed the best, then this would indicate an evolved shift in gene expression owing to selection – that is, genetic accommodation has occurred (sensu West-Eberhard, 2003; for a discussion of genetic accommodation in the context of gene expression, see Aubin-Horth & Renn, 2009; Renn & Schumer, 2013). Specifically, if origin is the best predictor, then this would indicate that overall expression is different between selective environments, but there is no diet-dependent plasticity in gene expression. The additive model (containing both diet and origin as fixed effects) being the best would show that there is parallel, diet-dependent plasticity in gene expression in both selective environments, but that the overall expression in the derived environment has evolved to be greater or less than in the ancestral environment. Finally, if the model containing the interaction between diet and origin is the best, then this would indicate nonparallel reaction norms between selective environments that may or may not have differences in elevation. Essentially, this is the catch-all model for any evolved change in the direction and magnitude of gene expression plasticity. As one example, if tadpoles from the allopatric (ancestral) selective environment show diet-dependent plasticity in gene expression, but tadpoles from the sympatric (derived) selective environment do not, then this would indicate evolution by genetic assimilation. Note, however, that genetic assimilation is only one of several alternative patterns of evolved gene expression associated with genetic accommodation of the carnivore ecomorph (see Fig. 1).

In addition to these tests on individual genes, we performed a comprehensive model selection procedure by constructing fifteen mixed-effects models to predict amount of gene expression (Cq'). These models contained individual as a random effect (to account for repeated measures on the same individual) and all possible combinations of the fixed effects 'gene identity', 'diet' and 'origin' (i.e. selective environment). We fitted models with maximum likelihood, calculated AICc as above and performed a likelihood ratio test (using the

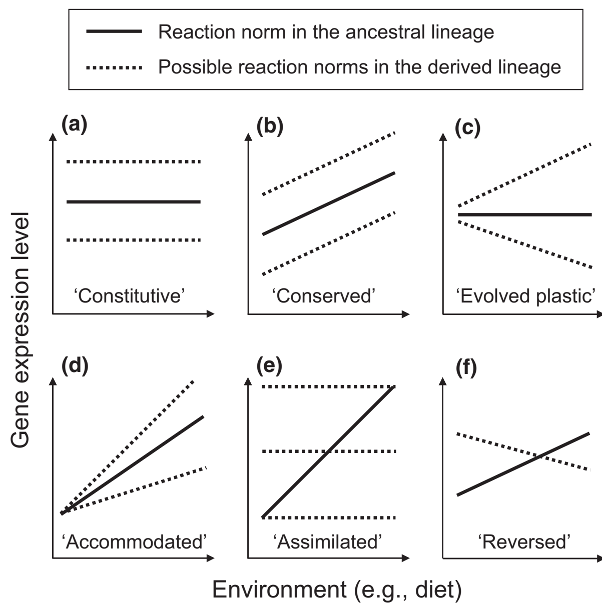

Fig. 1 Alternative hypothesized patterns of evolved gene expression associated with genetic accommodation of an ancestrally plastic phenotype. Each panel depicts the reaction norms of ancestral (solid line) and derived lineages (dashed line [s]). Note that, within each category, the derived lineage(s) might exhibit more than one possible reaction norm. Modified from Renn & Schumer (2013).

function 'anova') to determine how each model performed relative to a null model containing no fixed effects. The best model was selected as above.

We then evaluated the importance of diet on overall gene expression between the two selective environments. For these tests, we expected reduced sensitivity to diet differences for individuals from sympatry because tadpoles from this selective environment do not utilize alternative food resources as often as individuals from allopatry and, therefore, selection might have acted to canalize their patterns of gene expression. We performed a principal component analysis (using the function 'prcomp' in the 'stats' package in R) on a cross-correlation matrix of all family independent Cq′ prime values to obtain a composite metric of each individual's gene expression (i.e. PC1 and PC2; hereafter: 'gene expression profile'). No varimax rotation was used.

We first tested the expectation that individuals from the sympatric selective environment have reduced sensitivity to diet by generating 99% confidence ellipses around the gene expression profiles for the different diet treatments and comparing the number of individuals in the region of overlap between the allopatric and sympatric selective environments in JMP Pro (version 12.0.1). The density ellipses are computed from the bivariate normal distribution fit to the X and Y variables (i.e. PC1 and PC2, respectively). The bivariate normal density is a function of the means and standard deviations of the X and Y variables and the correlation between them. In short, the ellipses show where a given percentage of the data is expected to lie, assuming the bivariate normal distribution. Tadpoles found in the ellipse of only one diet treatment were considered to have more diet-dependent gene expression than individuals who were found in the region of overlap between ellipses. We used Fisher's exact test to determine whether the number of tadpoles with diet-dependent (i.e. only in one ellipse) versus diet-independent (i.e. in both ellipses) was significantly different in allopatry than in sympatry.

For each selective environment, we then performed 1000 iterations of randomized residual permutation procedure (RRPP; Collyer *et al.*, 2015) to determine whether our diet treatments differed in their gene expression profile in R (version 3.1.2). Briefly, RRPP acts as a nonparametric version of an ANOVA with a post hoc test by extracting the residuals of a null model, randomly pairing them with fitted values, and using these pseudorandom data to calculate pairwise distances using the full model. By repeating this process 1000 times, we were able to determine the probability of finding differences equal to or greater than our observed distances. This procedure generates an *F*-statistic that is the ratio of error variance between the reduced and a full model and the error variance of the full model, which quantifies the variation explained by the inclusion of additional variables.

Results

Our analyses revealed that our nine biased genes differed in how their expression level was affected by diet and selective environment (Table 1). For two genes (*Pm20d2* and *Btf3*), we found both an effect of diet as well as evidence of evolved shifts in gene expression between selective environments. In other words, for these two genes, we found evidence of genetic accommodation. However, this genetic accommodation was manifested in different ways in each of these genes. For *Pm20d2* (a carnivore-biased gene) both selective environments showed the expected pattern of diet-dependent expression, but individuals from sympatry had lower overall expression than individuals from allopatry (Fig. 2). By contrast, *Btf3* (also a carnivore-biased gene) had the expected diet-dependent expression pattern among individuals from only one of the two selective environments (i.e. allopatry). In other words, for this gene, there was a significant *interaction* between diet and selective environment. This interaction was driven by an increased expression on shrimp than on detritus among individuals from allopatry, but no such diet-dependent difference in expression among individuals from sympatry; that is, the diet-dependent plasticity present in the ancestral allopatric populations was lost in the derived sympatric populations.

For three additional genes (*Tbx15*, *Pnlip* and *Mug1*), diet was the only significant predictor of expression level. For two of these genes, the diet-induced expression patterns were consistent with the prior morph-biased designations of Leichty *et al.* (2012). In particular, *Tbx15* (a carnivore-biased gene) showed higher expression levels in tadpoles that were fed shrimp, whereas *Pnlip* (an omnivore-biased gene) showed higher expression levels in tadpoles that were fed detritus alone. By contrast, for *Mug1* (an omnivore-biased gene), the diet-induced expression patterns were *opposite* of that predicted: this gene showed a higher expression level in tadpoles that were fed shrimp compared to tadpoles that were fed detritus only. Thus, *Mug1* showed a pattern suggestive of a *carnivore*-biased gene, not an omnivore-biased gene (Fig. 3).

Finally, for the remaining four genes (*Amy2a, Col2a1, Col9a1* and *Pglyrp1*), we found that expression level did not differ between diets or selective environments. For these genes, the null model was the best fit. However, for *Col2a1*, the diet model was nearly significantly the best: this model had the lowest AICc value and was nearly significantly different from the null ($\chi^1_2 = 3.4696$, $P = 0.06251$).

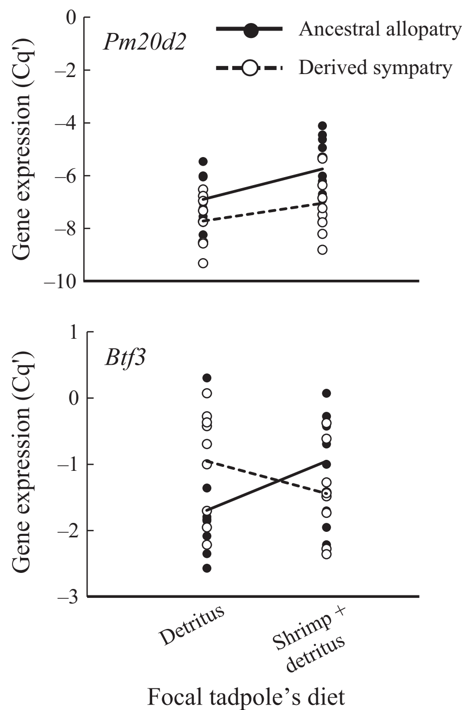

Fig. 2 Reaction norm plots for two carnivore-biased genes showing evidence of genetic accommodation: *Pm20d2* (top) and *Btf3* (bottom). The reaction norms were parallel with different elevations for *Pm20d2* (compare to Fig. 1b); the derived reaction norm was statistically flat for *Btf3* (compare to Fig. 1e). Points represent values for individual tadpoles.

Our comprehensive model selection procedure largely corroborated these observations. The top model indicated that there was a significant gene*diet interaction (Table S7). As noted above, different genes responded to diet in different ways. In addition, the second best model also had the gene*diet interaction term, but included the origin (i.e. selective environment: allopatry or sympatry) term as well. This model had a ΔAICc

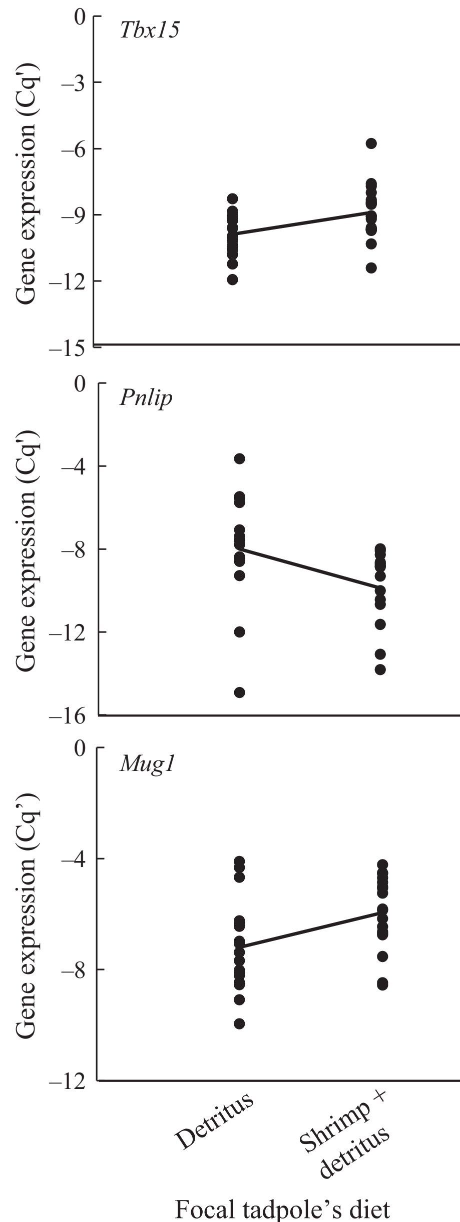

Fig. 3 Diet-induced plasticity in gene expression for a carnivore-biased (*Tbx15*) and two omnivore-biased (*Pnlip* & *Mug1*) genes. *Mug1* was the only one of these genes whose diet-induced expression levels were opposite of that predicted. Points represent values for individual tadpoles; both selective environments are pooled for each diet.

from the top model of 0.819 and was also significantly better than the null model (Table S7). This also corroborates the above observations because only two of our genes (*Pm20d2* and *Btf3*) showed any significant effect of origin. If more genes had a significant effect of origin, then this model (or the 3-way interaction model) would likely have had greater support.

We also evaluated whether the gene expression profile of tadpoles from sympatric (derived) populations had evolved to be less sensitive to diet than tadpoles from allopatric (ancestral) populations. In constructing each individual tadpole's gene expression profile (see Methods), we found that PC1 and PC2 explained 44.39% and 22.93% of the variation, respectively. Our first assessment revealed that diet had a greater influence on gene expression profile of tadpoles from the ancestral allopatric populations than tadpoles from the derived sympatric populations (Fisher's exact $P = 0.0178$). Specifically, whereas 40% of tadpoles from allopatric populations had gene expression profiles falling in the 99% confidence ellipse of only one diet treatment (i.e. they were diet-specific), none of the gene expression profiles of tadpoles from sympatric populations were in a single ellipse (i.e. they were diet-independent; Fig. 4).

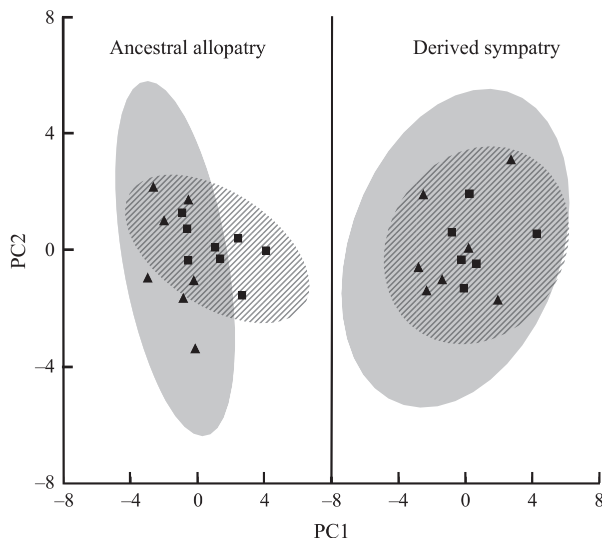

Fig. 4 Gene expression profiles and 99% confidence ellipses for individual tadpoles from allopatry (left) vs. sympatry (right) reared on a diet of detritus vs. shrimp + detritus. Each filled triangle and square represent the gene expression profile for individual tadpoles reared on a diet of detritus or shrimp and detritus, respectively; grey shading and diagonal line stippling represent the 99% confidence ellipses for tadpoles reared on a diet of detritus and shrimp and detritus, respectively. The gene expression profile for 6 of 15 (40%) tadpoles from allopatry was outside of the region of overlap between diet treatments. Conversely, none of the tadpoles (0/13) from sympatry had gene expression profiles outside the region of overlap between diet treatments.

Our additional test had similar results: the gene expression profiles of tadpoles from allopatric populations reared on different diets were significantly different ($F_{1,13} = 5.1862$, $P = 0.02$), but the gene expression profiles of tadpoles from sympatric populations were not significantly different ($F_{1,11} = 0.7792$, $P = 0.445$). For reference, diet-related differences in gene expression profile separated out primarily along PC1 (8/9 genes loaded heavily with PC1), and selective environment differences were primarily along PC2 (5/9 genes loaded heavily with PC2) (Table S6). The differences due to diet appeared greater than those between selective environments. These data suggest that tadpoles from the derived sympatric populations have lost the diet-induced gene expression plasticity present among tadpoles from the ancestral allopatric populations.

Discussion

We evaluated the 'plasticity-first' hypothesis for the evolution of character displacement (Pfennig & Pfennig, 2012b), which holds that character displacement evolves from an initial phase in which trait divergence is environmentally induced to a later phase in which divergence undergoes genetic assimilation (sensu Waddington, 1953). We did so by exploring the evolution of gene expression plasticity in natural populations of spadefoot toads, *Spea bombifrons*, that have undergone character displacement with a congener, possibly via genetic assimilation (see Pfennig & Martin, 2010). Using this system, we asked whether evolved shifts in gene expression plasticity mirror shifts in production of alternative ecomorphs (i.e. omnivores and carnivores) during character displacement. We found that, individually, different genes displayed different patterns of gene regulatory evolution, including genetic assimilation. However, the combined gene expression profiles revealed that individual tadpoles from the derived, sympatric populations had indeed lost the diet-induced gene expression plasticity present in individual tadpoles from the ancestral, allopatric populations. In other words, the *overall* gene expression profiles showed evidence of genetic assimilation. Our data therefore: (1) provide one of the few examples from natural populations in which genetic accommodation/assimilation can be traced to regulatory changes in specific genes; and (2) support the 'plasticity-first' hypothesis for the evolution of character displacement. We discuss these results in greater detail below.

The main goal of our study was to determine whether any of the candidate genes we used showed evidence of evolved shifts in gene expression reaction norms (as in Alaux *et al.*, 2009; Scoville & Pfrender, 2010; Morris *et al.*, 2014; Ghalambor *et al.*, 2015). Specifically, we evaluated whether any of the previously identified carnivore and omnivore-biased genes (Leichty *et al.*, 2012) showed an evolutionary increase

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or decrease in gene expression plasticity. We found evidence of such a molecular signature of genetic accommodation in two of the nine biased genes that we examined. However, these two genes differed from each other in how this genetic accommodation was manifested (for a classification scheme of different patterns of gene regulatory evolution that might contribute to genetic accommodation of a derived phenotype, see Fig. 1).

In particular, the carnivore-biased gene *Pm20d2* has apparently evolved *'conserved' gene expression plasticity* (sensu Renn & Schumer, 2013), but at a reduced level in the sympatric (derived) selective environment (compare Fig. 2a with Fig. 1b). By contrast, the carnivore-biased gene *Btf3* has apparently undergone *genetic assimilation*. For individuals from populations representing the ancestral condition (allopatry; see Rice & Pfennig, 2008), this gene showed diet-dependent plasticity in gene expression: it was expressed more highly among individuals reared on both shrimp and detritus than among individuals reared on detritus alone (Fig. 3). However, this same gene was environmentally insensitive among individuals whose parents were from sympatry: in these derived populations, it was fixed at mid-level compared to its ancestral plastic expression in allopatric populations (compare Fig. 2b with Fig. 1e).

It is unclear why these two genes have evolved different reaction norms (i.e. undergone different forms of genetic accommodation), even though both are associated with the *same* complex phenotype undergoing genetic assimilation (the distinctive carnivore ecomorph). One possible answer is that these genes might lie at different positions along the gene regulatory network (GRN). Although the exact signalling pathway leading to the alternative ecomorphs in *Spea* remains to be determined, morph development likely begins with signal reception and physiological changes induced by diet (e.g. gut cell proliferation; Ledón-Rettig *et al.*, 2008) and then proceeds through a signal cascade of transcription factors and hormones (Pfennig, 1992; Boorse & Denver, 2003; Ledón-Rettig *et al.*, 2008, 2009, 2010). Although the nine biased genes that we examined might only play a functional role in the fully developed ecomorph – and not in triggering a particular ecomorph's development – they likely lie at different points in the same GRN and might, therefore, be subject to different patterns of selection. Indeed, studies of other systems have found similar opposing patterns of gene expression reaction norms during genetic accommodation (e.g. Scoville & Pfrender, 2010; Matzkin, 2012), and these differences have also been attributed to GRN complexity (Alaux *et al.*, 2009; Scoville & Pfrender, 2010; Snell-Rood *et al.*, 2010; Hodgins-Davis *et al.*, 2012).

In the present case, *Pm20d2*, a carnivore-biased metabolic gene encoding a protein with a peptidase domain (carnivores consume more protein that omnivores), might have evolved reduced overall expression if selection has increased the efficiency with which these proteins break down peptides (which would presumably require fewer peptidases). This is certainly possible as a key prediction of genetic accommodation is that phenotypes should experience increased adaptive refinement (i.e. functional improvement) in derived lineages that are more frequently exposed to selection (e.g. our sympatric populations; West-Eberhard, 2003; Levis & Pfennig, 2016). The carnivore-biased transcription factor *Btf3* might be important for regulating expression of other carnivore-biased genes. The loss of diet-induced plasticity in gene expression in this gene might ensure that tadpoles in the sympatric selective environment develop into carnivores, regardless of their early diet. Such a loss of diet-induced plasticity in gene expression is likely favoured in sympatry, given that directional selection strongly favours the carnivore phenotype among the *S. bombifrons* in this selective environment (Pfennig *et al.*, 2007). Further study is needed to explain the exact role these genes play in carnivore ecomorph function and why they evolved these particular expression patterns.

In addition to evidence of genetic accommodation – and even of genetic *assimilation* – in specific genes, we found evidence that character displacement has also led to an *overall* pattern of genetic accommodation (and potentially genetic assimilation) for the *combined* gene expression in sympatric (derived) populations (Figs 2 and 4). First, there were no individuals from sympatric populations whose gene expression profiles were unique to a particular diet (Fig. 4). Moreover, the mean (centroid) gene expression profiles for each diet were not significantly different in the sympatric populations (Fig. S1). These patterns are in contrast to allopatric populations, where diet-dependent differences in gene expression profile were present. Thus, overall, individuals from the derived, sympatric populations seem to have lost the diet-induced gene expression plasticity present in ancestral allopatric populations, just as we had predicted (see Introduction).

Lastly, as part of this study, we also sought to determine whether diet-dependent shifts in gene expression were present in carnivore- and omnivore-biased genes, and in the direction predicted. Specifically, because omnivores in the wild eat abundant detritus (Paull *et al.*, 2012), we expected to find that the omnivore-biased genes that we examined would be expressed more highly when our tadpoles were fed a detritus diet. By contrast, because carnivores in the wild eat mostly shrimp (Paull *et al.*, 2012), we expected to find that the carnivore-biased genes that we examined would be expressed more highly on the shrimp diet. As shown in Table 1, we found evidence for these predicted diet-dependent shifts in gene expression in four genes: three carnivore-biased genes (*Btf3*, *Tbx15* and *Pm20d2*) and one omnivore-biased genes (*Pnlip*). Interestingly, these

genes have been implicated in regulatory (*Btf3* and *Tbx15*) or metabolic (*Pm20d2* and *Pnlip*) functions. By contrast, we found either no such diet-dependent plasticity in gene expression (*Col2a*, *Col9a*, *Amy2a* and *Pglyrp1*) or plasticity in the opposite direction of our expectations (*Mug1*). These genes have been implicated in structural (*Col2a* and *Col9a*), metabolic (*Amy2a*) or immunity (*Mug1* and *Pglyrp1*) functions. Although the significance (if any) of these differences between genes belonging to different functional groups is unclear, future studies should examine whether genes belonging to certain functional categories are more likely to be environmentally responsive (e.g. see Aubin-Horth & Renn, 2009; Hodgins-Davis & Townsend, 2009; Snell-Rood *et al.*, 2010).

In sum, our data thereby provide one of the few examples in which genetic accommodation/assimilation in natural populations can be traced to regulatory changes of specific genes (see also Scoville & Pfrender, 2010). This finding is significant, because, despite evidence for genetic assimilation in the laboratory (e.g. Waddington, 1953; Walworth *et al.*, 2016), its relevance in natural populations has been questioned (Orr, 1999; Wray *et al.*, 2014). Although there are a growing number of possible examples of genetic assimilation from the wild (Badyaev, 2005; Aubret & Shine, 2009; Schwander & Leimar, 2011; Diggle & Miller, 2013; Schlichting & Wund, 2014; Levis & Pfennig, 2016), studies have only recently begun to explore the mechanisms underlying genetic assimilation in natural populations (Badyaev, 2009; Scoville & Pfrender, 2010; Rohner *et al.*, 2013; Martin *et al.*, 2016; Parsons *et al.*, 2016; Schrader *et al.*, 2016). Our data suggest that genetic assimilation of gene expression plasticity might be more common in mediating adaptive evolution than is generally appreciated.

More generally, if genetic assimilation is relatively common (Schwander & Leimar, 2011), then it might be relevant in many cases of ecological character displacement. Indeed, a scenario in which ecological character displacement has evolved from an initial phase in which trait divergence was environmentally induced to one in which divergence became genetically canalized might explain many well-known examples of character displacement, including in *Anolis* lizards (Losos *et al.*, 2000; Losos, 2009), sticklebacks (Schluter & McPhail, 1992; Wund *et al.*, 2008), Darwin's finches (Grant & Grant, 2006; Lamichhaney *et al.*, 2016) and African cichlids (Parsons *et al.*, 2016). Further studies are needed to determine whether such a plasticity-first scenario (and, hence, genetic assimilation) is indeed a common pathway to character displacement.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Concentration and purity values from RNA extractions of each tadpole.

Table S2 Reaction components for qPCR.

Table S3 Reaction components for qPCR.

Table S4 Genbank accessions, Gene ID, and qPCR primer sequences for each gene.

Table S5 Results from our model selection procedure for each gene.

Table S6 Loadings of each gene's expression onto PC1 and PC2 of the gene expression profile.

Table S7 Results from our comprehensive model selection procedure.

Figure S1 Distribution of gene expression profiles for individuals (small shapes) and treatment groups (large shapes).

Data S1 RNA Extraction Protocol.

Data deposited at Dryad: https://doi.org/10.5061/dryad.r0mg6

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