Complex pleiotropic genetic architecture of evolved heat stress and oxidative stress resistance in the nematode *Caenorhabditis remanei*

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1   **ABSTRACT**

2   The adaptation of complex organisms to changing environments has been a central question in

3   evolutionary quantitative genetics since its inception. The structure of the genotype-phenotype

4   maps is critical because pleiotropic effects can generate widespread correlated responses to

5   selection and potentially restrict the extent of evolutionary change. In this study we use

6   experimental evolution to dissect the genetic architecture of natural variation for acute heat stress

7   and oxidative stress response in the nematode *Caenorhabiditis remanei*. Previous work in the

8   classic model nematode *C. elegans* has found that abiotic stress response is controlled by a

9   handful of genes of major effect and that mutations in any one of these genes can have

10   widespread pleiotropic effects on multiple stress response traits. Here, we find that acute heat

11   stress response and acute oxidative response in *C. remanei* are polygenic, complex traits, with

12   hundreds of genomic regions responding to selection. In contrast to expectation from mutation

13   studies, we find that evolved acute heat stress and acute oxidative stress response for the most

14   part display independent genetic bases. This lack of correlation is reflected at the levels of

15   phenotype, gene expression, and in the genomic response to selection. Thus, while these findings

16   support the general view that rapid adaptation can be generated by changes at hundreds to

17   thousands of sites in the genome, the architecture of segregating variation is likely to be strongly

18   parcellated by the pleiotropic structure of the underlying genetic networks.

Introduction

Biological organisms are complex, integrated systems. From an evolutionary point of view, a central consequence of this integration is that natural selection acting on one feature of an organism has the potential to have cascading effects across the whole organism (Lande 1979; Cheverud 1984; Phillips and McGuigan 2006). Evolutionary quantitative genetics has created a powerful statistical framework for analyzing complex phenotypes, even in the absence of knowledge of seemingly important factors such as the exact number or identity of genes or the distribution of their phenotypic effects (Barton and Turelli 1989). The advent of whole genome analyses would seem to herald the advent of a new era in which we have the power to understand the genetic basis of complex, multifactorial traits. However, even now, the field has achieved remarkably little success identifying the genetic basis of most adaptive phenotypes within natural populations, aside from traits where a majority of the variation is due to a handful of well-characterized mutations, such as coat color and oxygen affinity in deer mice (Steiner *et al.* 2007; Linnen *et al.* 2013; Natarajan *et al.* 2013). There may be a small handful of cases in which natural variation is generated by simple genetics, but it is clear that for many, if not most, traits this will not be the case (Phillips 2005; Rockman 2012). For example, human height is a well-studied, highly heritable, quantitative trait and yet despite sample sizes that now stretch into the millions, mapped loci still only account for a small fraction of the total genetic variance (Lango Allen *et al.* 2010; Nolte *et al.* 2017). Similarly, studies of complex diseases such as schizophrenia are able to map hundreds of genes associated with disease prevalence but still account for less than 10% of the variation in disease risk (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

However, genes rarely affect only a single, isolated trait within an organism. The idea that genetic coupling across functional systems should be the rule rather than exception led

43   Sewall Wright (1968) to posit the idea of "universal pleiotropy" for genetic systems. More

44   recently, Boyle *et al.* (2017) proposed an 'omnigenic' model for complex diseases that posits that

45   complex diseases could be affected by variation in genes in any tissue that affects that disease,

46   i.e. such diseases are affected by variation in sets of core and peripheral genes. To a large extent,

47   the omnigenetic paradigm is simply a translation of one of the central conceits of evolutionary

48   quantitative genetics to the setting of human association mapping. At its limit, extensive

49   pleiotropy can serve to "lock" the response to selection, creating a genetic constraint that limits

50   the rate of evolutionary change (Lande 1979; Charlesworth 1990; Arnold 1992). On the other

51   hand, variation in the functional specificity across the network of genes affecting complex traits,

52   such as differences in tissue-specific regulation (Carroll 2008), holds the possibility that the

53   genetic changes that actually lead to evolutionary divergence could be biased towards subsets of

54   genes with either more or less pleiotropy, depending on the specific pattern of selection (Phillips

55   and McGuigan 2006). Pleiotropy may therefore play a two-fold role in the response to selection.

56   First, following traditional quantitative genetic approaches, patterns of pleiotropy underlying

57   standing variation may lead to correlated responses to selection across broad suites of characters.

58   Alternatively, following a developmental-genetic perspective, long-term evolutionary change

59   may be strongly dependent on variation in pleiotropy, and may subsequently lead to a biased

60   subset of genes exhibiting reduced pleiotropic effects, leading to the evolutionary change in of

61   the pattern of pleiotropy itself (Phillips and McGuigan 2006; Arnold *et al.* 2008; Jones *et al.*

62   2014). The balance between these factors is largely an empirical question.

63   Several studies have found, depending on the organism, median levels of pleiotropy to

64   be between 4–7 traits affected by every gene (Wagner and Zhang 2011; Paaby and Rockman

65   2013), but there are many cases in which a single mutation can affect dozens of traits (Knight *et*

66   *al.* 2006). These studies tend to focus on what Paaby and Rockman (2013) have called

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67   "molecular gene pleiotropy": how many functions does a given locus affect when that gene is

68   knocked out. There is substantially less information on the extent of pleiotropy within

69   populations generated by naturally segregating variation within populations, or "mutational

70   pleiotropy" (Wagner and Zhang 2011; Paaby and Rockman 2013). Here the focus is on the entire

71   spectrum of pleiotropy generated by segregating alleles as opposed to genetic knockouts. Thus it

72   is the allele – and not the gene – that is the appropriate unit of variation for evolutionary change

73   (Wright 1968; Lande 1984; Phillips and McGuigan 2006; Wagner and Zhang 2011). Studies that

74   use forward genetic screens may find a large fraction of potential targets of those genes, but that

75   does not necessarily inform us as to how a trait of interest will respond to selection.

76   How can we understand the balance between molecular and mutational pleiotropy,

77   especially in the context of naturally segregating variation influencing complex correlated traits?

78   Experimental evolution coupled with whole genome sequencing, or evolve and re-sequence, has

79   emerged as a powerful method for studying the genetic architecture of complex traits (Kofler and

80   Schlötterer 2013). Selection on complex traits can leave weak or ambiguous signals of selection

81   on the genome (Kemper *et al.* 2014), but evolve and re-sequence facilitates direct comparison

82   between ancestral and evolved populations and quantification of evolutionary change across the

83   genome. In particular, replicated experiments within a well-defined hypothesis testing

84   framework allow sampling variance to be accounted for in a way that historical contingency

85   renders impossible within natural populations. While we have learned a great deal from

86   experimental evolution in microbial populations initiated from a single fixed genetic background,

87   questions related to the influence of complex segregating variation on the response to selection

88   can only be addressed by capturing a broad array of natural genetic variation in the initial

89   ancestor population (Schlotterer *et al.* 2015).

90   The nematode *Caenorhabditis remanei* is ideal for addressing questions relating to

91   complex traits via experimental evolution (Sikkink, Reynolds, *et al.* 2014; Castillo *et al.* 2015;

92   Teotónio *et al.* 2017). It is a genetically diverse, sexually reproducing organism (Jovelin *et al.*

93   2003; Cutter *et al.* 2006). Large populations can easily be maintained in a lab, with upwards of

94   2000 individuals living on one 10cm agar plate, so an entire experiment can occupy one shelf in

95   an incubator. Additionally, its close relationship to the model nematode *C. elegans* allows both

96   genomic and functional information to be applied across species.

97   Abiotic stress response in nematodes provides an interesting model system of interacting

98   traits. In particular, many of the important stress response pathways are well conserved

99   throughout animals and have important pleiotropic effects on aging and longevity (Kenyon

100   2010), with a handful of stress response pathways being know to affect the response to many

101   different abiotic stressors (Rodriguez *et al.* 2013; Murphy and Hu 2013). For example, as

102   elucidated within numerous studies in the well-studied insulin/insulin-like growth factor

103   signaling pathway in *C. elegans*, mutations often affect resistance to a wide variety of stressors

104   including heat stress, oxidative stress, ultraviolet radiation and pathogen stress (Murphy and Hu

105   2013). However, the role that natural genetic variation plays in individual variation in these traits

106   is less clear (Reynolds and Phillips 2013; Jovelin *et al.* 2014).

107   Here, we use experimental evolution and whole genome sequencing to dissect the genetic

108   architecture of acute heat stress and acute oxidative stress response in *C. remanei*. In previous

109   work, we saw a strong phenotypic response to selection for both acute heat stress and acute

110   oxidative stress but little to no correlated phenotypic response between selection for acute heat

111   stress and acute oxidative stress resistance (Figure 1; Sikkink *et al.* 2015). In this study, we seek

112   to align these global phenotypic patterns with a detailed analysis of gene expression and the
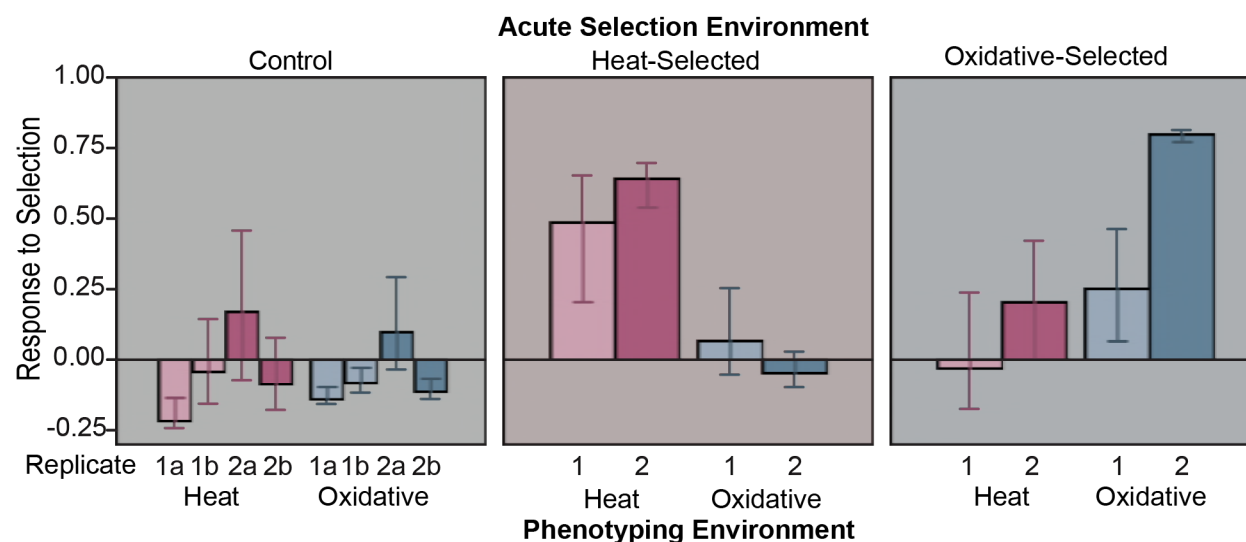
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Figure 1

Direct and correlated response to selection for each replicate of experimentally evolved lines. A direct response to selection is measured by the response to selection where the phenotyping environment matches the acute selection environment and a correlated response to selection is measured by the response to selection where the phenotyping environment does not match the acute selection environment. There is a strong direct response to selection seen in both acute heat and oxidative selected populations but no correlated response to selection. Modified from Supplementary Figure S1 in (Sikkink *et al.* 2015).

113   genomic response to selection at single nucleotide resolution in order to test the complexity and

114   coherence of genotypic and phenotypic responses to selection.

MATERIALS AND METHODS

EXPERIMENTAL EVOLUTION

117   As described in (Sikkink, Reynolds, *et al.* 2014), the base "ancestral" population used to initiate

118   the experimental evolution replicates was created using 26 isofemale strains, to create a

119   population representative of the naturally segregating genetic variation of a single population of

120   *C. remanei* collected in Ontario, Canada. This population (PX443) was used to establish each of

121   the replicate selection and control lines and was frozen soon after creation to prevent adaptation

122   to the lab environment. Using the methods described below, we determined that this population

123   had high initial levels of genetic diversity (genome wide average $\pi = 0.0178$, Figure S1). All

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124   natural isolates and experimental lines were raised on Nematode Growth Medium-lite (NGM-

125   lite, U.S. Biological) seeded with *Escherichia coli* strain OP50 (Brenner 1974).

126   Evolved populations of *C. remanei* belong to one of two replicate experimental blocks,

127   each derived from an independently thawed subset of the ancestral population. Within each

128   replicate block populations were evolved in parallel to one of three acute environments: control

129   (2 lines/block), acute heat stress (1 line/block) and acute oxidative stress (1 line/block) as

130   previously described (Sikkink, Reynolds, *et al.* 2014; Sikkink *et al.* 2015). Here, an acute stress

131   is one that the worms experienced for four hours as L1 juveniles before returning to the

132   laboratory control environment. The acute heat stress assay induced ~70% mortality in the

133   ancestor population and the acute oxidative stress assay induced ~80% mortality in the ancestor

134   population (Sikkink *et al.* 2015). Populations were subjected to acute stress conditions every two

135   generations or whenever the population produced ≥ 24,000 eggs, whichever happened later.

136   Control populations were randomly culled to 1000 worms during selective generations. For all

137   acute stress events, each population (control evolved, acute heat stress evolved and acute

138   oxidative stress evolved) was age synchronized via a bleach treatment (Stiernagle 2006). All

139   experimentally evolved populations were subject to a total of ten acute stress events. As

140   described in (Sikkink, Reynolds, *et al.* 2014) each population was frozen after about every

141   second generation of acute stress selection to ensure that the worms did not lose the ability to

142   survive a freeze and to provide a record of evolutionary changes. All told, there were a total of

143   eight experimentally evolved populations created from the ancestor population: four control

144   replicates, two acute heat stress replicates and two oxidative stress replicates.

145   **PREPARATION OF WHOLE GENOME SEQUENCING LIBRARIES**

146   We determined the genetic response to selection using a population-based pooled sequencing

147   (pool-seq) approach (Schlotterer *et al.* 2014). Ancestral and acute evolved populations were

148   sequenced via whole genome shotgun sequencing. Approximately 10,000 L1 individuals (~24

149   hours old juvenile) were pooled together for DNA extraction and sequencing library preparation.

150   Samples were prepared using the Nextera DNA Sample Preparation Kit (Illumina). Multiple

151   sequencing libraries were prepared from the original DNA sample as needed to ensure adequate

152   mean sequencing coverage (Table S1). Whole genome sequencing libraries were obtained for the

153   ancestor and all eight evolved populations. Samples were sequenced on an Illumina HiSeq 2000

154   and Illumina HiSeq 2500 at the University of Oregon Genomics and Cell Characterization Core

155   Facility. All sequences were deposited on NCBI's Sequence Read Archive, accession number

156   SRP126594 (https://www.ncbi.nlm.nih.gov/sra/SRP126594).

157   **WHOLE GENOME SEQUENCING ANALYSIS**

158   Raw sequence reads were quality filtered using the 'process_shortreads' component of Stacks

159   (Catchen *et al.* 2013). Low quality reads (mapping and base quality less than 20) were trimmed

160   or discarded; all reads that passed quality filtering were aligned to the *C. remanei* reference

161   genome assembled from the PX356 *C. remanei* inbred strain (Fierst *et al.* 2015) using the

162   alignment program *GMAP – GSNAP* (Wu and Nacu 2010). Single nucleotide polymorphism

163   (SNP) tolerant alignment parameters were used to ensure that divergent SNPs would align to the

164   reference genome. We used available gene annotation data for PX356 (Fierst *et al.* 2015) in

165   downstream data analysis. However, we also wanted to make use of the more extensive

166   functional annotation data available for *C. elegans*. To that end, we used the software

167   *OrthoFinder* (Emms and Kelly 2015) to identify *C. elegans* orthologs of *C. remanei* genes and

168   protein domains. The *C. elegans* protein annotations were obtained from Wormbase ParaSite

169   (Lee *et al.* 2018), version WS258.

170   We used the R (R Core Team 2017) package *Nest* (Jonas *et al.* 2016) to estimate effective

171   population size (N_e) of each experimental replicate using temporal changes in allele frequency

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172   based on existing theory (Krimbas and Tsakas 1971; Jorde and Ryman 2007; Waples *et al.*

173   2016), modified for samples taken from pooled population data. The input data, allele frequency

174   and coverage data for each SNP at generation 0 (ancestor population) and generation 30 (each

175   evolved population) was obtained from the program 'snp-frequency-diff.pl' from *Popoolation2*

176   (Kofler, Pandey, *et al.* 2011). Only SNPs with a minimum coverage of 10X in each population

177   were included in the effective population size estimator. Adding a maximum coverage threshold

178   did not affect the estimated N_e. We used a pool sample size (the number of individuals who went

179   into the original sequencing sample) of 1000 but increasing the pool size from 1000 to 10,000

180   individuals had a negligible effect on the estimated N_e. Expected N_e based on census population

181   sizes for the duration of the experiment were calculated based on Equation 3.5 in (Kimura 1983)

182   using the 'harmonic.mean' function in the R (R Core Team 2017) package *psych*

183   (https://CRAN.R-project.org/package=psych).

184   Nucleotide diversity in the ancestor population was calculated over 1000 base pair (bp)

185   windows using the program *Popoolation* (Kofler, Orozco-terWengel, *et al.* 2011). Allele

186   frequency differences were analyzed on a site by site basis between the ancestor and control, heat

187   stress evolved or oxidative stress evolved populations using the Fisher's exact test program from

188   the program *Popoolation2* (Kofler, Pandey, *et al.* 2011). Each SNP had to have a minimum of

189   10X coverage, a maximum coverage of 98% of total sequence coverage across the genome and

190   the minor allele had to have at least two copies at a site in order to be retained for further

191   analysis. Allele frequency differences were also analyzed over a range of sliding window sizes

192   where each sliding window overlapped with one third of the length of the previous window. For

193   each sliding window 75% or more of the bases in it had to meet the minimum and maximum

194   coverage thresholds used for the single locus Fisher's exact test comparison in order to be

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195   included. The geometric mean *p*-value of Fisher's exact test across the window was calculated

196   for each interval.

197   We then used a permutation test to determine whether or not a sliding window was

198   significantly diverged from the ancestor population using a random sampling program in which

199   windows were created at random by calculating the geometric mean *p*-value for a set of Fisher's

200   exact test outcomes from the pool of single-locus results (script available in File S1). The mean

201   number of SNPs in an empirical sliding window of a particular size was used to determine the

202   number of SNPs in each random sample window. Random sampling was repeated 1,600,000

203   times for each ancestor-experimentally evolved population pair. Random windows equivalent to

204   empirical 1000–20,000 bp windows were created, with the 5000 bp window size chosen for the

205   reported results because it is approximately the same size or slightly larger than nearly all genes

206   in *C. remanei*, ensuring that genic and near genic regions could be captured in one window (see

207   Figures S2 and S3 for a comparison of the distribution of empirical versus random sample

208   windows). The random sampling program code can be found in File S1. Every ancestor-evolved

209   population pair produced roughly 20,000 5 kp windows. We used the Bonferonni corrected *p*-

210   value for 20,000 tests (2.5×10^{-6}) as the cut-off for significance in each random sample window

211   file. Any empirical sliding window that had a mean Fisher's exact test value greater than or equal

212   to the 99.99975% of the random sample window distribution was considered to be significantly

213   differentiated from the ancestral population. A given window was defined as being significantly

214   differentiated within a given selection regime (acute heat or oxidative stress selected) if it did not

215   overlap with differentiated windows in the control populations and if the window was classified

216   as significantly differentiated within both replicates. *BEDTools* 'intersect' (move Quinlan and

217   Hall citation here) (Quinlan and Hall 2010) was used to identify overlapping genomic windows

218   and genes under significantly differentiated genomic windows.

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HAPLOTYPE PHASING USING SINGLE WORM RAD SEQUENCING

We tested for the potential buildup of linkage disequilibrium within selection lines by crossing

worms from the experimental evolution population with the reference genome strain (PX356),

thereby generating F1s heterozygous for a single ancestral population haplotype. Single worm

RAD sequencing libraries were prepared for 77 male F1 worms. Because the genotype of the

reference strain is known, the haplotype of the target crossed individual could be directly

determined in the F1 heterozygotes.

The RAD capture (Ali *et al.* 2016) method was used to genotype F1 worms. RAD capture

is a flexible reduced-representation sequencing method that allowed us to multiplex and

economically sequence all 77 genomes in a single Illumina lane. We digested genomic DNA

with the restriction enzyme *EcoRI*; ligated digested fragments to barcoded, biotinylated adaptors;

and sheared fragments with a BioRuptor. We enriched for fragments containing *EcoRI* cut sites

with streptavidin beads and prepared libraries for sequencing with the GE GenomiPhi V3 DNA

Amplification kit. Samples were sequenced on an Illumina HiSeq 4000 at the University of

Oregon Genomics and Cell Characterization Core Facility.

Raw fastq files were reoriented using flip2BeRAD

(https://github.com/tylerhether/Flip2BeRAD), which reorients read pairs such that all RAD tags

and inline barcodes are present as read 1. Retained reads were filtered and demulitplexed with

'process_radtags' from *Stacks* using standard stringencies. Reads were aligned to the *C. remanei*

PX356 reference sequence (Fierst *et al.* 2015) using *GSNAP* ultrafast settings. We only used read-

pairs that mapped to the reference in the expected orientation (pairs facing inward toward each

other) in downstream analyses. Individuals were genotyped using the 'HaplotypeCaller' and

'GenotypeGVCF' programs from *GATK* (https://software.broadinstitute.org/gatk/) (Van der

Auwera *et al.* 2013). *VCFtools* (Danecek *et al.* 2011) was used to filter sites based on missing data,

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243   minor allele frequencies and genomic position. *PLINK*

244   (http://pngu.mgh.harvard.edu/purcell/plink/; Purcell *et al.* 2007) was used to calculate linkage

245   disequilibrium on the 13 largest contigs, which account for ~54% of total genome length. The

246   squared inter-variant allele count correlations (r^2 values) were reported from *PLINK* (Purcell *et*

247   *al.* 2007).

248   **TRANSCRIPTIONAL PROFILING OF EVOLVED POPULATIONS**

249   RNA sequencing libraries were prepared as described in (Sikkink, Reynolds, *et al.* 2014).

250   Briefly, pooled samples of ~100,000 age-synchronized L1 larval worms were collected from the

251   ancestor population, one of the control populations from the replicate two block (Control line –

252   2), and the acute heat stress and acute oxidative stress evolved populations from the replicate two

253   block. Total RNA was isolated from each sample using standard TRIzol (Ambion) methods. All

254   tissue samples were collected from L1 larval worms. All worms were raised in the standard lab

255   environment of 20°C; the two stress evolved populations were not exposed to the stressor (either

256   heat stress or oxidative stress) prior to tissue collection. We collected 6–8 replicate samples from

257   each evolved line from at least two independently thawed populations from each evolved line,

258   except for the oxidative stress evolved line, where 6 replicate samples were collected from one

259   thaw. Samples were sequenced on an Illumina HiSeq 2000 at the University of Oregon

260   Genomics and Cell Characterization Core Facility. All transcriptome sequences have been

261   deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2001) and are accessible through

262   GEO Series accession number GSE108235.

263   Raw sequence reads were quality filtered as described in (Sikkink, Ituarte, *et al.* 2014).

264   Briefly, raw sequencing reads were quality filtered using the 'process_shortreads' component of

265   *Stacks* (Catchen *et al.* 2013) and all reads that passed quality filtering were aligned to *a C.*

266   *remanei* reference genome assembled from the PX356 *C. remanei* inbred strain (Fierst *et al.*

267   2015) using *GMAP – GSNAP* (Wu and Nacu 2010). We used the 'htseq-count' tool from the

268   Python *HTSeq* package (Anders *et al.* 2014) to count all reads that aligned to protein–coding

269   genes, using the criteria described in (Sikkink, Reynolds, *et al.* 2014).

270   Differential gene expression analysis was conducted using the R package *DESeq2* (Love

271   *et al.* 2014). Low variance genes were filtered using *DESeq2*'s automatic filter. We tested for

272   differences in gene expression between the ancestor and three experimentally evolved

273   populations (control line – 2, acute heat stress evolved and acute oxidative stress evolved). Genes

274   were only called as differentially expressed in each comparison if the *p*-value was less than 0.05

275   after Benjamini-Hochberg adjustment as implemented in *DESeq2*. We also tested for a

276   relationship between $\log_2$ fold changes in gene expression between acute heat stress–ancestor

277   and acute oxidative stress–ancestor comparisons using Model II linear regression. This analysis

278   was implemented in the *lmodel2* package in R (R Core Team 2017).

279   **DATA AVAILABILITY**

280   File S1 contains the python script used to create the random sampling windows. Files S2 and S3

281   contain the full list of genes identified as differentially expressed and/or divergent, including *C.*

282   *elegans* ortholog names. Table S1 contains sequencing coverage for all experimental

283   populations. Files S1-S3, Table S1 and all supplemental figures available on figshare

284   (https://figshare.com/projects/Supplemental_material_for_Complex_pleiotropic_genetic_archite

285   cture_of_evolved_heat_stress_and_oxidative_stress_resistnace_in_the_nematode_Caenorhabditi

286   s_remanei_/58364). Whole genome sequencing data are available at NCBI SRA database under

287   accession number PRJNA422140. The transcriptome data have been deposited in NCBI's Gene

288   Expression Omnibus (Edgar *et al.* 2001) and are accessible through GEO Series accession

289   number GSE108235. This Whole Genome Shotgun project has been deposited at

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290   DDBJ/ENA/GenBank under the accession LFJK00000000. The version described in this paper is

291   version LFJK02000000.

292   **RESULTS**

293   **ADAPTATION TO ACUTE STRESS INVOLVES HUNDREDS OF LOCI**

294   As demonstrated previously, selection for resistance to acute heat stress and acute oxidative

295   stress leads to the evolution of significantly increased survival within the acute heat stress and

296   acute oxidative stress environments (Sikkink *et al.* 2015). Using whole-genome resequencing of

297   the selected lines, we find that this phenotypic response is strongly echoed across the entire

298   genome: 13–27% of genomic windows within each population were more differentiated then

299   expected by chance (Figure 2). There was a large amount of genetic differentiation between not

300   only the ancestor and stress evolved populations, but also between the ancestor and control

301   evolved populations (Figure 2). Because there was essentially no detectable response to either an

302   acute heat stress or an acute oxidative stress in any of the control evolved populations at the

303   phenotypic level (Figure 1), genetic differentiation within the control populations is most likely

304   due to the effects of genetic drift and adaptation to the lab environment, since this population had

305   very recently been brought into the lab from nature (Sikkink, Reynolds, *et al.* 2014). For this

306   reason, we focused on differentiated genomic regions in the acute stress populations that were

307   not differentiated in any of the control populations (that is, unique) and were shared among the

308   evolutionary replicates (Figure 2). Those regions are referred to "differentiated windows" in

309   what follows. After this filtering, we were able to identify hundreds of reproducibly

310   differentiated windows: 495 and 491 differentiated 5 kp windows in the acute heat stress evolved

311   and acute oxidative stress evolved populations, respectively.
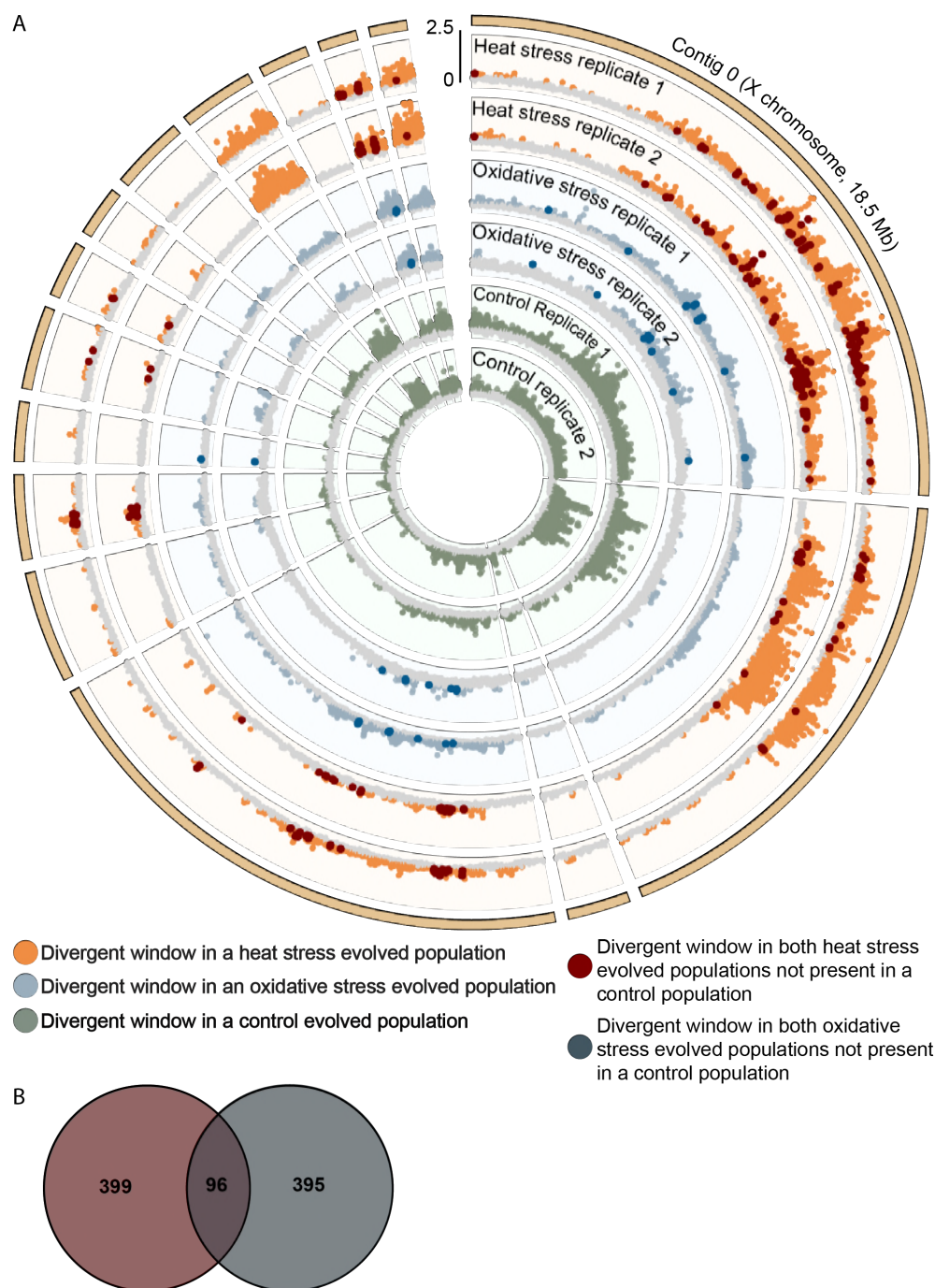
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Figure 2

(a) Location of significantly differentiated 5000 bp genomic windows. Mean $-\log_{10}$ of the Fisher's Exact Test p-value for each window shown. The two control populations for each replicate are plotted on same ring. Widespread divergence from the ancestor (non-grey dots) is seen in all evolved populations, including the control populations. (b) Overlap between unique differentiated windows between heat stress and oxidative stress evolved populations. Only around one fifth of the unique differentiated windows overlap in location.

17

GENOMIC DIFFERENTIATION IN HEAT STRESS AND OXIDATIVE STRESS

EVOLVED POPULATIONS IS LARGELY INDEPENDENT

Stress resistance, including resistance to heat and oxidative stress, is expected have a common

genetic basis in nematodes based on previous studies of *C. elegans* (Kenyon 2005; Rodriguez *et*

al. 2013). In contrast to these expectations, we observed little correlated phenotypic response to

either acute heat stress resistance or acute oxidative stress, even while there was a strong

evolutionary response within a given selective environment (Figure 1; Sikkink *et al.* 2015). This

lack of correlation at the phenotypic level is strongly reflected in the pattern of evolutionary

response at the genetic level. We identified 491 differentiated windows in lines evolved under

acute oxidative stress conditions and 495 in lines evolved under acute heat stress. Of these, only

96 are shared between the two selective regimes (Figure 2B). This indicates a general lack of

pleiotropy in the genes underlying the response to selection for heat and oxidative stress.

LINKAGE DISEQUILIBRIUM AS A POSSIBLE SOURCE OF COMPLEXITY IN

GENETIC RESPONSE

The large number of genomic regions that we saw responding to selection could be due to a large

number of genes underlying the traits of interest or, alternatively, due to the impact of linked

selection on a smaller number of causative loci during adaptation (Franssen *et al.* 2015). We

used single-individual SNP data in the ancestral population to estimate the degree of linkage

disequilibrium within these populations. We found that linkage disequilibrium in the ancestor

decays to background levels within 200 bp (Figures S4 and S5), very similar to the pattern

observed within natural populations (Cutter *et al.* 2006), and found no evidence of local or long-

distance linkage disequilibrium beyond that (Figures S6 and S7).

Even in the absence of haplotype structure in the ancestral population, selection itself

may generate linked responses to selection within specific chromosomal regions, especially those

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Table 1

Estimated effective population (N_e) size for each experimentally evolved population. N_e estimated for all eight evolved populations using allele frequency and coverage data estimated using *Popoolation2* (Kofler, Pandey, *et al.* 2011). N_e for all SNPs and just SNPs on the assembled X chromosome shown.

Population	N_e	X Chr N_e
Replicate 1 Control – 1	650	619
Replicate 1 Control – 2	781	479
Replicate 1 Heat stress	978	640
Replicate 1 Oxidative stress	1602	1449
Replicate 2 Control – 1	1051	1507
Replicate 2 Control – 2	821	805
Replicate 2 Heat stress	654	528
Replicate 2 Oxidative stress	379	557

Table 2

Expected effective population sizes for the three experimental evolution conditions given the cyclical census population sizes described in Sikkink *et al.* (2015). Ranges come from different intermediate generation census population sizes. N_e values calculated using equation 3.5 in Kimura (1983).

Population	Expected N_e
Control evolved	~2,500 – ~2,800
Heat stress evolved	~5,000 – ~6100
Oxidative stress evolved	~3,500 – ~4600

339   with reduced recombination rates (Burri *et al.* 2015).

340   We cannot directly address this with pool-seq data, which is one limitation of our resequencing

341   approach (Franssen *et al.* 2015). However, any form of linked selection would be expected to

342   bias the results in favor of finding apparent pleiotropy, since linkage is another important source

343   of genetic correlation among traits (Lande 1984). The larger the haplotype block, the higher the

344   probability that two genes with independent effects on a suite of traits display a correlated

345   response to selection. It is therefore likely that we

346   have actually overestimated the degree of shared pleiotropy

347   within the 5kb genomic windows of our analysis.

348   **EFFECTIVE POPULATION SIZE**

349   Effective population size is an important parameter in experimental evolution, as effective

350   population size is directly related to the relative opportunity and strength of random genetic drift
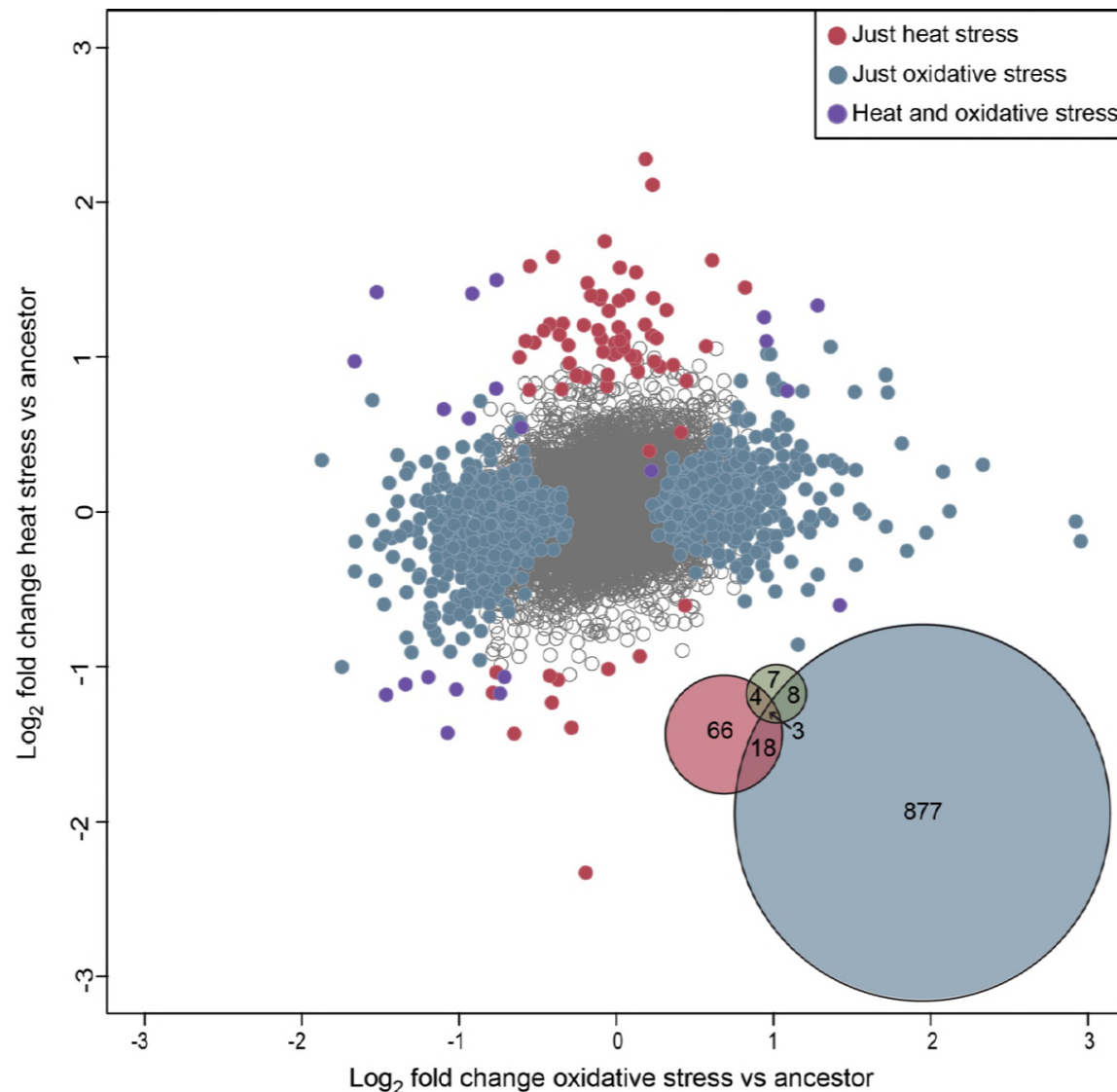
Figure 3

Correlation between $\log_2$ fold change of heat stress evolved vs. ancestor and oxidative stress evolved vs. ancestor tests for differential gene expression. The Venn diagram in the lower right shows the overlap in identity of significantly differentially expressed genes in the heat stress evolved, oxidative stress evolved and control evolved populations.

351   versus selection in a population (Kimura 1983). Estimates of effective population size (N_e) for

352   each of the evolved populations ranged from 379-1602 (Table 1). This is much smaller than the

353   expected N_e given our census population sizes (Table 2), which fluctuated between 1,000–24,000

354   worms (Sikkink *et al.* 2015). We also obtained N_e estimates using SNPs found only on the X

355   chromosome. *C. remanei* has an XX/XO sex determination system (Hodgkin 1987; Thomas *et*

356   *al.* 2012) so we would expect N_e on the X chromosome to be around 75% of the N_e on the

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357   autosomes. However, our estimates show X chromosome N_e values that range from 60% to

358   146% of the all SNP N_e estimates (Table 1).

359   **PLEIOTROPIC STRUCTURE OF GENE EXPRESSION**

360   The lack of correlated response to selection in the stress resistance phenotypes is echoed at the

361   level of the molecular phenotype of gene expression as well. In this case, however, many

362   thousand responses can be interrogated simultaneously. Here, changes in expression are based on

363   the comparison of baseline expression in the evolved populations versus those in the ancestor

364   population. The numbers of differentially expressed genes greatly differ across selective

365   environments (906 in the oxidative stress evolved population versus 91 in the heat stress evolved

366   populations), and 21 genes are in common between the two groups (Figure 3). One of the

367   common significantly differentially expressed genes is a putative member of the HSP70 family

368   that promotes protein binding in response to stress and constitutively, but the other shared

369   differentially expressed genes were not part of any canonical stress response pathways (File S2).

370   In addition, we observed an interesting pattern in the directionality of the evolution of

371   gene expression. Genes differentially expressed in the heat stress evolved population were more

372   likely to be up-regulated than down-regulated compared to both the oxidative stress evolved

373   population (Fisher's exact test, $p = 1.7 \times 10^{-8}$) and the control population (Fisher's exact test, $p =$

374   1.9×10^{-4}). However, the proportions of up-regulated versus down-regulated differentially

375   expressed genes in the oxidative stress evolved population were not significantly different from

376   the control population (Fisher's exact test, $p = 0.29$). There was also a weak correlation between

377   changes in gene expression between oxidative-stress evolved and heat-stress evolved populations

378   ($r^2 = 0.049$, $p = 0.01$), but this correlation appears to be largely driven by a relationship between

379   the genes that have not changed in expression very much (i.e., it is a weak aggregate response

380   over many genes).

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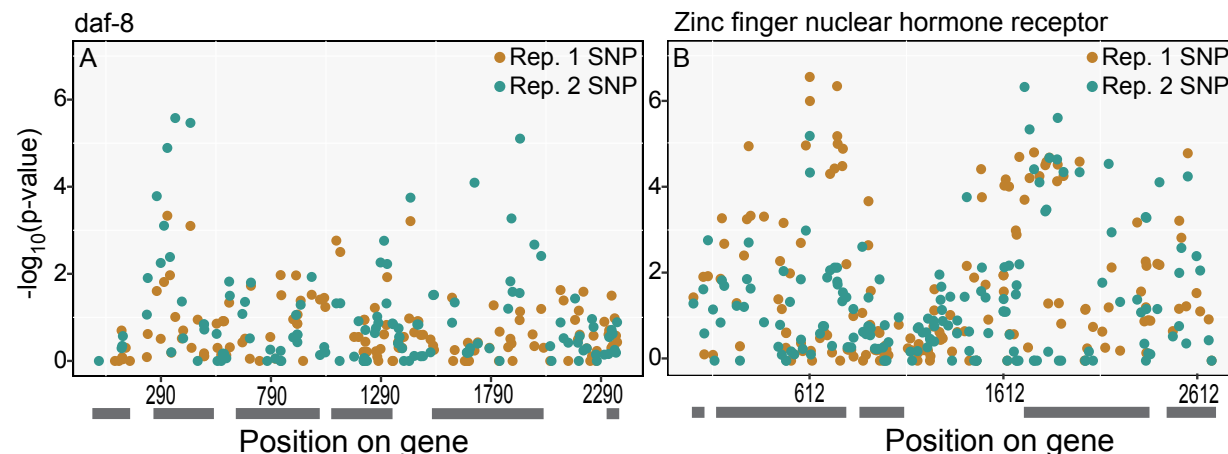**Figure 4**

(a) –log10 *p*-values of Fisher's Exact Test for each SNP in *daf-8* in the heat stress evolved populations. *daf-8* affects dauer development and is a component of the TGF-β signaling pathway (Savage-Dunn 2005). Grey bars indicate exons. (b) –log10 *p*-values of Fisher's Exact Test for each SNP in a zinc finger nuclear hormone receptor (*nhr-27*). This gene has lowest mean Fisher's Exact Test *p*-value of the differentiated genes in the heat stress evolved populations. Grey bars indicate exons.

CANDIDATE GENES UNDER SELECTION FOR ACUTE HEAT STRESS AND

ACUTE OXIDATIVE STRESS

Genes implicated as potentially being involved in the phenotypic response to selection, either via differences in gene expression or divergence due to allele frequency changes tended to create non-overlapping sets among the stress-response environments. Specifically, only three genes were found to be in common for the differential expression and genomic divergence gene sets within the heat stress evolved populations and 16 in common for the oxidative stress evolved populations. None of the shared genes had annotations associated with, or were orthologs of, genes known to be relevant to stress resistance (Files S2 and S3).

Differentiated genes due to allele frequency changes: There were 591 genes contained within significantly differentiated genomic windows in the heat stress evolved populations and 590 genes contained within significantly differentiated genomic regions in the oxidative stress evolved populations. Of those differentiated genes, 126 are shared in common between the heat stress and oxidative stress evolved populations. There are hundreds of other genes in these

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395   regions (File S3) and, again, for the most part they do not contain members of the canonical

396   stress response pathways that have been determined via mutagenesis approaches.

397   We did identify a small number of stress response pathway candidate genes found within

398   the differentiated genome regions in the heat stress evolved populations: a gene with a heat

399   shock chaperonin-binding motif, *daf-8*, three insulin-like growth factor binding proteins, and *Ras*

400   (File S3). The heat shock chaperonin-binding motif is found on the stress-inducible

401   phosphoprotein STI1, known to bind heat stress proteins (Song *et al.* 2009). *daf-8* (Figure 4A) is

402   a member of the TGF-β pathway, one of the signaling response pathways involved in heat shock

403   response in *C. elegans* (Rodriguez *et al.* 2013). Insulin like growth factor binding proteins

404   control the distribution and activity of insulin like growth factor receptor, an important stress

405   response transmembrane receptor protein (Rodriguez *et al.* 2013; Murphy and Hu 2013). Finally,

406   *Ras* is a member of the MAPK signaling pathway, which has been found to be responsive to

407   pathogen (Ewbank 2006) and endoplasmic reticulum stress (Darling and Cook 2014). There is

408   one candidate gene in the oxidative stress evolved populations with a known stress response

409   function: SKI-interacting protein, which is a member of the TGF- β signaling pathway that

410   promotes dauer formation when active (Savage-Dunn 2005).

411   **Differentially expressed genes:** Similar to the pattern described above for differentiated genes,

412   only a handful of the significantly differentially expressed genes have known or putative

413   functions in abiotic stress response. There were significantly up-regulated heat shock proteins

414   (HSPs) in both the heat shock evolved and oxidative shock evolved populations, and up-

415   regulated insulin like growth factor binding proteins in the oxidative shock evolved population.

416   Similar to previous work (Sikkink, Ituarte, *et al.* 2014), we find that up-regulated HSPs are

417   significantly enriched in the heat stress evolved population (Fisher's exact test, $p = 0.00146$), as

418   well as in the oxidative stress evolved population (Fisher's exact test, $p = 0.029$). In addition to

23

419   up-regulated HSPs, SKN-1, a transcription factor whose regulatory targets are important for

420   oxidative stress resistance (An and Blackwell 2003; Murphy and Hu 2013), was also

421   significantly up-regulated in oxidative stress evolved population.

DISCUSSION

423   Whole organism phenotypes are the combined result of the effects of and interactions

424   among tens of thousands of genes. The classic assumption within the field of quantitative

425   genetics is that majority of phenotypic variation found within natural populations is quantitative,

426   with the phenotypic differences among individuals likely caused by a large number of genes

427   operating within a complex environmental context (Hill 2009). Yet over the last two decades a

428   small controversy has emerged about whether we expect the genetic basis of adaptation to be

429   dominated by a few genes of major effect or whether it will be highly polygenic (Orr and Coyne

430   1992; Orr 2009; Rockman 2012). While this question is obviously most relevant in the context of

431   evolution within specific natural populations, there is an unavoidable conflation of adaptation,

432   demographic history, and chance events for any particular realization of an evolutionary lineage

433   that makes it impossible to test specific hypotheses within a single instance. For example, even

434   in the case of putatively adaptive armor loss in sticklebacks, in which it initially appeared that

435   the same syndrome had been replicated many times independently during adaptation to fresh

436   water, we now know that the genetic basis of this adaptation in each case is generated by a

437   complex set of alleles that have likely been forged by thousands of generations of migration-

438   selection balance (Colosimo *et al.* 2005; Nelson and Cresko 2018). Experimental evolution

439   provides a means of addressing these questions in a controlled, repeatable manner. Our previous

440   work has demonstrated that the nematode *C. remanei* is a powerful system for experimental

441   evolution of stress resistance phenotypes (Sikkink, Reynolds, *et al.* 2014; Sikkink *et al.* 2015).

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442   Here, we use an evolve and re-sequence approach to probe the genetic basis of these phenotypic

443   changes.

444   Our data demonstrate that both heat stress response and oxidative stress response are

445   complex, polygenic traits, where hundreds of regions in the genome respond to selection (Figure

446   2) and hundreds of genes show significant changes in gene expression (Figure 3). This fits the

447   pattern shown by many other experimental evolution and artificial selection studies (Turner *et al.*

448   2011; Orozco-terWengel *et al.* 2012; Pettersson *et al.* 2013; Hirsch *et al.* 2014), in which

449   hundreds of differentiated genes and/or genomic regions have been identified that influence traits

450   as varied as seed size, novel temperature regimes and body size.

451   The infinitesimal model, which allows researchers to predict the effects of selection on a

452   trait without knowing the identity of the genes that underlie variation in that trait (Fisher 1918;

453   Hill 2009), forms the cornerstone of quantitative genetics (Falconer and Mackay 1996) and has

454   been very successful in plant and animal breeding (Hill 2014). Recent work in human genetics

455   has found that that the vast majority of human traits and diseases are caused by variation in

456   hundreds if not thousands of loci (Wood *et al.* 2014; Visscher *et al.* 2017). Taken in the context

457   of this larger body of work, our study supports the view that much variation is quantitative in

458   nature and responses to selection are generated by changes in hundreds of genes, even when

459   selection itself is extremely strong (Johansson *et al.* 2010; Pettersson *et al.* 2013; Hirsch *et al.*

460   2014).

461   **THE GENOMIC BASIS OF MUTATIONAL PLEIOTROPY**

462   Despite the expectation set by many molecular biology studies of stress response pathways in *C.*

463   *elegans* (Kenyon 2010; Rodriguez *et al.* 2013; Murphy and Hu 2013), we found little evidence

464   overall for pleiotropy for the genetic basis of acute heat stress resistance and acute oxidative

465   stress resistance. There little to no correlated response to selection for heat stress or oxidative

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466   stress for the other stress at the level of survival (Figure 1 combine parentheses) (Sikkink *et al.*

467   2015) and mixed evidence for a correlated response for changes in gene expression (Figure 3).

468   Finally, at the level of genomic response to selection, we also find little evidence for pleiotropy

469   between the heat stress evolved and oxidative stress evolved populations at the level of

470   individual genes (Figure 2).

471   This of course does not mean that pleiotropy is not a characteristic of any of the genes

472   involved in stress response network, simply that it is not for those that respond to selection here.

473   This discrepancy could be due to a number of different factors. First, a correlated response to

474   selection is generated by the alleles that actually respond to selection, not all possible alleles for

475   a gene (Lande 1979). It is possible that many of the stress response genes that have been

476   identified via mutagenesis have broadly pleiotropic effects such that they cannot respond to

477   direct selection for increased stress resistance per se because of potential negative pleiotropic

478   effects on other traits (e.g., developmental rate, reproduction). That is, the genes that respond to

479   selection are not major genes with many pleiotropic effects, since they would likely have some

480   other negative pleiotropic effects. Second, molecular geneticists and quantitative geneticists

481   focus on related but subtlety different definitions of the term pleiotropy. Most fundamentally,

482   pleiotropy is a property of alleles, not genes (Phillips and McGuigan 2006), and so it is possible

483   for there to be variation in pleiotropy at a single locus such that some segregating alleles will

484   lead to a correlated response to selection while other will not. For stress resistance, we do not

485   find a clear signal for canonical stress response pathway members in the first place, and so

486   variance in pleiotropy at genes defined via measures of molecular pleiotropy still does not appear

487   to be the primary driver here, regardless of any potential variation in allelic effects.

488   **THE GENETIC AND GENOMIC BASIS OF THE RESPONSE TO SELECTION**

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489   While there are a handful of genetically differentiated and differentially expressed genes

490   with functions that are directly related to abiotic stress response, it is clear that the response to

491   acute heat stress and acute oxidative stress cannot be explained by these genes alone. We do not

492   find evidence that many of the canonical stress response genes, including *hsf-1* or *daf-16*

493   (Rodriguez *et al.* 2013; Murphy and Hu 2013) for heat stress and *pink-1, lrk-1* and *sod-1, -2* or *-3*

494   for oxidative stress (Rodriguez *et al.* 2013) responded to selection for acute heat stress or acute

495   oxidative stress survival, although *skn-1* is constitutively up-regulated in the oxidative stress

496   evolved population. This is further evidence that gene knockouts can paint an incomplete picture

497   of the genetic architecture of a phenotype of interest and the fact that the genes that respond to

498   selective pressures in a population are unlikely to be highly pleiotropic genes of large effect.

499   We found hundreds of genomic regions that appeared to respond to selection for either

500   acute heat stress or acute oxidative stress resistance. If we assume that regions are independent of

501   each other (i.e. in linkage equilibrium) then our results support the quantitative genetics

502   worldview that variation in most phenotypes is due to many alleles, presumably of small effect

503   (Falconer and Mackay 1996). However, some clustering of the genomic response can be clearly

504   seen in Figure 2. For example, 22% of the differentiated windows in the two heat stress evolved

505   populations are found on the X chromosome. When we look at strongly selected genes we do see

506   evidence of local selective sweeps, where multiple SNPs have similarly highly significant

507   Fisher's exact test *p*-values and changes in allele frequency (Figure 4B, Figure S8). This raises

508   the possibility that linkage disequilibrium is responsible for some of the response to selection in

509   the evolved populations. Although we found that linkage disequilibrium decays to background

510   levels within 200 bp within the ancestor population (Figures S4 and S5), it is possible that

511   linkage disequilibrium developed over the course of the experimental evolution project, due to

512   small effective population sizes (Table 1). However, the same clustering of genomic response is

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513   not seen in either of the oxidative stress evolved populations (Figure 2), where at most 10% of

514   differentiated windows were found on one contig (that contig not shown in Figure 2).

515   We find that most our estimated effective population sizes are about an order of

516   magnitude smaller than the post-selection census population size (Tables 1 and 2 combine

517   parentheses) (Sikkink *et al.* 2015). Fluctuating population sizes will reduce N_e (Kimura 1983),

518   and in our case very strong selection has the potential to strongly reduce the level of variation at

519   linked sites via the Hill-Robertson effect (Hill *et al.* 1966; Charlesworth 1996; Comeron *et al.*

520   2007; Charlesworth 2012). Even so, the reduction in N_e seems to be particularly severe relative

521   to the very large population sizes (for an animal) carefully maintained throughout the

522   experiment. For example, despite maintaining a census population size 10-20X larger than

523   similar experiments in *Drosophila melanogaster*, our estimated N_e values are only two–three

524   times higher than estimated N_e values for those populations (Orozco-terWengel *et al.* 2012;

525   Jonas *et al.* 2016). Differences in overall strength of selection may play a role here (strength of

526   selection is unknown in Jonas *et al*. 2016), but one additional source of reduction in N_e is

527   potential variance in mating such that male mating success and/or female reproductive output

528   displays a greater than Poisson variance (Wright 1931; Kimura 1983). This seems particularly

529   likely because the control populations have similar effective population sizes to the selected

530   lines. Variation in male mating ability has been previously documented within *C. remanei*

531   (Palopoli *et al.* 2015) and bears further investigation within the context of experimental evolution

532   within this species.

Conclusion

534   Organisms are complex, integrated systems and the manner in which natural variation percolates

535   though that system is likely to be highly structured by functional interactions. Although

536   pleiotropy may be ubiquitous throughout the genome, individuals with mutations in highly

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537 pleiotropic genes are likely to suffer from reduced fitness since those mutations will most likely

538 affect more traits than those under selection. As such, seeking to understand the genetic

539 architecture of traits solely via an analysis of gene knockouts is likely to leave an incomplete

540 picture of the overall structure of the pleiotropic network, especially with respect to segregating

541 variation within natural populations. Using standing genetic variation, experimental evolution

542 and whole genome sequencing therefore provides a powerful toolkit for understanding the

543 genetic architecture of complex traits, no matter how complex they ultimately prove to be.

Acknowledgments

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