

Evolution of resource generalism via generalized stress response confers increased reproductive thermal tolerance in a pest beetle

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Generalism should be favoured evolutionarily when there is no genetic constraint or loss of fitness across alternative environments. However, evolution of generalism can require substantial evolutionary change, which can confer a general stress response to other aspects of the environment. We created generalist lineages from an ancestral, resource-specialized laboratory population of seed beetles (*Callosobruchus maculatus*) by rearing lines over 60 generations on a mixture of both ancestral and novel host species to test for costs associated with the evolution of generalism involving evolutionary changes in gene expression and correlated phenotypic responses during a shift to generalism. Evolved lines had higher fitness on the novel resource, with no loss of fitness on the ancestral resource, indicating that they overcame initial fitness trade-offs. This involved upregulation of major stress response (heat shock protein) genes and genes coding for metabolic enzymes, suggesting an underpinning metabolic and physiological cost. Resource generalist populations also evolved greater thermal tolerance breadth, highlighting that the evolution of resource generalism might pre-adapt species to respond favourably to other environmental stressors, following selection for generalized stress response gene upregulation. The rapid gain of novel hosts during a pest invasion might also confer greater thermal resilience to ongoing climate change.

ADDITIONAL KEYWORDS: acetyl-CoA carboxylase – dietary niche breadth – generalist – genetic assimilation – heat shock protein – phenoloxidase – pyruvate carboxylase – specialist.

INTRODUCTION

Ecological generalization that leads to increased niche breadth (increased environmental tolerances) (Futuyma & Moreno, 1988; Holt, 2009) might provide considerable advantage in transitioning between or coping with changing, heterogeneous or variable environments, and reduces the chance of extinction (Levins, 1968; Gilchrist, 1995; Schluter, 2000). A generalist might evolve either by expressing a fixed phenotype that functions in diverse conditions or by expressing a plastic phenotype that responds flexibly to changing conditions (Via & Lande, 1985; Lynch & Gabriel, 1987; Gabriel & Lynch, 1992; Gomulkiewicz & Kirkpatrick, 1992; Gavrillets & Scheiner, 1993). In theory, owing to trade-offs among finding, obtaining, processing or competing over different types of

resources, genotypes that perform over a broader range of environments will perform worse in the optimal environment; that is, a jack of all environments is a master of none (Lynch & Gabriel, 1987). Thus, when environmental conditions are constant and homogeneous and when competition for resources is strong, lineages should evolve to be specialists (MacArthur & Levins, 1964; Kassen, 2002). However, in changing, heterogeneous or variable environments, selection should favour generalists that perform well over a wide range of conditions (Levins, 1968; Gilchrist, 1995; Schluter, 2000).

Although links between environmental heterogeneity and the evolution of generalism have been validated theoretically many times (MacArthur & Levins, 1964; Levins, 1968; Van Tienderen, 1991; Abrams, 2006; Chevin & Lande, 2010), there is less empirical evidence for these transitions and how they occur mechanistically (Forister *et al.*, 2012). Elucidating the links between genotype, gene expression, phenotype

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and environment should help us to gain a better understanding of the fundamental evolutionary processes underpinning generalization. Evolutionary shifting in the regulation of gene expression is a key candidate mechanism by which generalism can be achieved (Celorio-Mancera *et al.*, 2013; Snell-Rood *et al.*, 2013); in the case of dietary niche, different food sources can vary substantially in nutritional content and defence compounds and impose varying degrees of nutritional stress; thus, suites of genes associated with various biochemical pathways are predicted to be under selection to be expressed differentially in lineages regularly using alternative resources. This might include induction of enzymes for toxin metabolism (e.g. cytochrome 450s and P-glycoproteins; Keppler & Ringwood, 2001; Li *et al.*, 2007). Therefore, by characterizing shifts in gene expression responses associated with evolutionary shifts to generalism, we can gain a better understanding of the underlying genetic mechanisms and how these might impact correlated evolutionary or plastic responses to other aspects of the environment.

A complete understanding of the distinction between specialists and generalists requires evaluation along multiple dimensions of environmental and resource conditions (Peers *et al.*, 2012). Adaptation to a novel resource or thermal regime might simultaneously affect performance in other environmental regimes, including resources that the population might never have encountered (Martins *et al.*, 2014). Few studies have addressed the issue of the evolution of generalism in multiple environmental regimes. More examples are needed to determine whether evolution of, for example, resource or thermal generalism is mediated typically by highly specific changes in target traits or whether evolved changes involve a more generalized phenotypic response that enables organisms also to generalize to other environments. For example, generalization might involve shifts in the expression of genes involved in stress resistance, such as heat shock proteins (HSPs), which can confer resistance to multiple environmental pressures of temperature stress, oxidative stress and some toxins (Telemeco *et al.*, 2019; Sikkink *et al.*, 2020). The upregulation of stress resistance under selection in one environmental dimension might pre-adapt lineages for multidimensional niche evolution across different aspects of environmental stress (Sørensen *et al.*, 2003; Snell-Rood *et al.*, 2018). Such correlated evolutionary changes during transitions to generalism can have large but currently understudied implications for the predictability of correlated niche evolution along other dimensions of the environment, and thus the distribution and environmental responses of species in real-world, multidimensional environments. Understanding the nature of

generalism in phytophagous insects is crucial for the control of pest species and for the elucidation of the macroevolutionary mechanisms of speciation and diversification (Celorio-Mancera *et al.*, 2016).

In this study, we aimed to test whether individuals become resource generalists during adaptation to a multiple-resource environment and whether this involves overcoming trade-offs in the ability to use multiple resources successfully. We also explore the phenotypic changes accompanied by plastic, evolved or no changes in expression of metabolic or stress response genes, and furthermore, whether these genetic changes in regulatory gene expression during the evolution of generalism confer a general stress response to other aspects of the environment. To test this, we used laboratory populations of the model organism *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae: Bruchinae), which is a global crop pest on stored legumes, with multiple host switches and associated geographical range expansions reported in the wild (Tuda *et al.*, 2014). We created a replicated set of generalist populations from an ancestral, specialized lineage for 60 generations, and then conducted a common garden experiment to test fitness parameters in the different evolved and developmental rearing conditions and across two niche dimensions: resource and temperature. We used gene expression profiling to compare levels of candidate genes among these different evolved and rearing conditions. This combined approach allowed us to test how different physiological pathways are involved in resource generalization, and whether and how these physiological shifts are correlated with evolved or plastic changes in phenotypes and fitness.

MATERIAL AND METHODS

SOURCE POPULATION

Callosobruchus maculatus is a globally relevant crop pest that is spreading poleward under current global warming, facilitated by the global legume trade (Tuda *et al.*, 2014). Females attach eggs singly to the surface of the bean. Larvae hatch and burrow into the bean, where they complete development and only emerge as adults. Thus, larvae are unable to move between resources during their feeding stage (Messina & Durham, 2013). The *C. maculatus* beetles used in this experiment were sourced from Niamey, Niger and have been maintained in an outbred laboratory population on a diet of cowpeas (*Vigna unguicularis*), at a constant 27 °C thermal regime and 35% relative humidity with a 12 h–12 h light–dark photoperiod, for 19 years or ~300 generations (Eady *et al.*, 2000; Price *et al.*, 2017; Paul Eady, personal communication). Given the region

of ancestry and long-term rearing conditions, our beetles are therefore well adapted to these constant, narrow resource conditions and laboratory conditions that approximate its 'natural' habitat (Messina & Gompert, 2017).

EXPERIMENTAL ESTABLISHMENT OF HOMOGENEOUS AND HETEROGENEOUS LINES

To select experimentally for resource generalism, we created five replicate outbred lines, each placed in containers (10 cm × 14 cm) with a mix of two host species: 50 g of the ancestral host (cowpeas) and 50 g of a novel host, dried peas (*Pisum sativum*). Fifty grams of resources amounted to ~250 beans of cowpeas and ~250 beans peas. Five replicate control lines were also created and offered only 100 g of cowpeas. Shifting to peas exerts moderate selection on beetles from our stock population. Pilot studies suggested that total offspring produced by a population introduced onto peas was 49 ± 0.06 (SE) adult offspring produced per female after two generations, compared with 67.9 ± 2.83 (SE) offspring per female on cowpeas.

Within each of our study lineages, we allowed adaptation to the multiple-host environment to evolve for 60 generations under quasi-natural selection, as a consequence of differential fitness of animals developing in the two potential host species. Each generation was provided with new resources of the same quantity and proportion as the previous generations, and the used legumes were removed only every two generations; this allows for different emergence times from the two types of legumes. New resources were mixed but kept separated from the resources of previous generations by placing them in adjacent plastic trays within each container from the resources of previous generations. Females were required to disperse between plastic trays within the colony (a distance of only a few centimetres) to move from their emergence legume to the new resources. Given that *C. maculatus* is generally extremely active, individuals were very likely to disperse over this distance. Dispersing adults would lay eggs and then die within a few days after colonizing the new resources, where the next generation of adults would emerge after a few weeks. Starting a new generation with a sample of colonized legumes, rather than a sample of emerged adults, ensured that we were not imposing inadvertent direct selection on development time or indirect selection on other, correlated traits. Population density was not controlled during the experiment (i.e. no live beetles were ever removed from the system except those laid on spent beans, although this was rare); therefore, the limited available resources were expected to impose strong selection to colonize the novel resource type. Control lines were created using

the same method as the experimental lines, using only the ancestral resource.

PHENOTYPIC VARIATION ASSAY FOR LIFE-HISTORY TRAITS

After 40 months (~60 generations) of rearing on either the ancestral host (cowpea) or the evolved line (mixed cowpea with pea), we conducted a common garden experiment to assess whether changes in fitness occurred on each host type and whether such changes were evolved or plastic. For this, 20 virgin males and 20 virgin females were isolated from each resource type in each replicate line and mated to individuals emerging from the same resource type and line but not the same individual legume (for the cowpea lines, $N = 20$ pairs per replicate line were obtained from cowpeas; for the evolved lines, $N = 20$ pairs were taken from each of the resource types; Fig. 1). Half of these ($N = 10$ virgin pairs per resource type per line) were then placed on the ancestral resource type to complete reproduction, and the other half ($N = 10$) were placed on the novel legume ($N = 400$ pairs total; Fig. 1), including individuals from the control lines, which were reared for one generation

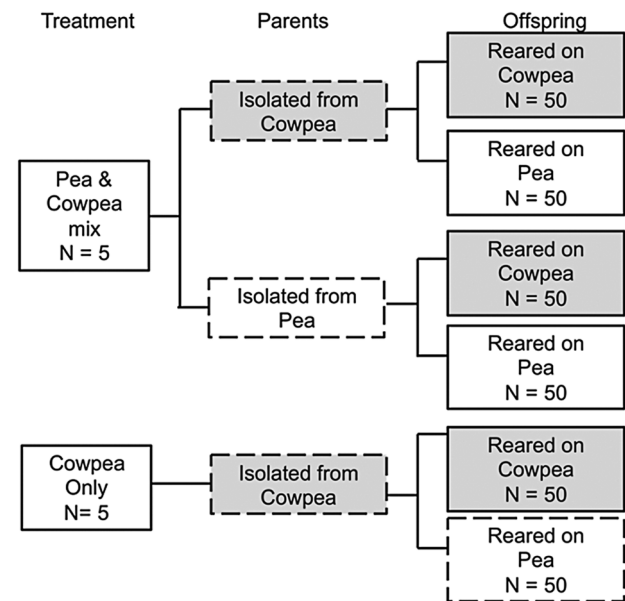


Figure 1. Two-generation rearing design used to evaluate evolved and rearing host effects in two evolved treatments i.e. adaptation to the multiple-host environment (pea and cowpea) or maintained on the ancestral cowpea for 60 generations (five replicate lines each) of the seed beetle *Callosobruchus maculatus*. For gene expression studies, six larvae were extracted from cowpeas and six larvae from peas within each line of the evolved treatments (dashed boxes). Pairs of beetles were also removed from treatments (dashed boxes) and reared on cowpeas at either 35 or 27 °C.

on peas, to facilitate a fully factorial design. We then counted the total number of offspring resulting from each pair. Given that *C. maculatus* has distinct generations, we waited until the offspring produced had died to ensure that we counted the total number of offspring produced over the lifespan of the females.

A second common garden experiment was conducted to assess the effect of evolved line and developmental resource on thermal performance of reproduction, for three of the evolved lines and three control lines. Virgin beetles were isolated from cowpeas and peas within the pea–cowpea mix treatment, and from cowpeas within the cowpea-only lines and from peas after individuals from the control lines were reared for one generation on peas (Fig. 1, dashed lines). The virgin beetles were paired, and their production of offspring (on cowpeas) was tested under two temperature regimes, the ancestral 27 °C and a novel 35 °C ($N = 120$ pairs). We then counted the number offspring that emerged from each pair in the alternative thermal conditions. The temperature of 35 °C was chosen because pilot studies suggest that although it was higher than their optimal temperature (27 °C), hence they exhibited the expected loss of fitness, it was in the upper limit of the thermal tolerance of the species (Leonard AM & Lancaster LT, unpublished data).

GENE EXPRESSION ASSAY: RNA EXTRACTION, COMPLEMENTARY DNA SYNTHESIS AND QUANTITATIVE PCR

Candidate genes were identified from the published literature as those considered to have specific metabolic and defence functions required for the development and survival of insects that grow and feed on starch- and lipid-containing seeds. This approach provides an accessible and useful tool for providing insights into the mechanisms of evolved or developmental dietary shifts, in addition to evolutionary insights, and reveals key details about their potential role in resource generalism. Candidate genes included genes coding for enzymes involved with regulation of carbohydrate metabolism [Acetyl-CoA carboxylase (*ACAC*)] (Grossi De Sa & Chrispeels, 1997; Franco *et al.*, 2002) and lipid metabolism [Pyruvate carboxylase (*PYC*)] (Crabtree *et al.*, 1972); an enzyme involved in cellular defence, detoxification and immunity [Phenoloxidase (*PO*)] (González-Santoyo & Córdoba-Aguilar, 2012); and genes for chaperone proteins associated with protection from thermal and oxidative stress [Heat shock proteins 70 and 90 (*HSP70* and *HSP90*)] (King & MacRae, 2015). Furthermore, HSPs prevent dangerous aggregations of denatured proteins in the presence of multiple types of stressors (e.g. heat, desiccation and caloric restriction) (Telemecco *et al.*, 2019).

Tissue collection

Beetle larvae from each replicate line were extracted from cowpeas and from peas within the pea–cowpea mix treatment, in addition to being isolated from cowpeas within the cowpea-only lines and from peas after individuals were forced through one generation on peas (Fig. 1). Larvae were size matched at a late instar (~3 mm) to ensure a comparable developmental stage and were immediately frozen at –70 °C until molecular analysis. Six larvae per evolved/developmental condition were pooled within resource types for each replicate line for later RNA extraction. In total, this resulted in $N = 20$ pools of six larvae distributed across all four combinations of evolved and developmental conditions ($N = 5$ replicate pooled samples per treatment, with each sample corresponding to one of the five replicate lines).

RNA extraction

RNA was extracted from each pool of six larvae using a Trizol–chloroform method, following the manufacturer's protocol (ThermoFisher Scientific) (Lynch *et al.*, 2016). Pooled larvae were homogenized in 1 µL of Trizol to extract the RNA. Two hundred microlitres of chloroform was added, and samples were centrifuged at 4 °C for 20 min at 13.5×1000 r.p.m. to separate the RNA, DNA and protein. The aqueous RNA layer was isolated, and 500 µL of isopropanol was added to each pooled sample of RNA, which was centrifuged again at 4 °C for 20 min at 13.5×1000 r.p.m. Pure RNA was again centrifuged with 500 µL of 75% ethanol, and the RNA was re-suspended in 30 µL of DNase-free water. Extracted RNA was analysed for quality and quantity using Nanodrop spectrophotometry (ThermoFisher Scientific), and concentrations were adjusted using RNase-free water to achieve 2.0 µg per sample. Complementary DNA (cDNA) was synthesized using 2.0 µg total RNA in a 20.0 µL reaction with an oligo(dT) primer, according to the instructions provided by nanoScript2 Reverse Transcription Kit (Primerdesign, London, UK). Complementary DNA was stored at –20 °C until its use in quantitative PCR.

Quantitative PCR

Complementary DNA was prepared for quantification by combining 5 µL of cDNA solution with 10 µL of iQ Sybr Green fluorescent dye (Bio-Rad, UK), 4 µL RNase-free DNase-free water and 1 µL of primer mix. Primers (Table 1) were designed to target *ACAC*, *PO*, *PYC*, *HSP70* and *HSP90*. An 18S reference gene primer (Wang *et al.*, 2019) was used as the internal control because it does not change between treatments (effect of F1 resource = -0.73 ± 0.72 SE, $t_{1,10} = -1.02$, $P > 0.05$; effect of F0 resource = -0.79 ± 0.72 SE, $t_{1,10} = -1.10$, $P > 0.05$). For *ACAC* and *PYC*, we used

Table 1. Forward and reverse sequences for primers used and the temperature at which they were annealed according to the quantitative PCR protocol

Gene	Forward	Reverse	Annealing temperature (°C)	Reference
<i>HSP70</i>	CGTTCGACATCGACGCCAA	TGGTGATGGTGATCTTGTTCTCC	58	This study
<i>HSP90</i>	CAGAGGGAAGACAGACTTCAGAG	CAGTGACAAGATGAATGGAATAGCA	60	This study
<i>PO</i>	GGTGTATTGATATACCTGTGCAC	CGAACCACGCCCTTTAGG	60	This study
<i>ACAC</i>	TGCTGTTGCTCTTGCTGCTGTT	GTGGCGTTCTGGAACCTGAAGG	60	Cui <i>et al.</i> (2019)
<i>PYC</i>	CGTGGGTGTGGATGTGAATGGG	GACGAGCTGGTGAAGGCTGGTA	60	Cui <i>et al.</i> (2019)
18S	ATGGTTGCAAAGCTGAAACT	TCCCGTGTGAGTCAAATTA	60	Wang <i>et al.</i> (2019)

published primer sets previously developed for *Callosobruchus* (Cui *et al.*, 2019), and for *PO*, *HSP70* and *HSP90*, we commissioned primers to be designed by Primerdesign (using sequences obtained from the reference transcriptome (Sayadi *et al.*, 2016), with assistance in obtaining upstream sequences by Ahmed Sayadi, based on their assembly (Sayadi *et al.*, 2019).

Quantitative PCRs were performed with 2.0 µL of cDNA, primer and Sybr Green solution using a Bio-Rad Laboratories CFX96 system with the following conditions: denaturing of cDNA (95 °C for 30 s), followed by 39 cycles of 95 °C for 30 s annealing of primers at their annealing temperatures for 30 s (see Table 1 for temperatures) and cDNA extension at 72 °C for 30 s. The number of cycles until amplification, detected by Sybr Green fluorescence, determined the cycle thresholds (cT). At the melting curve specificity stage, the temperature was reduced to 55 °C for 5 s, then gradually increased by 0.5 °C every second until it reached 95 °C. Assessment of the temperature(s) at which the cDNA denatures will verify whether the qPCR has amplified one specific product. After the final PCR cycle, all reactions were subjected to melting curve analyses to determine the quality and specificity of each reaction. Real-time PCR Miner Software (Zhao & Fernald, 2005) was used to calculate the PCR efficiencies (*E*) and cycle thresholds (*C_T* values) from the raw fluorescence data. In accordance with MIQE guidelines (a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments) (Bustin *et al.*, 2009), samples with *E* values < 0.8 or > 1.2 were excluded from subsequent analyses. All qPCR runs were performed in technical duplicate, and *C_T* values were averaged within technical duplicates. The fold expression of the genes was then calculated in relationship to the average cycling times of 18S rRNA reference gene using the formula $2^{\Delta\Delta C_T}$ (relative to the reference gene, 18S) (Lynch *et al.*, 2016).

STATISTICAL ANALYSIS

Evolution of resource generalism

We ran two sets of models to evaluate the evolution of generalism. First, we tested for the effect of evolved history on fitness and fitness trade-offs. For this, we evaluated the total number of offspring as a function of evolved treatment i.e. adaptation to the multiple-host environment (pea and cowpea) (vs. controls), offspring developmental resource type (ancestral or novel) and their interaction, as fixed effects. All parents were isolated from cowpeas to facilitate comparison of evolved and control lines. We next tested for fitness trade-offs within the evolved lines in a common garden to understand the role of phenotypic plasticity of the ability of a generalist population to produce offspring in a heterogeneous environment. We evaluated the number of offspring produced (lifetime reproductive success) as a function of parental developmental resource (i.e. the legume type from which each mated pair emerged), offspring developmental resource (i.e. the legume type that mated females were provided on which to lay their eggs), and the interaction of parental and offspring resource environments. To test how developmental resource and evolved resource treatment impacted reproductive thermal tolerance, we modelled offspring production (on the ancestral resource) as a function of evolved treatment, parent developmental resource type, temperature and their interaction, as fixed effects. For all tests, general models were applied using the *Lme4* (v.1.1-25) (Bates *et al.*, 2015) and *LmerTest* (v.3.1-3) (Kuznetsova *et al.*, 2017) packages for R (v.3.5.0) (Bates *et al.*, 2015; R Core Team, 2013) with a quasi-Poisson error structure, because a Poisson generalized linear model revealed an overdispersion parameter value of $1 < \theta > 15$. The replicate experimental line was included as a fixed effect in each model. Additive and interactive models

were compared using the Akaike information criterion (Burnham & Anderson, 1998).

Further analyses were run to test for the effect of evolved treatment on the number of offspring produced in peas and cowpeas separately, by running generalized linear models on the data subset by developmental resource, with evolved treatment and replicate line as fixed effects. All post hoc models were fitted with replicate line as a fixed effect and a quasi-Poisson error distribution to account for overdispersion in the data.

Gene expression

Gene expression levels (fold change) were \log_{10} -transformed for each locus to meet the assumption of normality. Five separate models were run for gene expression levels of each locus. To assess for evolved effects vs. effects of developmental plasticity on gene expression patterns, we fitted fixed effects for the experimental treatment (evolved vs. control) and the resource type (cowpea vs. pea) from which larvae were extracted, in addition to the interaction between evolved treatment and developmental resource type. We used linear models because each replicate line was represented once in the analysis. When the interaction between evolved treatment and developmental conditions was

not significant, it was removed from the model, and only direct effects of these variables are reported.

RESULTS

EVOLUTION OF RESOURCE GENERALISM

We found a significant positive interaction between offspring developmental resource type and evolved treatment (parental developmental resource was cowpea for both evolved treatments) on the number of offspring produced (Table 2). Assessing the effect of evolved treatment separately within each offspring developmental resource type revealed that our main result stemmed primarily from an evolved ability to produce more offspring from peas in the evolved lines, but no change in the ability to produce offspring from cowpeas between evolved and control lines (effect of evolved treatment on number of offspring produced from peas: 0.44 ± 0.21 SE, $t_{2,98} = 2.01$, $P < 0.05$; effect of evolved treatment on offspring produced from cowpeas: -0.04 ± 0.08 SE, $t_{2,98} = -0.49$, $P = 0.63$; Table 2; Fig. 2B). There was another significant main effect of offspring developmental resource type, in that more offspring emerged from cowpeas irrespective of the evolved treatment (Table 2).

Table 2. Fixed effects of the best-fitting model explaining total offspring produced by F1 individuals as a function of evolved line (evolved lines vs. control, cowpea-only lines) and F1 resource type (cowpea vs. pea; all F0 individuals were isolated from cowpeas)

Fixed effects	Estimate*	SE	<i>t</i> -value	Pr(> <i>t</i>)
Offspring developmental resource (pea)	-1.35	0.16	-8.29	< 0.001
Treatment (evolved)	-0.04	0.11	-0.38	> 0.05
Offspring developmental resource × treatment	0.48	0.21	2.24	< 0.05

*Full model reported where interaction is significant, otherwise interaction is dropped and main effects only reported.

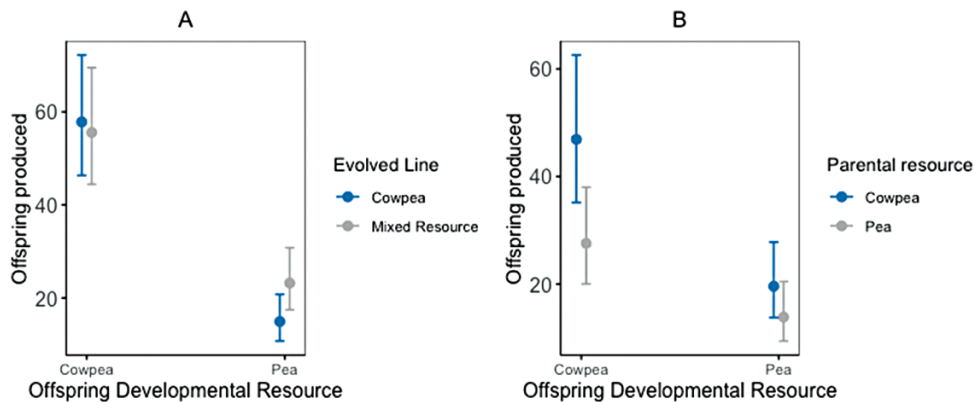
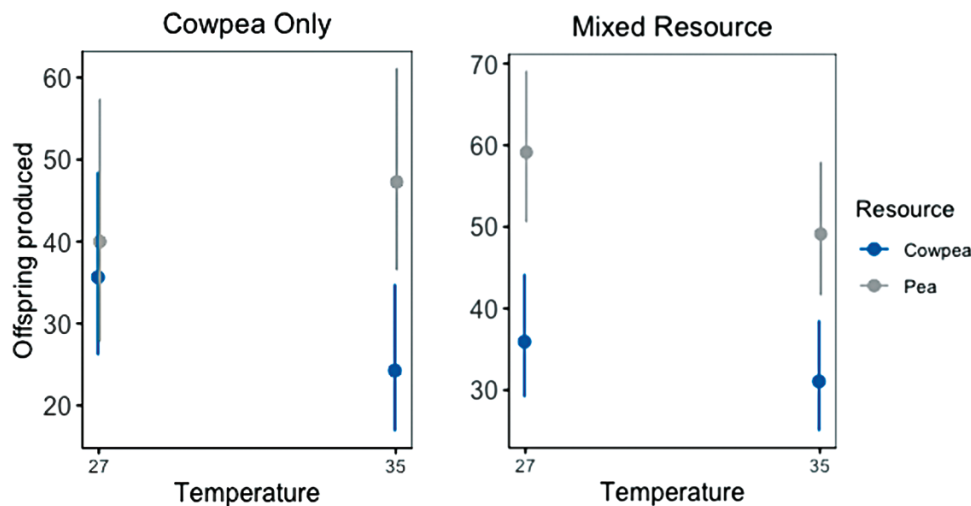


Figure 2. Marginal effects plots of total number of offspring produced from the evolved lines only (pea–cowpea), as a function of the interaction between evolved treatment and the resource type in which offspring developed when parents were reared on cowpeas (A) and as a function of the developmental resource of the parents and the developmental resource of the offspring (B).

Table 3. Fixed effects of the best-fitting model explaining F1 total offspring produced by individuals within the evolved (pea–cowpea mix) lines, as a function of F0 (maternal) and F1 (own) developmental resource type

Fixed effects	Estimate*	SE	<i>t</i> -value	Pr(> <i>t</i>)
Offspring developmental resource (pea)	−0.87	0.16	−5.49	< 0.001
Parent developmental resource (pea)	−0.53	0.14	−3.74	< 0.001
Offspring × parent developmental resource	0.19	0.25	0.74	> 0.05

*Full model reported where interaction is significant, otherwise interaction is dropped and main effects only reported.

**Figure 3.** Marginal effects plots of total offspring produced in specialist, cowpea-only lines (left panel) and in lines evolved for 60 generations (right panel) as a function of the interaction between the resource when the parents developed on peas (grey symbols) or cowpeas (blue symbols) and the temperature at which offspring developed on cowpeas. The ancestral temperature was 27 °C and the novel temperature 35 °C. Error bars represent 95% confidence intervals.

Within the pea–cowpea mix evolved lines, there were further significant, additive effects of both parental and offspring developmental resource types on offspring produced, whereby developing in peas or being born to parents that had developed in peas both had strong negative effects on the number of offspring produced (Table 3; Fig. 2A). Interestingly, there was a strong effect of parental developmental resource on the number of offspring produced (and not significantly smaller than the effect of the offspring developmental resource; Table 3; Fig. 3), indicating the presence of strong, deleterious carryover effects of suboptimal resources across multiple generations in this system. For additional analyses on fecundity and survival, please see the supporting Information.

COMPARISON OF OFFSPRING PRODUCED AT DIFFERENT TEMPERATURES

Consistent with higher *HSP* expression in response to novel resources and evolved lines, there were significant interactions between rearing temperature

and developmental resource on total offspring produced (Table 4).

EXPRESSION OF MRNA

There were no significant interactions between evolved line and developmental resource type gene × environment interactions ($G \times E$) on larval gene expression at any of our selected loci, hence interaction terms were removed from all reported models. There were significant main effects of evolved treatment on the expressions of *HSP70* and *ACAC*, both of which were upregulated in individuals which had adapted over ≥ 60 generations to the evolved lines, after controlling for effects of resource type (Table 5; Fig. 4). Moreover, there was a significant additive effect of rearing conditions (developmental resource type) on expression of *HSP90*, which was upregulated in individuals developing in peas, after controlling for evolved vs. control population history (Table 5; Fig. 4). Of the loci tested, only *PO* and *PYC* showed no significant responses to evolved treatment or

Table 4. Fixed effects of the best-fitting model explaining total offspring produced by F1 individuals as a function of temperature, evolved line (evolved, pea–cowpea lines; and control, cowpea-only lines) and developmental resource (cowpea and pea; all F0 individuals were isolated from cowpeas)

Fixed effects	Estimate*	SE	<i>t</i> -value	Pr(> <i>t</i>)
Temperature	−0.38	0.20	−1.85	0.067
Developmental resource (pea)	0.08	0.21	0.38	0.70
Treatment (evolved)	0.01	0.24	0.03	0.97
Temperature × developmental resource (pea)	0.59	0.28	2.07	0.04
Temperature × treatment (evolved)	0.23	0.28	0.81	0.42
Treatment (evolved) × developmental resource (pea)	0.41	0.27	1.54	0.13
Temperature × treatment (evolved) × developmental resource (pea)	−0.63	0.37	−1.69	0.095

*Full model reported where interaction is significant, otherwise interaction is dropped and main effects only reported.

Table 5. Fixed effects of the best-fitting model explaining gene expression levels (fold change) of the five candidate genes considered here

Locus	Effects*	Estimate	SE	<i>z</i> -value	Pr(> <i>z</i>)
<i>HSP70</i>	Resource (pea)	1.1033	0.5242	2.104	0.0515
	Evolved treatment	1.3495	0.5242	2.574	0.0204
<i>HSP90</i>	Resource (pea)	−1.1583	0.4843	−2.391	0.0303
	Evolved treatment	0.868	0.4813	1.803	0.0915
<i>ACAC</i>	Resource (pea)	0.5569	0.4658	1.196	0.2492
	Evolved treatment	1.9609	0.4658	4.21	0.0007
<i>PO</i>	Resource (pea)	−0.4705	0.2712	−1.735	0.101
	Evolved treatment	0.4134	0.2712	1.524	0.146
<i>PYC</i>	Resource (pea)	0.9865	0.8071	1.222	0.2433
	Evolved treatment	0.6764	0.8071	0.838	0.4171

*The full model is reported when the interaction is significant; otherwise, the interaction is dropped and only main effects are reported.

developmental resource type (Table 5; Fig. 4; see Supporting Information).

DISCUSSION

We integrated phenotypic assays across two niche dimensions with gene expression studies to examine the potential mechanisms and provide insight into the regulatory changes that occur during the evolution of generalization and tested the hypotheses that (1) there is a cost to the evolution of generalization involving evolutionary changes in gene expression, and (2) changes in regulatory gene expression during the evolution of generalism have (potentially beneficial) consequences for other aspects of the niche of the organisms.

Previous studies have shown that generalism in phytophagous insects is more rare than specialism, and this is presumably attributable to the trade-offs associated with multiple host use (Bernays & Minkenberg, 1997; but see Lancaster, 2020; Singer & Parmesan, 2021). However, we found that individuals from evolved lines evolved increased fitness on peas,

with no loss of fitness on the ancestral resource. Thus, adaptation to multiple host environment resulted in a generalist population with no apparent fitness trade-off between use of the two alternative resources. Nonetheless, we observed significant upregulation of major stress response genes (heat shock proteins) and genes coding for metabolic enzymes in the evolved lines, and this occurred irrespective of developmental resource type, suggesting that there is an underpinning metabolic and physiological cost of becoming generalized that is not observed in the fitness data. Changes in gene expression of metabolic and stress response genes might regulate survival on a novel resource and are likely to represent a key mechanism for the evolution of generalism in a multi-resource environment. Counter to the ‘jack of all trades, master of none’ theory, the number of offspring produced in the ancestral temperature and resource conditions was higher in generalist individuals from the evolved lines than in the control lines. Thus, evolved generalist beetles exhibit an increased resource niche breadth without experiencing a trade-off in peak performance within the narrow range of ancestral specialization.

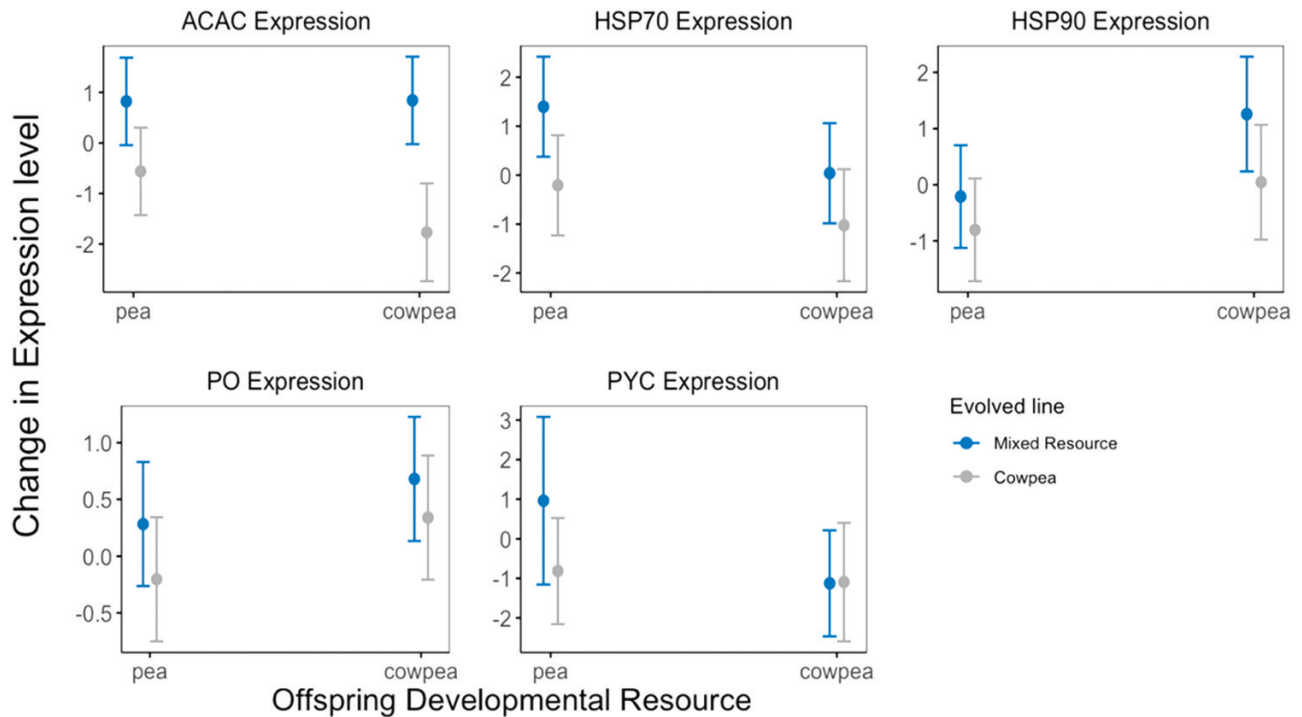


Figure 4. Gene expressions for \log_{10} -transformed *ACAC*, *HSP70*, *HSP90*, *PO* and *PYC* from larvae developing within cowpeas or peas, from evolved lines (blue symbols) or control lines (grey symbols). Error bars represent 95% confidence intervals. Marginal effects of evolved and rearing conditions are depicted after accounting for replicates. Note the different axis scales.

Addressing our second hypothesis, our results indicated that evolved or plastic increases in resource niche breadth simultaneously conferred increased niche breadth in another environmental axis, which was not under selection or exposed to acclimation conditions (temperature). This result suggests that evolutionary and plastic niche shifts between and within specialists and generalists might generally be far more complex and multidimensional than the traditional understanding of niche dimensions might suggest. By exploring evolved changes in gene expression, we can begin to make suggestions regarding the mechanisms contributing to generalism across multiple niche dimensions, provide the mechanism for the generalized stress response that has been described in other studies (Sørensen *et al.*, 2003; Snell-Rood *et al.*, 2018; Sikkink *et al.*, 2020) and provide evidence for potential mechanisms of a correlated generalized response across multiple niche dimensions. This study highlights the important consequence that if genotypes or populations are pre-adapted to tolerate novel resources, they might be more likely to colonize new environments.

Our gene expression data, combined with fitness data across multiple environments, also highlight that evaluating the costs of specialism is not straightforward.

We found: (1) no fitness costs of resource generalism in the niche axis under selection; (2) a fitness benefit of resource generalism in an orthogonal niche dimension (thermal); and (3) evidence of a metabolic cost of generalism that did not manifest as a fitness cost in our study, but which might negatively impact resilience in other ways, such as reduced fitness in other niche dimensions (e.g. humidity). These patterns of gene expression are similar to other results showing divergent patterns of the regulation of differential gene expression between closely related generalist and specialist species found in the wild (Lee *et al.*, 2006; Govind *et al.*, 2010; Torregrossa *et al.*, 2012).

The individual candidate genes considered each have specific metabolic and defence functions, and the resulting changes in gene expression at each of these loci in response to evolved or developmental dietary shifts reveal key details about their potential role in resource generalism and might provide fundamental insight into simultaneous generalism in indirect niche dimensions. Heat shock proteins 70 and 90 act to protect cells from thermal and oxidative stresses, which normally act to damage proteins (King & MacRae, 2015). Our analysis of HSPs emphasizes the importance of these proteins in host shifts, and upregulation might be a specific response to toxins and nutritional

changes in peas. Larvae must acquire enough nutrition and metabolize toxins adequately in the novel host environment to pupate successfully, and this requires a plastic, and later evolved, physiological adjustment, whether to respond actively to stressors or to suppress physiological ‘noise’ and maintain homeostasis. The pattern of upregulation of *ACAC* gene expression exhibited in response to the evolutionary shift to incorporate peas into the diet might reflect differences in macromolecular content of the two legume species. The function of *ACAC* is to regulate the metabolism of fatty acids, which are required for the development and survival of insects that grow and feed on starch-containing seeds (Telemeco *et al.*, 2019). We observed only evolved effects of the shift to generalism on *ACAC* expression, with no developmental effects of the different resource types, suggesting that *ACAC* levels are an evolved response to generalism (or peas) per se, rather than being upregulated as a short-term response to nutrient stress.

Despite observed evolved acceptance of peas, fitness remained relatively lower on this resource overall. Concomitantly, we saw upregulation of HSPs and (very marginally) defensive enzymes (Phenoloxidase) in response to rearing on peas, irrespective of evolved treatment. These results confirm our pilot results that peas are a challenging resource for beetles from our source population, and that ≥ 60 generations are insufficient for them to become adapted completely. The fact that HSPs became upregulated in response to developmental rearing on peas and also became upregulated in the evolved lines suggests that the stress response might have become canalized as a part of the adaptive response within the evolved lines (a form of genetic assimilation; Pigliucci *et al.*, 2006; Schneider & Meyer, 2017). In essence, differences in transcript levels of stress-related genes between the two evolved treatments suggest that larvae are stressed more permanently when they have evolved generalism in a multiple host environment as opposed to being specialists on cowpeas.

There was no evolved or rearing effect of host type on expression of Pyruvate carboxylase, a key enzyme involved in gluconeogenesis that is thought to be important for mobilizing dispersal in insects (Crabtree *et al.*, 1972) and has previously been implicated in the response to stress induced by hypoxia/hypercapnia in seed beetles (Cui *et al.*, 2019). These results suggest either that this gene is not involved in resource trade-offs or that our laboratory colony has lost the ability to upregulate this gene, but not *ACAC*, following their long-term exposure to laboratory conditions. We found only marginal effects of rearing resource environment on Phenoloxidase expression. This enzyme is involved in immunity and homeostasis (González-Santoyo & Córdoba-Aguilar, 2012) and has previously been shown

to be upregulated in response to dietary generalization in honeybees, a species that is already generalist (Alaux *et al.*, 2010). However, there were no evolved changes in *PO* associated with the shift to generalism in our laboratory lines, suggesting that immunity is not part of the evolved generalism syndrome.

Together, the results indicate that fatty acid metabolism and stress protein expression levels are under positive selection during the evolutionary shift to generalism, whereas metabolic or immune genes are less likely to be involved in evolved changes. Among these genes showing evolved changes in expression, only HSPs are also involved in plastic responses to stressful new resources (genetic assimilation). Further exploration of additional genes will be required in combination with studies of differentiation in allele frequency, to understand the exploitation of a novel hosts which may contribute to speciation in this fascinating system.

CONCLUSIONS

Here, we have identified patterns of genetic adaptations that might have enabled at least partial generalization a heterogeneous environment. Moreover, we have illustrated, through common garden experiments, evolved physiological change at the level of gene expression that is consistent with genetic assimilation. We have shown that such differences can evolve within the same species in as little as 60 generations and might reflect, in part, genetic assimilation of developmental stress responses, and these physiological shifts impact changes in niche breadths in a non-specific way, broadening both the selected (resource) and non-selected (temperature) niche dimensions. This study shows the importance of investigating gene expression in niche evolution studies, because this might be associated with ecologically important phenotypes not evident from morphological or life-history traits and might indicate cryptic costs of generalism that are subtle and therefore not immediately apparent in fitness data obtained in the study conditions.

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DATA AVAILABILITY

The data are available as Supporting Information.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Fixed effects of the best-fitting model explaining F1 survival and fecundity of individuals within the evolved (pea–cowpea mix) lines, as a function of F0 (maternal) and F1 (own) developmental resource type.

Table S2. Fixed effects of the best-fitting model explaining survival and fecundity of F1 individuals as a function of evolved line (evolved, cowpea–pea lines vs. control, cowpea-only lines) and F1 resource type (cowpea vs. pea; all F0 individuals were isolated from cowpeas).

Figure S1. Marginal effects plots of fecundity (A) and survival of offspring (B) from the lines evolved on mixed resources (pea–cowpea), as a function of the developmental resource of the parents and the developmental resource of the offspring. The interaction was not significant, but each of these effects impacted larval survival additively in approximately equal measure. Error bars represent 95% confidence intervals.

Figure S2. Marginal effects plots of fecundity (A) and survival of offspring (B) as a function of the interaction between evolved treatment (blue symbols indicate experimental lines evolved for 60 generations on the mixed resource environment; red symbols indicate control lines) and the resource type in which offspring developed. Error bars represent 95% confidence intervals.