Signals of selection beyond bottlenecks between exotic populations of the bull-headed dung beetle, *Onthophagus taurus*

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Melissa H. Pespeni[1, 2] * and Armin P. Moczek[2]

1 Department of Biology, University of Vermont, 109 Carrigan Drive, Burlington, Vermont 05405
2 Department of Biology, Indiana University, 915 E. 3rd Street, Bloomington, IN 47405

Author of correspondence:
Melissa H. Pespeni
e-mail: mpespeni@uvm.edu

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Abstract

Colonization of new environments can lead to population bottlenecks and rapid phenotypic evolution that could be due to neutral and selective processes. Exotic populations of the bull-headed dung beetle (*Onthophagus taurus*) have differentiated in opposite directions from native beetles in male horn-to-body size allometry and female fecundity. Here we test for genetic and transcriptional differences among two exotic and one native *O. taurus* populations after three generations in common garden conditions. We sequenced RNA from 24 individuals for each of the three populations including both sexes, and spanning four developmental stages for the two exotic, differentiated populations. Identifying 270,400 high quality SNPs, we revealed clear genetic differences between the three populations, and evidence of recent bottlenecks within and an excess of outlier loci between exotic populations. Differences in gene expression between populations were greatest in pre-pupae and early adult life stages, developmental stages during which differences in male horn development and female fecundity manifest. Finally, genes differentially expressed between exotic populations also had greater genetic differentiation and performed functions related to chitin biosynthesis and nutrient sensing, possibly underlying allometry and fecundity trait divergences. Our results suggest that beyond bottlenecks, recent introductions have led to genetic and transcriptional differences in genes correlated with observed phenotypic differences.

Keywords: population genomics, developmental transcriptomics, allometry evolution

Introduction

Population colonization is likely to result in both bottlenecks and new selective pressures. Thus both neutral and selective processes can result in phenotypic differences between native and introduced populations. Understanding the efficacy of, interactions between, and phenotypic consequences of both neutral and selective processes in shaping early population differentiation are of fundamental interest to population biologists, as well as to those seeking to link micro- and macro-evolution of development to its population-biological context (Hendry, 2013). Recently established populations of exotic species present opportunities to detect signatures of both neutral genetic drift and selection, and their respective consequences, over short timescales. Such introductions often involve small propagule sizes and/or are followed by severe population contractions, while newly invaded locales commonly present novel or divergent selection pressures (Dlugosch and Parker, 2008; Estoup et al., 2016). Therefore, exotic populations provide a unique study system to link recent phenotypic differentiation to the underlying molecular mechanisms.

Differentiation in gene regulation can be an important developmental mechanism to enable successful colonization of, and rapid adaptation to, novel environments. Modest regulatory changes can enable substantial phenotypic change, potentially facilitating rapid adaptation to different environments (King and Wilson, 1975; Carroll, 2005; Whitehead and Crawford, 2006). In particular, genes that are expressed in a context dependent manner have reduced pleiotropic constraint and thus may be well positioned to contribute to the early stages of population adaptation to novel conditions via modifications of their regulation (Snell Rood et al., 2010; Hunt et al., 2011). Indeed, several studies have identified signatures of rapid divergence between species in conditionally expressed genes, such as sex-biased genes in *Drosophila melanogaster* (Perry et al., 2014), and tissue- and sex-specific genes in horned beetles (Snell Rood et al., 2010; Warren et al., 2014; Pespeni et al., 2017). However, few studies have taken advantage of recent population colonizations to assess potential relationships between geographic variation in allele frequencies and context-dependent gene expression and its evolution. Sequencing RNA presents the opportunity to generate and analyze both gene expression and genetic variation data simultaneously (De Wit et al., 2015) and recently has been used to identify polymorphisms associated with gene expression phenotypes in a variety of organisms (Fraser, 2011; Ishikawa et al., 2017; Rose et al., 2018). In addition, the maintenance of populations in common environmental conditions for multiple generations is a tool to reveal persistent, genetically controlled differences in gene regulation between populations (Kawecki and Ebert, 2004).

Native to the Mediterranean, the recently established populations of the bull-headed dung beetle *Onthophagus taurus* in Australia and Eastern North America represent an excellent system to investigate the role of neutral and selective forces in contributing to rapid population differentiation. Between 1975 and 1984, several thousand *O. taurus* were bred from a mix of Mediterranean stock populations and released on Western Australian farms to aid in the biological control of dung and dung breeding flies (Tyndale-Biscoe, 1996). Around the same time, *O. taurus* was also introduced to the Eastern United States where it was first recorded in northern Florida in 1974 (Fincher and Woodruff, 1975). Unlike the introduction to Western Australia, *O. taurus*' appearance in the Eastern US seemed to be the product of a single, accidental introduction involving a small founding population whose precise origin is unknown (Fincher and Woodruff, 1975). Further, while Western Australian *O. taurus* were subject to extensive re-harvesting and re-distribution, no such efforts were carried out in the Eastern US. *Onthophagus taurus* is now well established in both exotic ranges and in the Eastern US has undergone remarkable range expansion and climatic niche evolution (Silva et al., 2016). Most importantly for the purposes of this study, Eastern US and Western Australian populations have, since establishment, differentiated in opposite directions from native Mediterranean populations in a range of heritable traits (reviewed in (Casasa and Moczek, 2018)), with some of the most dramatic differences in (i) the scaling relationship between male horn length and body size (Fig. S1) and (ii) timing and degree of ovarian development in females and the resulting differences in female fecundity. Specifically, male *O. taurus* in Western Australia exhibit a higher body size threshold between horned, fighting morphs and hornless, sneaking morphs compared to their Eastern US counterparts (Fig. S1), while Western Australian females initiate ovarian development significantly earlier, invest more into ovarian maturation, and exhibit significantly higher fecundity compared to Eastern US females (Macagno et al., 2015). Both population differences are canalized, maintained in common garden conditions across generations (Beckers et al., 2015), and rival differences normally seen between species (Kijimoto et al., 2013).

In this study, we reared beetles from three populations, derived respectively from within the native range (Italy) and one from each of the two exotic ranges (Western Australia, Eastern

US, Fig. S1) in common garden conditions for three generations. We then sampled and sequenced RNA from 24 individuals from each population to identify genetic variants and their relative population frequencies and to measure transcriptome-wide gene expression variation under common garden conditions. For the divergent exotic populations, we sampled three beetles of each sex for each of four developmental stages chosen based on the developmental timing of the traits that have diverged between populations (late 3rd larval instar, pre-pupae day 1, pupae day 1, and adult day 4), whereas poor breeding performance of the native population restricted us to sampling adult stages only. Male body size-horn size allometries become first established during prepupal development and are measurably different among pupae of different exotic populations, whereas differential ovarian development does not manifest until females reach the young adult stage (Macagno et al., 2015). This experimental framework allowed us to test simultaneously for genetic, developmental, and transcriptional mechanisms underlying population differences and to assess the relative contributions of both neutral and selective processes to population differentiation. Specifically, we tested three main hypotheses: (i) that invasion history has left a neutral signature on transcriptome-wide population genetic variation, (ii) that gene expression variation between exotic populations matches predictions based on the sex- and developmental-stage specific timing of trait differences, and (iii) that the integration of gene function with genetic and transcriptional variation data reveals signals of rapid evolution that implicate the targets of natural selection. We generated, analyzed, and integrated genetic variation data for approximately 270,400 SNPs and gene expression data for 17,483 genes to address these hypotheses.

Methods

Beetle Collection and Husbandry

Adult beetles (~500 individuals) were collected from within their native Mediterranean range near Milan, Italy (IT), as well as near Busselton, Western Australia (AU) and Chapel Hill, North Carolina, U.S. (US) and sent to Bloomington, IN to initiate laboratory colonies. We reared beetles in common garden conditions in a walk-in insectary at 25°C, 40% humidity, and a 16:8 L:D photoperiod as previously described (Beckers et al., 2015; Macagno et al., 2015) for three generations, ensuring that different generations of the same populations were kept in separate colony containers, before setting up breeding pairs to collect offspring of different developmental stages for the present study. Each population was maintained in 2-3 insectary containers (54 cm length x 30 cm height x 34 cm width) filled half full with a 2:1 moist sand:soil mixture and fed twice per week with ~0.5 L defrosted, homogenized cow dung. The density of beetles in each colony container was maintained at 200-500 individuals with a 50:50 sex-ratio. This density is intermediate to the low natural densities in the U.S. and high densities in Australia.

To collect individuals at appropriate developmental stages, we set up cylindrical, light-impermeable breeding containers (1.5 L, 27 cm heigt x 7.2 cm diameter; further described in (Beckers et al., 2015)) with one female and two males per container and approximately 30 containers per population. After 4 days, brood balls from each container were collected. Offspring were allowed to develop within their native brood ball until they reached the second larval instar at which point they were transferred to 12-well plates containing standardized artificial brood balls as described in (Shafiei et al., 2001), and stored in incubators at 25°C and a 16:8 L:D photoperiod. From this point, we monitored developmental stages daily. This approach allowed us to collect sufficient samples for each developmental stage as described below for both Western Australian and Eastern US populations. In contrast, while we were able to maintain the colony successfully in the lab, parents from Italy did not produce a sufficient number of brood balls to allow a comparable developmental sampling from this population. Subsequent work has since shown that native and exotic populations appear to have diverged in their reliance on an adult diapause (Macagno et al., in preparation) which may explain the poor breeding performance of native Italian *O. taurus* during this study.

Developmental Stages and Sampling

We sampled three individuals per sex for each of four developmental stages for both the Western Australian and Eastern US populations, for a total of 24 individuals per population. We chose this sampling scheme to maximize the diversity of transcripts expressed and sampled across a range of developmental stages that could underlie differences among populations. We selected the following stages due to their relationship with the timing of horn development in males and reproductive investment in females: late 3rd larval instar (L3L), pre-pupa day 1 (PP1), pupa day 1 (PD1), and young adults 4 days after eclosion to adult stage (AD4). L3L instars are characterized by having nearly completed their primary feeding stage. PP1 have completed the gut purge and begin the proliferation of future pupal and adult tissues as well as the breakdown of larval specific structures. While externally resembling larvae, it is at this PP1 stage that head development undergoes rapid remodeling and future horn tissue is undergoing especially rapid cell proliferation. PD1 then marks the first day post pupation. At this stage the specification of pupal morphology is complete, pupal horns are externally visible, and remodeling of pupal epidermis toward the final adult shape and size is initiated (Moczek, 2007; Kijimoto et al., 2010)). Lastly, the AD4 stage marks the stage of extensive gonadal differentiation and maturation (Macagno et al., 2015). We sampled only one individual per breeding pair in order to maximize genetic diversity sampled for population genetic and gene expression analyses. For L3L and PP1 stages, the head and thorax were separated from the abdomen (to exclude the gut and associated gut microbiome) and flash frozen. For PD1 and AD4 stages, whole bodies were flash frozen.

In contrast, due to the poor breeding performance of the Italian population (see above) we were only able to sample six individuals from the AD4 stage for this population. To keep sample sizes across populations for population genetic analyses (n = 24), we randomly sampled an additional 9 males and 9 females from the lab colony. We did not observe any indications of inbreeding in the colony or genomic data among sampled individuals.

RNA Extraction, Library Preparation, and Sequencing

We homogenized the tissue in 600 ul RLT buffer from the Qiagen RNeasy kit (Valencia, CA) plus 10 ul beta-mercaptoethanol using three metal beads in the GenoGrinder (Spex, Metuchen, NJ) in manufacturer tubes for 2 minutes at 1500 rpm, allowed the samples to cool for 1 minute, and homogenized an additional 30 seconds at 1500 rpm. We pelleted the debris and used the supernatant to continue with total RNA extraction following manufacturer recommendations. RNA quality was validated using NanoDrop (Thermo Scientific, Wilmington, DE) and gel electrophoresis for all samples, and BioAnalyzer (Illumina, San Diego, CA) for a random subset of each developmental stage. High quality RNA went into cDNA library preparations using the TruSeq stranded mRNA library prep kit following manufacturer recommendations (Illumina, San Diego, CA). Each sample was individually barcoded and library quality for each sample was validated by BioAnalyzer showing a fragment size distribution between 200 and 700 bp in length, with the mode at 300 bp on average. We sequenced the 72 libraries across six Illumina

HiSeq2000 lanes at the Genome Sequencing Facility at the University of Texas Health Science Center at San Antonio with an additional two lanes of sequencing for samples that yielded fewer reads. This resulted in an average of 18.7 million paired-end 100 base pair reads per sample for a total of 1.35 billion 2 x 100 bp paired-end sequences distributed across the 72 samples – deposited into the NCBI Sequence Read Archive (BioProject accession PRJNA594858).

Quality Filtering and Mapping

We cleaned raw sequence reads for base quality (minimum quality score 28) and length, removing any Illumina adaptors and retaining only matched and properly oriented left and right reads (Trimmomatic v. 0.33, (Bolger et al., 2014)). See supplemental file data_processing_commands.pdf for a list of all programs, versions, commands, and parameters for reproducing this work. We aligned the cleaned reads to the reference transcriptome (downloaded from the i5k genome project: https://i5k.nal.usda.gov/Onthophagus_taurus) using BWA aln function with default parameters (v. 0.7.12-r1039; Li and Durbin 2009). We used resulting sequence alignment files to generate both gene expression and SNP data for downstream analyses. To extract read count information for gene expression analyses (the number of reads that mapped uniquely to each gene), we used a custom Python script available from (http://sfg.stanford.edu/; De Wit et al. 2012). To prepare alignment files for SNP identification and genotyping, we merged individual sequence alignment files, sorted reads, and removed potential PCR duplicates using samtools and sambamba functions (see data_processing_commands.pdf for commands, program, and version details). We then used samtools mpileup and bcftools for SNP calling and individual genotyping. We used vcftools to filter the raw variant call format (.vcf) file for biallelic SNPs with a minimum depth of 10 reads and a minimum genotype quality of 20. We also filtered for zero missing data using the '--max-missing' flag in vcftools. Filtering to only include variants with no missing samples is an important step to exclude potential erroneous genotype calls due to differential splicing among sexes, developmental stages, or populations. Code for data processing can be found on GitHub: https://github.com/PespeniLab/otau_popgenomics.

Population Genomic and Gene Expression Analyses

We used vcftools to calculate several population genetic statistics: Weir and Cockerham's F_{ST} for each population pair, and Tajima's D and nucleotide diversity (pi) for each population. To visualize how variance in genotype partitioned among individuals from different populations, we used Principal Components Analysis implemented using the R package SNPrelate (Zheng et al., 2012) and only used SNPs with low linkage disequilibrium (excluded SNPs with $R^2 > 0.2$ between adjacent SNPs) to be more conservative. To identify potential outlier loci, we used BayeScan v2.1 (Foll and Gaggiotti, 2008; Fischer et al., 2011). To use BayeScan, we first used PGDSpider (v. 2.0.9.0) to make eigensoft files from the vcf files for each population pair (Lischer and Excoffier, 2011). We ran BayesScan with parameters of the chain left to default values (20 pilot runs, burn in = 50,000 generations, thinning interval =10) but adjusted the prior odds for the neutral model to 1000 to be more conservative, which set the prior probability that a given SNP was under selection to 1 out of 1,000. We calculated the Population Branch Statistic to quantify the relative allele frequency differentiation for each population relative to the other two populations based on the methods described in Yi et al. 2010 (Yi et al., 2010). Briefly, for each SNP locus, we calculated divergence time (T) between populations using Weir and Cockerham's F_{ST} for each population pair using the following equation, $T = -\log(1 - F_{ST})$. For each population, the pairwise divergence time estimates were used to calculate the Population Branch Statistic, e.g., $PBS_{AU} = (T_{AUvsUS} + T_{AUvsIT} - T_{USvsIT})/2$. PBS distributions among populations were compared using a Wilcoxon rank sum test.

To identify genes differentially expressed between the Eastern US and Western Australia populations, we used DESeq2 to set up models to test specific hypotheses and contrasts (v.1.14.1; (Love et al., 2014)). Gene expression analyses did not include samples from Italy because we were unable to collect all developmental stages from this population and because we were primarily interested in differences in expression between Eastern US and Western Australia populations, as these two populations have diverged morphologically and physiologically in opposite directions from their native Mediterranean range. To identify genes differentially expressed between populations and sexes at each developmental stage, we subset the data by stage and tested for an interaction between population and sex using the model ~ sex + population + sex:population. This approach, to subset the data by the devstage factor and test for a two-way interaction, yielded more accurate results compared to testing for a three-way interaction in DESeq2. To identify genes differentially expressed between populations controlling for differences between sexes and developmental stages, we used the model ~ devstage + sex + population and pulled out the contrast on population. Similarly, we pulled out the contrasts for sex and for developmental stage while controlling for each other factor. These models yielded similar results to using a group model (~ group) with the factors of population, devstage, and sex combined and pulling out specific contrasts of interest.

Exotic populations are likely to differentiate due to neutral processes such as founder effects, bottlenecks, and other demographic differences. To evaluate the extent to which gene expression differences between populations are the result of neutral versus selective processes, we compared the distributions of genetic differentiation (F_{ST}) among null models of gene expression variation to expression variation partitioned between populations, similar to Q_{ST}-F_{ST} comparisons (Feiner et al., 2017; Rose et al., 2018). To test if genes differentially expressed between populations showed greater levels of genetic differentiation (F_{ST}) than expected under neutrality, we tested for differences in F_{ST} distributions among genes differentially expressed between AU and US populations (overall, between females, and between males) versus genes expected to diverge neutrally (all genes and genes differentially expressed between developmental stages and sexes).

To test for non-random association of genetic and expression variation with specific functional classes of proteins, we first annotated the reference transcriptome using the NCBI blastp algorithm to align to the non-redundant (nr) database and to the UniProt (uniref90) database (provided through the Trinotate package from Trinity, (Grabherr et al., 2011)). We linked genes with protein functions by using UniProt identifiers from the blastp to uniref90 results to match the UniProt identifiers with Gene Ontology (GO) categories using the UniProt database (http://www.uniprot.org/). To test for functional enrichment, we used a rank-based Mann-Whitney U test to identify Biological Process categories that contained genes with higher than expected values for F_{ST} and positive association between individual genotype and gene expression phenotype (Kenkel and Matz, 2016).

To test for a relationship between genotype and gene expression phenotype, we used a publically available scripts in the repository vcf2eqtl (https://github.com/noahrose/vcf2eqtl) that tests for allelic imbalance in heterozygous individuals using beta-binomial distribution and likelihood ratio tests, then uses regression (lm) in R (R Core Team, 2015) to test for an association between genotype and gene expression. Code for data analyses can be found on GitHub: https://github.com/PespeniLab/otau_popgenomics.

Results

Genetic differentiation among native and exotic populations

We identified on average of 270,400 SNPs across 17,483 genes between pairs of population after quality control filtering. Considering only a conservative set of 59,375 independent SNPs after filtering for linkage disequilibrium, we found strong partitioning of genetic variation among the three populations with PC1 separating Italy (IT) and Western Australia (AU) from Eastern US (US) and PC2 separating IT and AU (Fig. 1).

To test for signatures of population contractions or bottlenecks, we used Tajima's D to compare observed (nucleotide diversity, pi) versus expected (segregating sites, S) levels of genetic diversity. We found that the transcriptome-wide average Tajima's D was close to zero for the native population from Italy, consistent with neutral evolution, while both exotic populations exhibited average Tajima's D values greater than zero, indicating excess pairwise nucleotide differences and recent population contractions (Table 1, Fig. S2). The Eastern US population, likely founded by the smallest number of individuals, had the greatest number of genes with Tajima's $D > 2$, due to fewer rare alleles than expected, suggestive of a bottleneck (Table 1, Fig. S2). All three populations had different Tajima's D distributions (KS test, $P < 0.0001$, Fig. S2). We used F_{ST} to identify loci with genetic variation partitioned among population pairs. The exotic populations, Western Australia versus Eastern US, had the most F_{ST} outlier loci (49) despite having the same mean F_{ST} as the Italy-Eastern US comparison (Table 2). This F_{ST} distribution was also the only to show enrichment for high F_{ST} in genes with specific functional roles (Table 2). Enriched GO biological process categories were related to translation, and metabolic and biosynthetic processes (Table 3). To quantify relative allele frequency divergence among population pairs, we calculated the Population Branch Statistic (PBS). We found that the Eastern US population showed longer branch lengths overall and in specific loci, suggesting more rapid evolution in this population (Fig. S3).

Transcriptional differences between exotic populations after three generations in common conditions

After filtering for depth, we tested for differences in expression for 16,851 genes among populations (Eastern US and Western Australia only), developmental stages and sexes. Partitioning gene expression variance using principal components analysis, we found that developmental stage followed by sex at later developmental stages shaped global transcription patterns (Fig. 2). PCA revealed an arch of transcriptional profiles through the developmental trajectory starting with larvae (L3L), followed by pre-pupae, where sex-specific development is initiated, then pupae, where sexes further differentiate, culminating in the fully elaborated differences between the sexes at the early adult stage (Fig. 2).

Using a model including population, sex, and developmental stage, we found that after multiple generations in common garden conditions 306 genes were differentially expressed between populations (1.8% of all genes), 1,331 genes were differentially expressed between sexes (7.9% of all genes; 89% of which were more highly expressed in males relative to females), and an average of 9,921 genes were differentially expressed between pairs of developmental stages (59% of all genes). Genes differentially expressed between populations were enriched for two functional classes of proteins: glucosamine-containing compound metabolic process (GO:1901071), whose protein members perform functions related to chitin exoskeleton biosynthesis, and vesicle-mediated transport (GO:0016192), involving 32 and 44 genes, respectively (FDR $P_{adj} < 0.05$).

Recall that male body size-horn size allometries first become established during prepupal development (Moczek and Nagy, 2005) and are measurably different among pupae of different exotic populations (Moczek and Nijhout, 2002a). In contrast, differential ovarian development between exotic populations manifests in females later during the young adult stage (Macagno et al., 2015). To test for gene expression differences that may underlie these developmental differences, we tested for interactions between sex and population for each of the four developmental stages. We identified 28 genes with sex by population interactive differences in expression, 21 genes at the early adult stage, four at the pre-pupal stage, two at the L3L stage, and one at the pupal stage (Fig. 3, FDR $P_{adj} < 0.05$). In adults, the patterns of expression showed strong upregulation in Eastern US compared to Western Australia females and strong downregulation in Eastern US males compared to Western Australia males (Fig. 3). In prepupae and pupae, we observed a strong signal of upregulation in Western Australia females (Fig. 3). Genes with a population by sex interaction perform functions related to metabolism and regulation of growth, development, and immune response, though over 40% of the genes perform uncharacterized functions (Table S1), suggesting they may be rapidly evolving or may be sufficiently novel in this species to not be recognized by sequence homology to the well-characterized genomes of *Drosophila melanogaster* and *Tribolium castaneum*.

Association between genetic and transcriptional differentiation between populations

Transcriptional phenotypes can diverge between populations due to neutral or selective processes. To test if expression differences between populations matched neutral expectations, we compared F_{ST} distributions of genes differentially expressed between populations to the transcriptome-wide F_{ST} distribution as well as to genes differentially expressed between sexes and developmental stages. We found that all categories of genes expressed in a population-specific manner showed a higher mean F_{ST} and a broader F_{ST} distribution than expected compared to the transcriptome-wide null distribution and to genes differentially expressed between sexes or developmental stages (Fig. 4, KS-test, $P < 0.0001$). Genes that were differentially expressed between populations were two times more likely to have high F_{ST} SNPs in the 10% quantile of the F_{ST} distribution compared to the transcriptome-wide null (Fisher's Exact Test: Chi-square = 36.08, df =1, $P < 0.0001$). Specifically, twenty one percent of genes differentially expressed between populations had high F_{ST} (65 out of 306), while only 10% of genes not differentially expressed between populations had high F_{ST} (812 out of 8,449).

This positive association between transcriptional and genetic differences between populations prompted us to test for associations between individual genotype and gene expression phenotype. Excluding reads that map to SNP sites, we identified 27 SNPs in 19 genes where the genotype of the individual was associated with the expression level of the gene (FDR $P < 0.05$; Table S2). One particular gene of interest was phosphatidylinositol N-acetylglucosaminyltransferase, which is a membrane protein important in sensing nutrients and coordinating transport, signaling and metabolism (Moussian, 2008, Fig. 5A). We also found that the 27 expression-associated SNPs were closer to the transcription start site in their respective genes compared to the positions of all other SNPs not associated with expression phenotypes (Fig. 5B; $P < 0.05$), suggesting an increased likelihood for a regulatory role for these polymorphisms.

Lastly, we used functional enrichment to test if the positive association between expression and allelic differences between populations was due to neutral versus selective processes. Matching predictions for differentiation driven by selective processes, we found that genes that showed a positive relationship between expression and allelic differentiation between populations were non-randomly concentrated in six functional classes of proteins related to transport, translation, amino acid modification, and localization in the cell (Table 4, Mann-Whitney U, FDR $P_{adj} < 0.05$).

Discussion

After the planned and accidental introductions of *O. taurus* to Australia and the Eastern United States, respectively, the exotic populations have differentiated in opposite directions relative to their native Mediterranean ancestors in fitness-related traits including male horn polyphenism and female fecundity (Moczek and Nijhout, 2002b; Macagno et al., 2015). While behavioral and ecological studies support the hypothesis that these trait differences reflect selective responses to differences in intra- and interspecific competition for breeding opportunities (Moczek, 2003), the potential contribution of neutral processes including population bottlenecks has been difficult to assess. Moreover, even though we know when during development trait divergences are initiated (Moczek and Nijhout, 2002b; Moczek and Nagy, 2005; Macagno et al., 2015), little is known about their transcriptional underpinnings. Taking a population transcriptomic approach, we were able to support our three main hypotheses, (i) that invasion history has left a neutral signature on transcriptome-wide population genetic variation, (ii) that gene expression variation between exotic populations shows sex- and developmental-stage specific differences in expression that parallel differences in trait differentiation between exotic populations in potentially informative ways, and (iii) that the integration of gene function with genetic and transcriptional variation data reveals signals of rapid evolution that implicate putative targets of natural selection. Specifically, we found strong genetic differentiation among populations and signatures of recent population bottlenecks in the introduced populations (Fig. 1 and Table 1). We also identified a portion of the transcriptome (2-5%) that was differentially expressed between exotic populations (Figs. 3-4) and found that these genes had greater genetic differentiation between populations than expected under neutral evolution (Figs. 4 and 5). These differentiated genes perform functions related to glucosamine metabolism and transport (Tables 3 and 4), potentially underlying phenotypic differences as they relate to chitin biosynthesis and nutrient sensing. In sum, these results identify allelic and expression variation that has evolved under both neutral and selective processes to contribute to the rapid population differentiation among exotic *O. taurus* populations.

Signatures of neutral and selective processes in population genetic variation

Our results from an average of 270,400 SNPs per population pair suggest that there are clear genetic differences between populations (Fig. 1) but that these differences are likely due to their recent population contractions with their introductions as evidenced by the elevated Tajima's D -- both transcriptome-wide and in specific genes -- a pattern found only in the two exotic populations (Table 1). For a given gene, Tajima's D compares the observed average number of pairwise nucleotide differences between all sequenced individuals in a population (pi) to expected levels of diversity based on the number of segregating sites (S) across a gene sequence considering all individual sequences at once. Under neutrality, these values should be equal. However, a recent population contraction will result in a loss of rare variants yielding an excess of pairwise nucleotide differences relative to few segregating sites (Tajima's D >> 0). Matching expectations based on their differences in introduction, the signature of a bottleneck is stronger in the accidental introduction to the Eastern United States in contrast to the deliberate introduction to Western Australia. Reassuringly, genetic patterns in our population sampled from the native distribution (Italy) match expectations based on neutrality (Tajima's D = 0).

Even though most of the genetic variation across the transcriptome reflects a neutral demographic signature of population contractions, some genetic variation could be differentially segregating between exotic populations due to natural selection. We find that exotic populations had 2.5 times more F_{ST} outliers when compared to each other (49) than when the Italian population was compared to either Eastern US (20), or 16 times more when compared to Western Australian *O. taurus* (3), respectively. The greater number of F_{ST} outliers between exotic populations taken together with the signatures of population bottlenecks described above suggests that F_{ST} outliers between exotic population could be driven by reduced effectiveness of purifying or background selection due to smaller effective population sizes (Charlesworth, 1994, 2009). However, if genetic differences were driven solely by neutral demographic processes, we would not expect differentiated loci to be concentrated in specific functional classes of genes. In addition, matching non-neutral expectations, we find that only the F_{ST} distribution comparing exotic populations shows functional enrichment (Table 2). Lastly, we find a stronger signal of differentiation in the Eastern US population considering pairwise Population Branch Length statistics. This result is congruent with recent studies that suggest that the US population has undergone niche expansion and potential local adaptation (Silva et al., 2016; Rohner and Moczek, 2020).

Estimating allele frequencies from RNAseq data has the potential to introduce bias due to differences in allelic expression or post-transcriptional modifications among individuals (Konczal et al., 2014). However, the potential impact of any bias in genotype data can be minimized by sequencing from individuals rather than pools of individuals and by using sufficient SNP filtering strategies including minimum depth of coverage, genotype quality, and no missing data across samples, the latter of which precludes the potential for biased expression due to factors such as tissue, developmental stage, sex, or population (Su et al., 2008; Rogier et al., 2018). We utilized each of these measures in the present study. In addition, allelic ratio deviations greater than 70:30 have been shown to be rare (Serre et al., 2008), which suggests that if genotyping from RNAseq data from individuals with sufficient sequencing depth, genotypes of heterozygotes are unlikely to be impacted by biased allelic expression (Skelly et al., 2011). Lastly, in the present study, we did not see extensive evidence for cis-eQTL, which suggests no evidence of systemic bias in genotype data based on sequencing RNA. Future work however, could further investigate top gene candidates and validate population allele frequency estimates.

Signatures of both neutral and selective processes in transcriptional variation between populations

Variation in developmental stage contributed the greatest amount of transcriptional variation, followed by sex (Fig. 2). However, approximately 5% of the transcriptome showed differential expression between populations. In testing for interactions between sex and population at each stage, we found the strongest differences at the adult stage with upregulation of genes in Eastern US versus Western Australia females (Fig. 3). Because adult Western Australian females invest significantly more into reproduction than their Eastern US counterparts (Macagno et al., 2015) and reproductive effort is generally associated with increased gene expression (Baker and Russell, 2009), we predicted adult Western Australian females to exhibit higher expression than Eastern US females. However, we observed the opposite - relatively lower expression in Western Australian females compared to Eastern US females (Macagno et al., 2015). Such a signature would, however, be consistent with an evolved change in inhibitor function during ovarian maturation, delaying it in Eastern US females but accelerating it their Western Australia counterparts. Follow-up comparative functional studies would be needed to test this interpretation. Alternatively, this result may be a consequence of assessing gene expression based on whole body RNA extractions. Gene expression in *O. taurus*, like most organisms, is highly tissue-specific (Snell Rood et al., 2010; Pespeni et al., 2017). RNA extractions for the present study included the entire head and thorax for larvae and pre-pupae and whole bodies for pupae and adults – a design chosen to maximize sequencing coverage across the whole transcriptome to be able to generate SNP and expression data for as many genes as possible. Yet a potential downside of this design is its reduced ability to reveal possible differences in the expression of key regulators operating in a specific tissue.

Positive association between genetic and transcriptional variation

Sequencing expressed transcripts allows the simultaneous generation of both genetic variation and transcriptional variation data independent of each other (De Wit et al., 2015). For variant data, the unit of analysis is a given base pair across all samples, whereas for transcript abundance quantification the unit of analysis is represented by all the reads mapping to a given transcript for each sample. Here we used both types of data to test for a relationship between genetic and transcriptional variation using three different approaches, a simplified Q_{ST}- F_{ST} approach to compare variance in expression phenotypes to variance expected based on neutral genetic divergence, tests for functional enrichment, and a test for expression QTLs linking expression phenotypes to individual genotypes. For a given gene, a positive relationship between genetic and transcriptional variation would suggest that variants segregating between populations are likely in linkage disequilibrium with putatively causal *cis*-regulatory variants that result in differences in gene expression.

We found that genes differentially expressed between populations had a significantly higher mean F_{ST} and broader F_{ST} distribution than expected based on the transcriptome-wide neutral null distribution. Functional enrichment tests showed that genes with both genetic and transcriptional differences were more likely to perform functions related to vesicle-mediated transport, ion and proton transport, translation, amino acid modification, and localization in the cell suggesting non-neutral or selective processes driving both genetic and transcriptional differences in given genes. In addition, we identified 27 SNPs in 19 genes where expression patterns differed based on individual genotype (*cis*-eQTL; Fig. 5). One particularly interesting gene with a cis-eQTL was phosphatidylinositol N-acetylglucosaminyltransferase (PIGA), a gene that plays a key role in the formation and function of extracellular matrices (Moussian, 2008) as well as the regulation of basic developmental signaling pathways including signaling via the Hedgehog and Decapentaplegic pathways (Nishihara, 2010) known to affect beetle horn development in *O. taurus* (Wasik and Moczek, 2011; Kijimoto et al., 2012).

Efforts in *Drosophila* have found extensive evidence for divergence in cis-regulatory variation within and between species (Wittkopp et al., 2008, 2009; Wittkopp and Kalay, 2012). Recent work in other study systems has also found *cis*-regulatory variation driving species divergence in coral (Rose et al., 2018) and ecotype divergence in cichlid and stickleback (Parsons et al., 2016; Ishikawa et al., 2017). Work in these fish in particular suggests that expression QTL are specific to environmental conditions in which they are measured, different salinities or modes of feeding, respectively (Parsons et al., 2016; Ishikawa et al., 2017). However, even in common garden conditions, we identify 27 *cis*-eQTLs. Further studies measuring gene expression in hybrid individuals across multiple conditions could reveal the relative contributions of *cis*- versus *trans*-regulatory elements.

Conclusions

Exotic *O. taurus* populations have diverged heritably in diverse morphological, physiological, and life history traits (reviewed in Casasa and Moczek 2018) and several studies have begun to examine the role of population phenology (e.g. Moczek 2003), ancestral plasticity (Casasa and Moczek 2018, Rohner and Moczek 2020) or physiological and developmental mechanisms (e.g. Macagno et al. 2011, 2015, 2018; Newsom et al. 2019) to population differentiation. Here we add a first understanding of the relative contributions of both neutral and selective forces to population divergences, and show that selective processes alongside signatures of neutral differentiation due to bottlenecks have contributed to the rapid differentiation among these populations. Sequencing RNA from individuals following generations of common garden rearing allows the generation of both transcriptome-wide genotype and genetically controlled gene expression variation data. Integration of these data with assessments of gene function then has the power to reconstruct the developmental evolution of key traits in response to both neutral and selective processes. Predicted and novel genes and pathways identified can be manipulated in future investigations to link genotype or expression phenotypes to ecologically and evolutionarily important traits, meeting one of the grand goals for understanding the origins of diversity in nature.

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Table 1. Population genetic diversity summary statistics.

Population	Tajima's D	No. genes with Taj.D > 2	No. genes with Taj.D < -2
Italy	-0.05	56	9
Western Australia	0.11	117	5
Eastern US	0.22	168	3

Table 2. Population genetic differentiation statistics.

Population comparison	No. SNPs	Mean F_{ST}	No. F_{ST} outliers	No. categories enriched for high F_{ST}
IT - AU	271,891	0.03	3	0
IT - US	275,708	0.05	20	0
AU - US	263,601	0.05	49	6

Table 3. Functional classes of genes enriched for high F_{ST} between Western Australia and Eastern US.

Category	GO ID	No. genes	$P_{adj.}$
translation	GO:0006412	68	0.000
regulation of cellular catabolic process	GO:0031329	7	0.015
ribonucleoside triphosphate metabolic process	GO:0009199	13	0.028
single-organism biosynthetic process	GO:0044711	71	0.028
regulation of autophagy	GO:0010506	5	0.034
nucleoside triphosphate biosynthetic process	GO:0009201	9	0.048

Table 4. Functional classes of genes enriched for positive association between gene expression variation and population genetic differences between Western Australia and Eastern US.

Category	GO ID	No. genes	$P_{adj.}$
vesicle-mediated transport	GO:0016192	43	0.016
translation	GO:0006412	68	0.035
ion transport	GO:0006811	42	0.035
peptidyl-amino acid modification	GO:0018193	9	0.035
establishment of localization in cell	GO:0051649	76	0.035
proton transport	GO:1902600	26	0.035

Figure legends

Figure 1. Statistical summary of population genetic structure based on principal component analysis of 59,375 SNPs with low linkage disequilibrium from individuals sampled from three populations.

Figure 2. Statistical summary of gene expression variation based on principal component analysis of 16,851 genes expressed in males and females from both exotic populations at four developmental stages, ordered from earliest to latest: late 3rd larval instar (L3L), pre-pupa day 1 (PP1), pupa day 1 (PD1), and adult day 4 (AD4; three individuals per developmental stage per sex). Population is not represented as a factor since most variation in gene expression was explained by developmental stage and sex.

Figure 3. Heat map of scaled gene expression for genes differentially expressed with population by sex interactions at each developmental stage ($P_{adj} < 0.05$).

Figure 4. Genetic differentiation (F_{ST}) in genes groups by differences in gene expression: all genes (null distribution; red), genes differentially expressed between developmental stages (orange), sexes (beige), and populations (blue). The transcriptome-wide null distribution represents neutrality for all genes that had both high quality genetic and gene expression data (N= 8,845) and genes that were differentially expressed between developmental stages, sexes, or populations (FDR $P_{adj} < 0.05$). Maximum F_{ST} was used for genes with multiple SNPs. Genes differentially expressed between populations had a significantly broader F_{ST} distribution compared to the neutral null transcriptome-wide F_{ST} distribution (KS test, $P < 0.0001$) while F_{ST} distributions for stage- and sex-specific genes did not differ from the null (KS test, $P > 0.05$).

Figure 5. Positive association between genotype and gene expression phenotype for 27 SNPs in 19 genes. (A) Normalized gene expression counts plotted by genotype at SNP position 1940 in the gene phosphatidylinositol N-acetylglucosaminyltransferase; colors indicate population of origin ($F_{ST} = 0.52$; DGE $P_{adj} < 0.0001$). (B) Distance from transcription start site (TSS) for SNPs significantly associated with expression phenotype and those not associated with expression phenotypes.

Figure S1. Distributions of native and introduced populations of *Onthophagus taurus* beetles and colony collection localities (A) and morphological differentiation in horn length to body size allometry in the Eastern US (B) and Western Australian beetles (C both relative to native Mediterranean beetles represented by Italy (in green). Panels b and c reproduced by permission from Springer, Ecological Genomics (Moczek et al., 2014).

Figure S2. Density plot of Tajima's D for all genes for each population: Italy from the native species range (green), Western Australia (red), and Eastern US (blue); rug plot below shows Tajima's D of individual genes; shading fills distributions. All three distributions are different from one another (KS test, $P < 0.0001$).

Figure S3. Scatter plots of Population Branch Statistics for each pair of populations where color corresponds to F_{ST} between the population pair (A-C) and a density plot comparing all three distributions (D; Wilcoxon rank sum test, $P < 0.0001$).