Signatures of rapid evolution in urban and rural transcriptomes of white-footed mice

*Peromyscus leucopus* in the New York metropolitan area

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Abstract

Urbanization is a major cause of ecological degradation around the world, and human settlement in large cities is accelerating. New York City (NYC) is one of the oldest and most urbanized cities in North America, but still maintains 20% vegetation cover and substantial populations of some native wildlife. The white-footed mouse, *Peromyscus leucopus*, is a common resident of NYC’s forest fragments and an emerging model system for examining the evolutionary consequences of urbanization. In this study, we developed transcriptomic resources for urban *P. leucopus* to examine evolutionary changes in protein-coding regions for an exemplar ‘urban adapter’. We used Roche 454 GS FLX+ high throughput sequencing to derive transcriptomes from multiple tissues from individuals across both urban and rural populations. From these data, we identified 31,015 SNPs and several candidate genes potentially experiencing positive selection in urban populations of *P. leucopus*. These candidate genes are involved in xenobiotic metabolism, innate immune response, demethylation activity, and other important biological phenomena in novel urban environments. This study is the first to report candidate genes exhibiting signatures of directional selection in divergent urban ecosystems.
Introduction

Urbanization dramatically alters natural habitats, and its speed and intensity will increase as nearly two-thirds of the world’s human population is predicted to live in urban areas by 2030 (Ibanez-Alamo et al. 2010). Understanding how natural populations adapt to ecologically divergent urban habitats is thus an important and immediate goal for urban ecologists and evolutionary biologists. Few ecological and evolutionary studies are conducted in urban environments (Martin et al. 2012), but recent attitude shifts and technological advancements have removed many of the obstacles to working on urban wildlife. Multiple studies have demonstrated that urban areas are biologically diverse, productive, and viable (Pickett et al. 2011), and the development of next generation sequencing (NGS) has facilitated the generation of genomic resources for uncharacterized species in natural environments (Hohenlohe et al. 2010; Rice et al. 2010; Storz et al. 2007). Understanding the genetic basis of adaptation in successful urban species will aid in future conservation efforts and provide insights into the effects of other anthropogenic factors, such as global climate change and evolutionary trajectories in human-dominated environments (Grimm et al. 2008; Pickett et al. 2011; White et al. 2005).

Cities typically experience a substantial decrease in biodiversity of many taxonomic groups as urban ‘avoiders’ disappear, accompanied by a rise in urban ‘exploiters’ that are primarily non-native human commensals such as pigeons or rats. Urban ‘adapters’ are native species that favor disturbed edge habitats such as urban forest fragments, relying on a combination of wild-growing and human-derived resources (Blair 2001; McKinney 2002, 2006). This last group is of primary interest for examining genetic signatures of recent evolutionary change in novel urban environments. Severe habitat fragmentation is one of the primary impacts of urbanization and often leads to genetic differentiation between populations (Wandeler et al. 2003; Shochat et al. 2006; Bjorklund et al. 2010). Introductions of new predators and
competitors alter ecological interactions (Peluc et al. 2008), and new or more abundant parasites or pathogens influence immune system processes (Sih et al. 2011). Air, water, and soil pollution typically increase in local urban ecosystems, and selection may favor previously rare genetic variants that more efficiently process these toxins (Francis & Chadwick, 2012; Whitehead et al. 2010; Yauk et al. 2008). Recent studies provide some evidence of local adaptation and rapid evolution in urban patches. Brady (2012) found rapid adaptation to roadside breeding pond conditions in the salamander, Ambystoma maculatum, and the weed, Crepis sancta, exhibited a heritable increase in production of non-dispersing seeds over only 5-12 generations in extremely fragmented urban tree pits (Cheptou et al. 2008). The genetic architecture of the phenotypes under selection has not been described for either of these urban ‘adapters’, but outlier scans of transcriptome sequence datasets are one promising approach (Sun et al. 2012).

Peromyscus spp. are an emerging model system for examining evolution in wild populations (Storz et al. 2007; Linnen et al. 2009; Weber et al. 2013), but large-scale genomic resources are not yet widely available. The genus contains the most widespread and abundant small mammals in North America, and Peromyscus research on population ecology, adaptation, aging, and disease has a long, productive history (Metzger 1971; O’Neill et al. 1998; Vessey & Vessey 2007; Wang et al. 2008). An increasing number of studies have demonstrated that Peromyscus spp. rapidly (i.e. in several hundreds to thousands of generations) adapt to divergent environments. These examples include adaptation to hypoxia in high altitude environments (Storz et al. 2007) and adaptive variation in pelage color on light-colored soil substrates (Linnen & Hoekstra, 2009; Linnen et al. 2009; Mullen & Hoekstra, 2008). P. leucopus is the sole Peromyscus spp. in New York City (J. Munshi-South, unpublished data), where it occupies most small patches of secondary forest, shrublands, and meadows within NYC parklands (Puth & Burns 2009; Munshi-South & Kharchenko 2010). The smallest patches in NYC often contain the highest population densities of white-footed mice (Ekernas & Mertes 2007), most likely due to
ecological release and obstacles to dispersal (Nupp & Swihart 1996; Barko et al. 2003).

Consistently elevated population density in urban patches compared to surrounding rural populations is predicted to result in density-dependent selective pressures on traits related to immunology, intraspecific competition, and male-male competition for mating opportunities, among others (Lankau 2010; Lankau & Strauss 2011).

White-footed mouse populations in NYC exhibit high levels of heterozygosity and allelic diversity at neutral loci within populations, but genetic differentiation and low migration rates between populations (Munshi-South & Kharchenko 2010; Munshi-South 2012). This genetic structure contrasts with weak differentiation reported for Peromyscus spp. at regional scales (Yang & Kenagy, 2009), or even between populations isolated on different islands for thousands of generations (Degner et al. 2007; Ozer et al. 2011). High genetic diversity within and low to nonexistent migration between NYC populations suggests that selection can operate efficiently within these populations, either on standing genetic variation or de novo mutations. In this study we take steps to develop P. leucopus as a genomic model for adaptive change in urban environments.

Pooling mRNA from multiple individuals is an effective approach to transcriptome sequencing that avoids the prohibitive cost of sequencing individual genomes (Boitard et al. 2012; Futschik & Schlötterer, 2010). While pooling results in the loss of genetic information from individuals, the ability to identify SNPs in a population increases due to the inclusion of multiple individuals in the pool (Gompert & Buerkle 2011). By analyzing SNPs within thousands of transcripts, it is feasible to identify candidate genes underlying rapid divergence of populations in novel environments (Bonin, 2008; Rice et al. 2010; Stinchcombe & Hoekstra, 2008; Ungerer et al. 2008). Certain statistical approaches, such as the ratio between non-synonymous and synonymous ($p_N/p_S$) substitutions, can be applied to pooled transcriptome data to identify potential signatures of selection between populations (Sloan et al. 2012; Sun et al. 2012).
If positive selection is acting on a codon, then non-synonymous mutations should be more common than under neutral expectations (Ellegren, 2008; Nielsen & Yang, 1998).

Here, we describe the results of de novo transcriptome sequencing, annotation, SNP discovery, and outlier scans for selection among urban and rural white-footed mouse populations. We used the 454 GS FLX+ system to sequence cDNA libraries generated from pooled mRNA samples from multiple tissues and populations. Several de novo transcriptome assembly programs were used and the contribution of specific tissue types to the transcriptome assembly was examined. We then identified coding region SNPs between urban and rural populations, and scanned this dataset for signatures of positive selection using $p_N/p_S$. We report several candidate genes potentially experiencing directional selection in urban environments, and provide annotated transcriptome datasets for future evolutionary studies of an emerging model system.

Materials and Methods

Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee at Brooklyn College, CUNY (Protocol No. 247), and adhered to the Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research (Sikes & Gannon 2011).

Study Sites and population sampling

$P. leucopus$ were trapped and collected from each of four urban and one rural site ($N = 20-25$ / population) for sequencing and analysis (total $N = 112$; Fig. 1). The four urban sites (Central Park, Flushing Meadows-Willow Lake, New York Botanical Gardens, and the Ridgewood Reservoir) were chosen due to their large area, isolation by dense urban matrix, high population density of mice, substantial genetic differentiation, and genetic isolation from other populations.
(Munshi-South & Kharchenko 2010; Munshi-South 2012). The rural site, Harriman State Park located ~68 km north of Central Park, is one of the largest contiguous protected areas nearby and the most likely representative of a non-urban population of mice in proximity to NYC. Mice were trapped over a period of 1-3 nights at each site using four 7x7 transects of 3”x3”x9” Sherman live traps. Mice were killed by cervical dislocation and immediately dissected in the field. Livers, gonads and brains were extracted, rinsed with PBS to remove any debris from the surface of the tissue, and immediately placed in RNALater® (Ambion Inc., Austin, TX) on ice before transport and storage at -80°C. These tissue types were chosen for initial analysis due to their wide range of expressed gene transcripts (Yang et al 2006) and potential roles in adaptation to urban conditions.

**RNA extraction and cDNA library preparation**

Total RNA was extracted and cDNA libraries were pooled for all five populations for four multiplexed plates of 454 sequencing. The first plate of sequencing was normalized to produce equalized concentrations of all transcripts present, potentially allowing enhanced gene discovery and greater overall coverage of the transcriptome (Babik et al. 2010). However, the normalization process introduces additional steps and biases in library preparation (Sloan et al. 2012), and resulted in a relatively low number of total high-quality 454 reads. Thus, non-normalized libraries were prepared using a modified protocol for the last three 454 plates.

For plate 1, total RNA was isolated from ~60 mg of liver (eight males and eight females / population), ~60 mg of testis (eight males / population), and ~60 mg of ovaries (eight females / population) for two populations using RNaqueous® kits (Ambion, Austin, TX). Individual RNA extracts were pooled by population and organ type and selected for mature mRNA using the MicroPoly(A)Purist™ kit (Ambion, Austin, TX). Next, mRNA pools were reverse-transcribed using the SMARTer™ cDNA synthesis kit (Clontech, Mountain View, CA), and normalized
using the Trimmer-Direct cDNA normalization kit (Evrogen, Moscow, Russia). Then, normalized cDNA pools were sequenced with multiplex identifiers using standard 454 FLX Titanium protocols. This pilot plate contained cDNA pools for Harriman State Park and Flushing Meadows-Willow Lake.

For plates 2-4, total RNA using Trizol® reagent (Invitrogen, Carlsbad, CA) was extracted from ~70 mg of brain tissue (four males and four females / population), ~70 mg of testes (eight males / population), and ~15 mg of liver (four males and four females / population). After DNAse treatment (Promega, Madison, WI) and pooling individual samples in equimolar amounts by population and tissue, the samples were treated with the RiboMinus™ Eukaryote kit (Invitrogen, Carlsbad, CA) to reduce ribosomal RNA. RNA pools were then reverse-transcribed using the Roche cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN) and sequenced with multiplex identifiers using standard 454 FLX Titanium protocols. Plate 2 included brain cDNA pools for all five populations, plate 3 included liver and testis cDNA for Central Park, Ridgewood Reservoir, New York Botanical Gardens, and Harriman State Park, and plate 4 included liver cDNA pools from all five populations. All raw sequencing files have been deposited in the GenBank Sequence Read Archive (SRA) under accession number SRP020005.

Transcriptome assembly

Two methods were used to assemble the best transcriptome from all four 454 plates: Cap3 (Huang & Madan, 1999) a long-read assembler that performs well in transcriptome assemblies (Cahais et al. 2012), and Roche’s proprietary software, Newbler (Version 2.5.3), that was designed specifically for assembling 454 sequencing reads with additional features for cDNA sequence. Newbler’s cDNA options assemble reads into contigs, followed by assembly into larger ‘isotigs’ representing alternatively-spliced transcripts. Isotigs are then clustered into larger ‘isogroups’ representing full-length genes. Transcriptome assembly was attempted with the full
set of reads using Cap3 and Newbler with cDNA options, but due to computational limitations
the full dataset could not be assembled with either software program. We addressed this issue by
first assembling sequences from all four plates with Newbler using the genome assembly settings
and default parameters after trimming 454 adaptors and barcodes from the reads. Reads that were
either ‘assembled’ or ‘partially assembled’ in this pilot run were filtered and used as input for
cDNA assemblies in Newbler or Cap3. These reads were filtered from the raw sff files using a
locally-installed instance of Galaxy (Blankenberg et al. 2010). Before the cDNA assembly,
nucleotides with poor quality scores, primer sequences, and long poly(A) tails were removed
using cutadapt (Version 1.2.1 2012, Martin, 2011) and the trim-fastq.pl perl script implemented
in Popoolation (Kofler et al. 2011). The filtered fastq files were then used as input for Cap3 or
Newbler with the cDNA assembly option, using default parameters for both assemblies. These
assemblies (1. genome assembly with Newbler, 2. cDNA assembly with Newbler, and 3. cDNA
assembly with Cap3) were compared to identify the best full reference transcriptome for
downstream analysis.

For analyses of individual tissues, separate cDNA assemblies were performed. Tissues
were barcoded, and sequence reads originating from liver, gonads, or brains were parsed from
the raw 454 sequencing reads. These datasets were small enough to be assembled separately as
tissue-specific transcriptomes in Newbler using the cDNA option with default parameters.
Population-specific transcriptomes were also assembled, using the same methodology, to
examine population-specific statistical signatures of selection.

Alignment to model rodent genomes
Peromyscus assemblies were initially characterized and annotated by performing two separate
analyses using Mus musculus and Rattus norvegicus genomic resources. The first analysis was
used to determine the number of likely genes in each assembly. BLASTN searches were
performed against *Mus musculus* (NCBI Annotation Release 103) and *Rattus norvegicus* (NCBI build 5.1) cDNA reference libraries downloaded from NCBI. BLASTN matches were considered significant when sequence identity was greater than 80%, alignment length was at least 50% of the total length of either the query or subject sequence, and the e-value was less than $10^{-5}$. While significant, these hits may not be ideal for population genomic analyses due to inclusion of paralogous gene matches, matches between multi-gene families, and false positive orthologous gene matches. In order to identify individual isotigs representing a single gene with known function useful for statistical analysis, BLASTN results were further filtered by including query hits that matched only one subject ID (i.e. gene) and *vice versa*. These contigs were annotated as ‘Gene Candidates’.

The distribution of *P. leucopus* isotigs across model rodent genomes was analyzed. All *P. leucopus* isotigs were mapped to chromosomes in the *Mus* (GRCm38) and *Rattus* (RGSC 5.0) reference genomes. Default BLAT parameters were used with an exception for aligning mRNA to genomes across species (-q=rnax -t=dnax, Kent, 2002), and best BLAT hits were parsed based on percent identity and score (# match − # mismatch).

**Mapping and SNP discovery**

To generate a SNP library for downstream population genomic analysis, 454 reads were first mapped to the Newbler cDNA assembly using the BWA-SW (http://bio-bwa.sourceforge.net/) alignment algorithm for long read mapping (Li & Durbin 2010). We only used trimmed reads from the final assembly, removed singletons before mapping to reduce false positive SNP calls from sequencing errors or duplicate reads, and included reads with a mapping quality > 20 in SAMtools. The SAM file from BWA-SW was used in the SAMtools package (v. 0.1.17, Li et al. 2009) to call SNPs. The SNP calling pipeline implemented in SAMtools uses base alignment quality (BAQ) calculations to generate likelihoods of genotypes, can overcome low coverage by
using sequence information from multiple samples to call variants, and uses Bayesian inference to make SNP calls with high confidence (Altmann et al. 2012; Li et al. 2009; Nielsen et al. 2011). In addition to the default parameters in SAMtools, we included stringent additional filters by removing any potential INDELs, only including SNPs with a phred quality (Q-value) ≥ 20, a minimum occurrence of two, and coverage ≤ 200 to exclude alignment artifacts, duplicates, and paralogous genes (Kofler et al. 2011; Rubin et al. 2010; Altmann et al. 2012).

Functional annotation of transcriptomes

The reference transcriptome was annotated by performing a BLASTX search to identify homologous sequences from the NCBI non-redundant protein database, and then GO terms associated with BLASTX hits were retrieved using the annotation pipeline in Blast2GO (Conesa et al. 2005; Götz et al. 2008). Tissue-specific assemblies were also annotated in Blast2GO, and Fisher’s Exact Test was used to examine whether GO terms were over-represented between pairs of tissue types. Each pairwise tissue comparison (liver, brain, gonad) was analyzed for over-representation, and significant results were identified with a False Discovery Rate (FDR) ≤ 0.05.

Prediction of Open Reading Frames (ORFs) and \( p_S/p_S \) calculations

ORFs were identified using BLASTX searches of our assembled contigs against the NCBI non-redundant protein database. Only best hits with an e-value ≤ 10^{-5}, and when query transcripts hit only one subject sequence and vice versa, were kept. From these results, a general feature file (GFF) was created indicating the start and stop coordinates of the putative ORFs. The Perl script, Syn-nonsyn-at-position.pl, implemented in Popoolation v. 1.2.2 (Kofler et al. 2011) was used to define population-specific SNPs obtained from the SAMtools analysis above as either non-synonymous or synonymous.
The ratio of non-synonymous ($p_N$) to synonymous ($p_S$) SNP substitutions ($p_N/p_S$) was calculated between individual Newbler cDNA population assemblies to identify coding sequences potentially experiencing directional selection in urban $P. \text{leucopus}$ populations. For each population pair, the fastaFromBed command in bedtools (Quinlan & Hall 2010) was used to filter contigs and generate a fasta file of putative ORFs (identified above) for each population assembly. The USEARCH (http://www.drive5.com/usearch/) clustering and alignment software for genomic datasets (Edgar 2010) was used to create pairwise alignments between all population ORFs using an $e$-value $\leq 0.001$. Signatures of selection between aligned ORFs were identified using KaKs_Calculator1.2 (Zhang et al. 2006) to calculate the ratio of non-synonymous ($p_N$) to synonymous ($p_S$) SNPs in each population pair. Only transcripts with at least three SNPs were included. The maximum likelihood method was used that accounts for evolutionary characteristics (i.e. ratio of transition / transversion rates, nucleotide frequencies) of our transcriptome datasets. Contigs with elevated $p_N/p_S$ ratios were then annotated in Blast2GO as above. Fasta files of assembled contigs / isotigs, vcf files of SNP marker data, BLAST2GO files of functional annotations, and output files from population genetics tests are available on the Dryad digital repository (doi: XXXXXXX).

Results

Sequencing and comparison of assembly methods

454 Sequencing of four full plates of $P. \text{leucopus}$ cDNA libraries made from liver, brain, and gonad tissue produced 3,052,640 individual reads with an average length of 309 ± 122 bp (median = 341, Interquartile Range (IQR) = 188 bp). While the initial Newbler genomic assembly and Cap3 assembly produced more contigs, the mean length and N50 for both sets of contigs were lower than the Newbler cDNA assembly (Table 1). The Cap3 assembly and the genomic assembly included a much higher proportion of shorter contigs than the cDNA
assembly (Fig. 2). Coverage was calculated for all three assemblies, and all had similar median read coverage per contig (Newbler Genomic, median = 4.7 reads, IQR = 4.6; Newbler cDNA, median = 4.9 reads, IQR = 4.1; Cap3, median = 5.0 reads, IQR = 7.0, Fig. S1).

After filtering BLASTN searches against *Mus musculus* and *Rattus norvegicus* cDNA libraries, there was an average for all assemblies of 13,443 hits to known genes. The Cap3 assembly and Newbler genomic assembly produced the most hits, but the average alignment length was longest for the Newbler cDNA assembly (Table 2). The Newbler cDNA assembly had the highest proportion (47%) of BLASTN hits that were characterized as ‘Gene Candidates’ followed by the Cap3 assembly (42%) and the Newbler genomic assembly (41%). Assessments important for looking at \( p_N/p_S \) (longest average length of contigs, largest N50 value) and for reducing false positives (largest proportion of hits to one gene with known function) supported the assertion that Newbler’s cDNA assembly produced the best quality reference transcriptome, and all further analyses used this assembly.

cDNA transcriptome assembly

The final reference *P. leucopus* Newbler cDNA assembly produced 17,371 contigs with an average length of 613 ± 507 bp. These contigs were assembled into 15,004 isotigs and 12,464 isogroups with a combined length of 13,390,740 bp. Isotigs were constructed from an average of 1.6 contigs and isogroups from an average of 1.2 isotigs. The contribution of sequence reads from individual tissues to the final reference transcriptome was not equal. Liver and brain cDNA libraries produced higher numbers of total reads and a greater proportion of assembled reads compared to ovary and testis libraries. The average read coverage of contigs for each tissue type varied, but coverage from liver sequences was highest, nearly 2X more compared to brain, testes, and ovaries (Table S1). Among all contigs assembled, 70% contained reads from plate 1 (normalized), 57% contained reads from plate 2 (non-normalized), 79% contained reads from
plate 3 (non-normalized), and 89% contained reads from plate 4 (non-normalized). Comparison of normalized (Plate 1) and non-normalized (Plates 2-4) cDNA libraries indicated that non-normalization produced nearly twice as many total sequencing reads as compared to normalization, and non-normalized plates were able to sequence rare transcripts at a similar rate compared to the normalized plate (Table S1).

Mouse and rat genome comparisons

Assembled mRNA transcripts from *P. leucopus* successfully mapped to both *Mus* and *Rattus* reference genomes and were distributed across all chromosomes for both references (Fig. 3). There were 9,418 best BLAT hits between *P. leucopus* contigs and known *Mus* genes and 8,786 best hits with *Rattus* genes. The latest cDNA references include 35,900 genes for *Mus* (mm10) and 29,261 genes for *Rattus* (rn5), suggesting that full or partial coding sequence from approximately one-third to one-fourth of the *P. leucopus* transcriptome was sequenced.

Functional annotation

Among isotigs from the reference *P. leucopus* transcriptome, 11,355 (75.7%) had BLASTX hits to known genes, and 6,385 (42.6%) mapped to proteins and were annotated with known biological functions (GO terms) from protein databases. Top sources for these annotations were the model rodents *Cricetulus griseus* (3,686 BLASTX hits, 24.5%), *Mus musculus* (2,914 BLASTX hits, 19.4%), and *Rattus norvegicus* (1,671 BLASTX hits, 11.1%, Fig. S2). For cDNA assemblies of individual organs, the ovary transcriptome (1,589 isotigs) had the highest proportion (73.9%) of assembled contigs with GO annotations (Fig. 4). Liver (6,240 isotigs) and testes (5,728 isotigs) produced the largest number of total assembled contigs with similar proportions having GO term annotations (65.6% and 64.6%, respectively). The brain
transcriptome (2,613 isotigs) included a lower number of assembled contigs and percent GO
annotation (56.8%; Fig. 4).

One-tailed Fisher’s Exact tests (FDR ≤ 0.5) indicated that liver had the most GO terms
that were significantly over-represented compared to the other tissue types (Table 3). When
reduced to their most-specific terms, pairwise comparisons detected 64 over-represented GO
annotations for liver when compared to both of the other tissues, 20 for brain, and five for
gonads (Table 3). The full list of GO terms was examined between the tissue types and 1,320
annotations in liver were overrepresented when compared to brain and gonads, and 69
annotations in brain when compared to gonad and liver (Fig. 5). Gonads had the least number of
annotations (five) commonly overrepresented when compared to brain and liver (Fig. 5). Over-
represented GO terms in liver were related to metabolic processes including ATP binding, GTP
binding, NADH dehydrogenase, and electron carrier activity. Over-represented GO terms in
brain included regulation of behavior, actin binding, ion channel activity, motor activity, and
calcium ion binding. Significantly different gonad annotations were related to reproduction,
cilium (for sperm locomotion), the cell cycle, transcription regulation, and epigenetic regulation
of gene expression (See Table S2 for full list of overrepresented GO annotations in all pairwise
comparisons).

SNP calling and calculation of \( p_N/p_S \)

After mapping the reads used in the assembly back to the Newbler cDNA reference
transcriptome, 31,015 SNPs were called in 7,625 isotigs. The distribution of SNPs per isotig
ranged from 1 – 78 (mean = 4 ± 5.4; median = 2). ORFs were identified in 11,704 isotigs
comprising 5.6 Mb of sequence, and 2,655 putative ORFs contained 4,893 SNPs. Of these
SNPs, 1,795 (36.6%) were classified as non-synonymous and 3,098 (63.3%) were classified as
synonymous. Aligned ORFs with at least three SNPs were used to calculate \( p_N/p_S \) between each
pair of populations. The majority of the ORFs did not exhibit statistical signatures of positive selection (overall mean ± SE \( p_N/p_S = 0.28 ± 0.56 \)). From the 2,307 pairs of homologous cDNA sequences between populations that contained predicted ORFs and \( \geq \) three SNPs, \( p_N/p_S \) values for 19 (0.8%) contigs exceeded 1.0 (Table 4, Fig. 6). Nine contigs (0.4%) exhibited \( p_N/p_S \) values > 1 in urban to urban comparisons and 11 contigs (0.5%) in urban to rural population comparisons. 42 (1.8%) contigs were found with \( p_N/p_S \) between 0.5 and 1 (Table S3, Fig. 6); \( p_N/p_S > 0.5 \) is a less conservative filter for detecting positive selection, especially when using truncated ORFs (Swanson et al. 2004; Elmer et al. 2010).

Different genes showed strong \( (p_N/p_S > 1) \) signatures of selection when urban populations were compared to other urban populations than when urban and rural populations were compared. Candidate genes identified from the ORF pairs (i.e. \( p_N/p_S > 1 \)) in urban to rural comparisons were related to metabolic processes (including xenobiotic metabolism), the immune system, reproduction, and demethylation (Table 4). Three genes were involved in metabolic processes: cytochrome P450 2A15 (xenobiotic metabolism, HP_contig01783, \( p_N/p_S = 1.89 \)), camello-like 1 (HP_contig00870, \( p_N/p_S = 1.74 \)), and aldo-keto reductase family 1, member C12 (Xenobiotic metabolism, HP_contig01919, \( p_N/p_S = 1.18 \)). Another candidate gene was found in two independent pairwise population comparisons and is involved in the alternative pathway of the innate immune response: complement Factor B (HP_contig01699, \( p_N/p_S = 1.08 \)). Our analysis also identified a reproductive gene, histone H1-like protein in spermatids 1 (HP_contig02656, \( p_N/p_S = 1.07 \)) that is involved in transcriptional regulation during spermatogenesis. The gene phd finger protein 8 (HP_contig01778, \( p_N/p_S = 1.12 \)), codes for a demethylase that removes methyl groups from histones.

Candidate genes in urban to urban population comparisons were primarily involved in immune system processes. Two of these genes are involved in regulating the innate immune response, complement factor H (RR_contig00157, \( p_N/p_S = 6.50 \)) and alpha-1-acid glycoprotein
I (CP_contig00748, $p_N/p_S = 1.97$), through regulation of alternative pathway activation and modulating innate immune response while circulating in the blood, respectively. The other immune system genes are involved in blood coagulation and inflammation, *serine protease inhibitor a3c* (CP_contig00256, $p_N/p_S = 1.76$) and *fibrinogen alpha chain* (CP_contig00473, $p_N/p_S = 1.23$). We also identified *solute carrier organic anion transporter family member 1A5* (CP_contig01204, $p_N/p_S = 1.55$) that facilitates intestinal absorption of bile acids and renal uptake and excretion of uremic toxins.

For the 22 contigs with $p_N/p_S$ between 0.5 and 1 for urban to rural comparisons, genes are primarily involved in the innate immune response, metabolic processes, and methylation activity, and some of these genes are involved in the same biological pathways as genes listed above for contigs that exhibited $p_N/p_S > 1$ (Tables 4, S3). For the 20 contigs with $p_N/p_S$ between 0.5 and 1 for urban pairwise comparisons, genes are primarily involved with the innate immune response, metabolic processes (including xenobiotic), and reproductive processes.

**Discussion**

*De novo transcriptome assembly and characterization*

Compared to other NGS technologies, 454 transcriptome sequencing provides longer read lengths ideal for *de novo* assembly (Metzker 2010) and is especially useful for organisms without extensive genomic resources like *P. leucopus* (Vera *et al.* 2008; Meyer *et al.* 2009; Renaut *et al.* 2010; Santure *et al.* 2011; Sloan *et al.* 2012). We compared the relative merits of two established long-read assembly programs, CAP3 and Newbler, for assembling our transcriptomes (Mundry *et al.* 2012; Cahais *et al.* 2012). Despite the substantially fewer megabases per run generated by 454 FLX+ compared to Illumina or SOLiD sequencing (Glenn 2011), we still ran into computational limitations during assembly when using options for cDNA sequence. Similar to Cahais *et al.* (2012), we had the most success after compressing the raw reads into a smaller
number of partially assembled sequences using a genome assembler followed by another assembly method better suited for transcriptome data. While the CAP3 assembly produced more contigs, the Newbler v. 2.5.3 transcriptome assembly performed better based on assessments useful for downstream population genomic analyses (number of long contigs, average contig length, and proportion of assembled contigs representing a single gene). Newbler performed well at assembling full-length cDNA contigs, and our results are in line with Mundry et al's (2012) findings that Newbler outperformed other assembly programs in simulated experiments.

The N50 value reported here is comparable to de novo Newbler cDNA assemblies for other organisms: N50 = 1,735 bp in Oncopeltus fasciatus, Ewen-Campen et al. (2011); N50 = 1,333 bp in Silene vulgaris, Sloan et al. (2012); N50 = 1,588 bp in Spalax galili, Malik et al. (2011); and N50 = 854 bp in Arctocephalus gazella, Hoffman & Nichols (2011).

We sequenced samples using normalized and non-normalized cDNA pools and examined the influence each protocol had on gene discovery. At the time the libraries were prepared for the first pilot plate, Roche had not yet provided a protocol for cDNA library preparation. Following sequencing of the first normalized plate, the company released a preferred protocol excluding normalization of libraries, and we followed this preferred method for subsequent sequencing. Surprisingly, we found that normalization did not necessarily improve the number of uniquely assembled contigs. Theoretically, normalization reduces the sequencing of overly abundant transcripts and increases the discovery of rare sequences (Christodoulou et al. 2011; Davey et al. 2011), but normalization does not disproportionately influence gene discovery when enough sequencing coverage is achieved (Vijay et al. 2012). Normalization also reduces the read coverage per transcript and may lead to fragmented assemblies (Cahais et al. 2012), thus reducing the number of correctly-assembled transcripts and informative sites (SNPs) for downstream population genomic studies. We found that read coverage per transcript increased for our non-normalized plates compared to the normalized pilot plate. However, Ekblom et al.
suggest that differences in technologies and sequencing effort may ultimately affect comparisons between normalized and non-normalized cDNA libraries, and any differences we identify may be due to different protocols used to extract RNA and prepare pooled libraries.

Mapping to rodent genomes

The mammalian laboratory models Mus and Rattus have extensively annotated genomes that provide a good substitute reference for other rodent sequencing projects. We compared our Peromyscus transcriptome to both genomes and found 9,418 (62.8% of assembled transcriptome) and 8,786 (58.6%) putative homologous genes in Mus and Rattus, respectively. The New World Peromyscus and Old World Mus and Rattus lineages last shared a common ancestor ~25 million years ago (Steppan et al. 2004). Deep divergence and high rates of chromosome evolution across these lineages (Mlynarski et al. 2010) may have affected the percentage of identified homologous gene transcripts. Ramsdell et al. (Ramsdell et al. 2008) found the Peromyscus genome to be more similar to Rattus than Mus due to an enhanced level of genome rearrangement in Mus compared to ancestral muroids. Our results support these findings given that most Peromyscus transcripts mapped to different chromosomes (96.1%) between Mus and Rattus. Our homologous gene matches between Peromyscus and Rattus also represented a higher proportion (30.1%) of total Rattus genes than homologous gene matches between Peromyscus and Mus (25.7%). Non-homologous hits and mapping differences between reference genomes may also be due to highly variable or alternatively spliced transcripts, contamination by genomic DNA, or inclusion of low-quality data (Ferreira de Carvalho et al. 2013), although our assembly methods included measures to limit the influence of these artifacts.

Functional annotation and tissue comparisons
Over 75% of our assembled contigs produced significant BLASTX hits to known genes in NCBI’s nonredundant (nr) protein database. This rate of annotation is similar to studies on other non-model species with large amounts of genomic information available from closely-related model organisms, e.g. 66% in the rodent Ctenomys sociabilis (MacManes & Lacey 2012) and 79.7% in the plant Silene vulgaris (Sloan et al 2012). These rates are much higher than some other organisms with few model relatives, such as 19.58% in a bat, Artibeus jamaicensis, (Shaw et al. 2012), 18% in a butterfly, Melitaea cinxia, (Vera et al. 2008), and 29.2% in the gastropod, Pomacea canaliculata, (Sun et al. 2012). Phylogenetic analyses support Peromyscus spp. and Cricetulus spp. as a monophyletic clade that diverged separately from Mus and Rattus (Steppan et al. 2004), and C. griseus represented the highest proportion of BLASTX top-hits (Figure S2, Supplementary Material). Laboratory use of C. griseus is not as prevalent as Mus or Rattus, but Chinese hamster ovary (CHO) cell lines are commonly used in vitro to produce biopharmaceuticals with complex folding and post-translation modifications (Becker et al. 2011). A draft genome has also been sequenced (Xu et al. 2011). Research on protein pathways and interactions within CHO cell lines provides a future resource for investigating functional consequences of divergent genes between urban and rural populations of P. leucopus.

Transcriptome studies in model rodents provide useful context for understanding how much of each tissue-specific transcriptome we sequenced in this study. Yang et al. (2006) used microarray analysis to identify 12,845 active genes in Mus liver, and RNA-Seq using an Illumina HiSeq 2000 on Rattus liver identified 7,514 known genes (Chapple 2012). Our gene discovery was between 40-60% of these previously reported liver transcriptomes, and thus we may have detected nearly half the genes expressed in the liver in white-footed mice. In brain tissue, 4,508 genes were identified in Mus by Yang et al. (2006), and Chrast et al. (2000) report ~4,000 genes identified by SAGE analysis in Mus brain tissue. The 2,610 gene annotations from our brain cDNA libraries represent between 60-65% of the full P. leucopus brain transcriptome.
Microarray analysis of testis RNA identified up to 13,812 known genes (Shima et al. 2004) in *Mus*, and 454 sequencing of cDNA libraries from the biopharmaceutical CHO cell line in the closely related *C. griseus* identified 13,187 annotations in ovary (Becker et al. 2011). UniGene, a database of transcribed sequences by organism and cell type (Pontius et al. 2003), includes 8,946 genes for *Mus* testis, 5,285 for *Mus* ovaries, 4,355 for *Rattus* testis, and 5,093 for *Rattus* ovaries. The only cDNA library established in UniGene for *Peromyscus* spp. includes 635 putative genes from testis (Glenn et al. 2008). Our assembled libraries from gonad tissue fall within these ranges, and non-annotated transcripts could represent *Peromyscus*-specific genes. The total number of assembled isotigs for each tissue directly parallels the number of cDNA libraries sequenced for this study (Fig. 4). 454 sequencing produces less sequencing output, and genes transcribed at low levels might have been missed during library preparation and sequencing. Higher coverage from individual resequencing using short-read, high-throughput platforms should recover these rare transcripts. To recover 100% of each tissue transcriptome, samples would need to be prepared at various developmental stages and under various environmental conditions.

Fisher’s Exact Tests (FDR ≤ 0.05) allowed us to identify annotated transcripts over-represented in one tissue compared to the others. The brain transcriptome of the social rodent, *C. sociabilis*, exhibited highly expressed genes involved with behavior and signal transduction (MacManes & Lacey 2012). Over-represented GO terms in *P. leucopus* brain tissue were related to similar major functions in the brain, including regulation of behavior, cellular signaling, actin binding, ion transport and channel activity, motor activity, and calcium ion binding. In liver, over-represented GO terms were largely dedicated to metabolic processes including ATP binding, GTP binding, NADH dehydrogenase, and electron carrier activity. There were also several GO terms related to the immune response, hematopoietic processes, and nutrient binding;
these annotations are supported by microarray and RNA-seq analyses of liver in mouse and rat, respectively (Yang et al. 2006; Chapple 2012).

SNP discovery and characterization

Without a reference genome for the study species, aligning reads to assembled transcripts and assigning mismatches as SNPs (Barbazuk et al. 2007) is an acceptable substitute for generating sequence polymorphisms for non-model species (Collins et al. 2008; Renaut et al. 2010; Sloan et al. 2012). Difficulties may persist in distinguishing true SNPs from false positives created by sequencing errors or misaligned reads. Alignment of reads to paralogous genes can also generate false SNPs, thus affecting downstream population genomic analyses. Identifying true SNPs depends on assembly quality, filtering criteria of nucleotide mismatches during alignment, and statistical models used to call nucleotide variants (De Wit et al. 2012). Incorporating a probabilistic framework in SNP-calling algorithms greatly reduces false positives (Nielsen et al. 2011; Altmann et al. 2012).

We could not validate our SNP calls using reference genome resources for Peromyscus, but used conservative filtering criteria when calling SNPs to minimize false positives. SAMtools (Li et al. 2009) excels at SNP detection with low sequence coverage by incorporating prior information about the probability of a SNP occurring based on simultaneous comparisons of multiple samples (Nielsen et al. 2011; Altmann et al. 2012). We also filtered variants based on thresholds of quality and minimum occurrence, and restricted maximum coverage to filter out false positive SNPs from paralogous genes. Excluding transcripts with the highest coverage after mapping limits problems with gene duplications (McCormack et al. 2011). The thresholds we used for minimum SNP occurrence and nucleotide quality reduce error rates by several orders of magnitude for pooled data, ensuring the reliability of SNP libraries for downstream analyses (Kofler et al. 2011). Our SNP library represents highly confident variant calls and will serve as
an important resource for future population genetic studies of urban and rural populations of \textit{P. leucopus}. We cannot completely rule out paralogous genes or misalignments in our transcriptome assemblies, and thus future work will require sequencing of transcripts from multiple individuals to validate SNP calls in candidate genes of particular interest.

\textit{Positive selection and the transcriptome}

We used the ratio of non-synonymous to synonymous substitution rates (\(p_N/p_S\)) to identify several candidate genes that may have experienced positive selection in urban populations of \textit{P. leucopus}. Identifying ORFs in assembled transcriptomes and using SNPs to calculate the ratio of non-synonymous to synonymous substitutions (\(p_N/p_S\)) between populations can be a successful method for identifying the operation of natural selection on individual loci (Nielsen \& Yang 1998; Oleksyk \textit{et al.} 2010; Hohenlohe \textit{et al.} 2011). This approach has recently been used to identify genes under positive or purifying selection between cichlid fish lineages in Nicaragua (Elmer \textit{et al.} 2010), between lake whitefish species pairs (Renaut \textit{et al.} 2010), and within \textit{Pomacea canaliculata}, an invasive gastropod (Sun \textit{et al.} 2012). Studies traditionally identify positive selection in genes with \(p_N/p_S > 1.0\). We used this cutoff value, but also identified sequence pairs with \(p_N/p_S\) between 0.5 and 1.0 to avoid overlooking relevant non-synonymous substitutions in candidate genes that might be of interest for individual re-sequencing projects. Lack of full-length ORFs can decrease \(p_N/p_S\) values when non-synonymous substitutions are not sampled from non-sequenced codons (Swanson \textit{et al.} 2004; Elmer \textit{et al.} 2010). The \(p_N/p_S\) index can also be used when samples have been pooled prior to sequencing (Baldo \textit{et al.} 2011), unlike summary statistics that rely on minor allele frequency spectra. Pooling cDNA from multiple individuals introduces biases in allele frequencies because of variability in transcript presence and abundance (Sloan \textit{et al.} 2012).
Many ecological changes arising from urbanization may drive local adaption to novel conditions in fragmented urban populations. Based on the existing urban ecology literature, we made several predictions about the types of adaptive traits present in urban habitats. Genes involved in divergence of urban and rural populations of white-footed mice are likely associated with quantitative traits affected by crowded (i.e. high population density) and polluted urban environments (life history, longevity, reproduction, immunity, metabolism, thermoregulatory and/or toxicological traits). We identified candidate genes showing strong positive selection ($p_N/p_S > 1$) that supported these predictions between urban and rural populations of mice, but also between individual urban populations. The urban matrix is a strong enough barrier to dispersal that white-footed mouse populations in individual city parks may experience highly localized selective pressures in addition to selective pressures that are general to urban environments (Munshi-South 2012).

New predators, competitors, parasites, and pathogens can drive local adaptation of traits, especially those related to immunity, in novel urban environments (Peluc et al. 2008; Sih et al. 2011). We identified candidate genes involved in the innate immune system and activation of the complement pathway to identify pathogens. Additionally, two candidate genes were identified in comparisons of urban populations that function in blood coagulation and inflammation. The innate immune system is a biochemical pathway that removes pathogens by identifying and killing target cells (Kosiol et al. 2008), and positive selection is found to act on pathogen recognition genes within the complement activation pathway (Sackton et al. 2007). The introduction of invasive species, population growth of ‘urban exploiters’, and increased traffic, trade, and transportation within cities can introduce large numbers of novel pathogens (Bradley & Altizer 2007). Our results suggest that white-footed mice in NYC may be evolving to efficiently recognize and respond immunologically to such urban pathogens. We also identified several genes involved in metabolism that were divergent between populations, and a gene
expressed during spermatogenesis that was divergent between urban and rural populations. Rapid evolution has been identified in reproductive proteins between *Peromyscus* spp. affecting spermatogenesis, sperm competition, and sperm-egg interactions (Turner et al. 2008), and the intensity of sperm competition and reproductive conflict may be increasing in dense *P. leucopus* populations in NYC.

Increasing air, water, and soil pollution are all typical impacts of urbanization (Yauk et al. 2008; Whitehead et al. 2010; Francis & Chadwick 2012). One potential marker of increased exposure to pollutants is hypermethylation of regulatory regions of the genome (Somers et al. 2002; Yauk et al. 2008; Janssens et al. 2009; Somers & Cooper 2009). Positive selection may also be acting on genes involved in xenobiotic metabolism. Heavy metals including mercury, lead, and arsenic occur at increased concentrations within NYC park soils (S. Harris, unpublished data), and McGuire et al. (2013) found lower pH and higher concentrations of heavy metals in NYC parks compared to green roofs. In *Fundulus heteroclitus* from multiple polluted estuaries along the Atlantic coast, genetic mechanisms of recent PCB resistance were identified in multiple populations (Whitehead et al. 2010). Wirgin et al. (2011) also found that tomcod in the Hudson River rapidly adapted to increased PCB concentrations through positive selection for a two amino acid deletion that reduces binding affinity. In urban to rural comparisons we found two potential toxicological candidate genes: one gene involved in metabolizing foreign chemical compounds (i.e. xenobiotics), and a demethylase that removes methyl groups from histone lysines.

Comparing candidate genes from all pairwise analyses with \( p_N / p_S \) between 0.5 and 1 reveals several additional patterns. Four complement factor proteins involved in the innate immune system were identified: *complement factor H, I, B*, and *c3 precursor*. All proteins function in the alternative pathway, which acts continuously in an organism without antibody activation to clear foreign pathogens (Carroll 2004). The alternative pathway for complement-
mediated immunity has been found to be rich in positively selected genes across several mammalian genomes, including *Mus* and *Rattus* (Kosiol *et al.* 2008). Cell signaling and protein recognition genes within the innate immune system’s response to pathogens are likely under similar selective pressures as the adaptive immune response (Sackton *et al.* 2007). We also identified proteins that interact with the complement system in multiple population pair analyses in both urban-to-urban and urban-to-rural population comparisons. *Alpha1-acid glycoprotein* was identified in four separate population comparisons and likely modulates the innate immune system while circulating in the blood (Fournier *et al.* 2000). Four *cytochrome p450* genes, 2d27-like, family 2 subfamily B, subfamily polypeptide 13, and 2a15, exhibited $p_N/p_S$ between 0.5 and 1 in urban populations and between urban and rural populations. The *cytochrome p450* family of genes plays a major role in xenobiotic metabolism, including detoxification in variable environments (Su & Ding 2004; Büntge 2010). Patterns of divergence and positive selection have been robustly identified in *cytochrome p450* genes in natural populations of both *Mus musculus* ingesting toxins through their diet and *Tetrahymena thermophila* exposed to toxic environments (Fu *et al.* 2009; Büntge 2010). *P. leucopus* in NYC populations may be experiencing different dietary demands and exposure to pollutants, leading to selective pressures on detoxifying genes like the cytochrome p450 gene family.

We have identified several transcriptome-wide trends of divergence between urban and rural populations of white-footed mice, as well as between isolated urban populations within NYC. Identification of candidates from the same gene families indicates that positive selection may be acting directly on protein pathways of white-footed mice in urban populations. However, inferring the function of candidate genes and phenotypes influenced by selection should be done with an excess of caution after identifying statistical signatures of positive selection.

Purifying selection can sometimes lead to $p_N/p_S > 1$, and individual codons within a gene can have an excess of non-synonymous substitutions due to random biological processes.
(Hughes 2007). However, current statistical tests address these issues and are generally robust in identifying positive selection (Zhai et al. 2012). In the case of a single population, \( p_N/p_S > 1 \) may not represent positive selection. Kryazhimskiy & Plotkin (2008) demonstrated that the relationship between \( p_N/p_S \) and selection is radically different when samples are being compared that originated from the same population; \( p_N/p_S \) actually decreases in response to positive selection. To make inferences about selection between two samples using \( p_N/p_S \), samples must come from reproductively isolated populations with fixed substitution differences (Kryazhimskiy & Plotkin 2008). All samples used to calculate \( p_N/p_S \) for this study came from reproductively isolated and genetically structured populations (Munshi-South & Kharchenko 2010). We assembled transcriptome datasets individually for each population to identify fixed substitutions between populations and avoid randomly segregating SNPs in \( p_N/p_S \) analyses. Indices such as \( p_N/p_S \) identify genes with previously unknown signatures of selection, but candidates still need to be studied in a controlled setting to identify phenotype and function (Zhai et al. 2012).

The ability of \( p_N/p_S \) to detect genes under positive selection is limited in some situations, so it is likely that we have missed many candidate genes by using this single statistic. Additionally, such analyses do not identify adaptive variation in gene regulatory regions as opposed to transcribed cDNA (Prud’homme et al 2007). Amino acid changes may also be more representative of past rather than recent or ongoing selection (Elmer et al. 2010; Hohenlohe et al 2011), and \( p_N/p_S \) ratios can vary widely when there are relatively few mutations per gene (Hughes 2007; Renaut et al 2010). Given strong selection within populations, however, it is plausible that multiple substitutions may rise to high frequency or become fixed within a few hundred generations (i.e. in the timeframe of divergence for urban and rural populations of white-footed mice). The candidate genes identified herein can be confirmed in future work using multiple tests of selection that provide more statistical power and higher resolution when identifying types and age of selection in single candidate genes (Grossman et al 2010; Li et al.
Our ongoing work in this system uses transcriptomic and genomic libraries from individual mice from several populations, and multiple outlier statistics based on the allele frequency spectrum and linkage disequilibrium, to examine recent selection in both coding and non-coding regions of urban white-footed mouse genomes.

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Figure Legends

Figure 1. Location and number of individuals collected from five populations in the NYC metropolitan area. Urban populations are in shades of blue; light blue = male; dark blue = female. Rural population in orange and brown; orange = male; brown = female. Areas shaded red on the map indicate degree of urbanization (i.e. impermeable surface cover such as roads and rooftops) and green areas indicate vegetation cover from the 2006 National Landcover Database. (CP = Central Park; NYBG = New York Botanical Gardens; RR = Ridgewood Reservoir; FM = Flushing Meadows-Willow Lake; HP = Harriman State Park).

Figure 2. Frequency of contig lengths for three transcriptome assembly methods. Inset: Zoomed-in view of frequency of longer assembled contigs from 1,500-3,000 bp. Blue line = Newbler cDNA, Red line = Newbler genome, Green line = Cap3.

Figure 3. Transcriptome alignment to reference rodent genomes. Number and distribution of contigs from *P. leucopus* transcriptome (Newbler cDNA assembly) that aligned to each chromosome of the. (a) *Rattus norvegicus*. Blue = total number of genes per chromosome for *Rattus*. Red = number of aligned *Peromyscus* isotigs per *Rattus* chromosome. (b) Blue = total number of genes per chromosome for *Mus*. Red = number of aligned *Peromyscus* isotigs per *Mus* chromosome.

Figure 4. Annotation of final reference transcriptome. Number of assembled *P. leucopus* contigs from four different tissue types that had significant hits with known proteins on BLASTX, and GO term annotations from reference databases using Blast2Go; Blue = Total number of contigs, Red = BLASTX hits, Green = number of annotated contigs.
Figure 5. Over-represented GO terms from pairwise tissue comparisons (FDR ≤0.05). (a) Comparison of brain transcriptome to liver and gonad. (b) Comparison of liver to brain and gonad. (c) Comparison of gonad to liver and brain.

Figure 6. Non-synonymous ($p_N$) SNP substitutions plotted vs. synonymous ($p_S$) substitutions for 354 genes. Each circle represents one unique assembled contig. (a) Pairwise comparisons for all urban populations. (b) Pairwise comparisons for urban to rural populations. The dashed line denotes $p_N / p_S = 1$, and circles above the line ($p_N / p_S > 1$) indicate candidates for positive selection. The solid line shows the slope for $p_N / p_S = 0.5$. 
A

\begin{center}
\begin{tikzpicture}
  \tikzstyle{every node}=[font=\small]
  \node (A) at (0,0) {Brain};
  \node (B) at (2,0) {Liver};
  \node (C) at (4,0) {Gonad};
  \node at (1,-1) {62};
  \node at (2,-1) {69};
  \node at (3,-1) {19};
  \draw (A) circle (1.5cm);
  \draw (B) circle (1.5cm);
  \draw (C) circle (1.5cm);
  \draw (A) -- (B);
  \draw (B) -- (C);
  \draw (C) -- (A);
\end{tikzpicture}
\end{center}

B

\begin{center}
\begin{tikzpicture}
  \tikzstyle{every node}=[font=\small]
  \node (A) at (0,0) {Brain};
  \node (B) at (2,0) {Gonad};
  \node (C) at (4,0) {Liver};
  \node at (1,-1) {15};
  \node at (2,-1) {1,320};
  \node at (3,-1) {1,489};
  \draw (A) circle (1.5cm);
  \draw (B) circle (1.5cm);
  \draw (C) circle (1.5cm);
  \draw (A) -- (B);
  \draw (B) -- (C);
  \draw (C) -- (A);
\end{tikzpicture}
\end{center}

C

\begin{center}
\begin{tikzpicture}
  \tikzstyle{every node}=[font=\small]
  \node (A) at (0,0) {Brain};
  \node (B) at (2,0) {Liver};
  \node (C) at (4,0) {Gonad};
  \node at (1,-1) {13};
  \node at (2,-1) {5};
  \node at (3,-1) {24};
  \draw (A) circle (1.5cm);
  \draw (B) circle (1.5cm);
  \draw (C) circle (1.5cm);
  \draw (A) -- (B);
  \draw (B) -- (C);
  \draw (C) -- (A);
\end{tikzpicture}
\end{center}
### Tables

**Table 1. Results of transcriptome assembly using three different approaches.**

| Assembly Method         | No. Contigs | Mean Contig Length (bp) | Median Contig Length (bp) | N50* Length (Mb)** |
|-------------------------|-------------|-------------------------|---------------------------|--------------------|
| Newbler genome\(^a\)   | 20,570      | 630 ± 504               | 516                       | 830                | 12.95              |
| Cap3\(^b\)             | 27,497      | 653 ± 380               | 566                       | 732                | 17.95              |
| Newbler cDNA\(^c\)     | 15,004 (Isotigs) | 895 ± 752               | 683                       | 1,039              | 13.42              |

\(^a\)Newbler v. 2.5.3 large genomic assembly of total set of raw sequencing reads

\(^b\)Cap3 assembly using ‘assembled’ or ‘partially assembled’ reads from Newbler genome

\(^c\)Newbler v. 2.5.3 cDNA assembly using ‘assembled’ or ‘partially assembled’ reads from Newbler genome assembly

\(^*\)N50, The value where half the assembly is represented by contigs of this size or longer

\(**\)Total assembly length in Megabases.
Table 2. BLASTN search results of three *P. leucopus* transcriptome assemblies against reference cDNA libraries from *Mus* and *Rattus*.

| Assembly Method | Total Significant Hits; *Mus* | Total Significant Hits; *Rattus* | Gene Candidates, *Mus* | Gene Candidates, *Rattus* |
|----------------|-------------------------------|-----------------------------------|------------------------|--------------------------|
| Newbler genome | 12,932                        | 12,807                            | 8,568 (708 bp)         | 8,080 (714 bp)           |
| Cap3           | 17,333                        | 16,792                            | 11,662 (623 bp)        | 10,938 (638 bp)          |
| Newbler cDNA   | 10,699                        | 10,094                            | 7,048 (823 bp)         | 6,814 (847 bp)           |

* = Average alignment length in base pairs

Total significant hits represent sequence identity ≥ 80%, alignment length ≥ 50% of the total length of either the query or subject sequence, and e-value ≤ $10^{-5}$. Gene candidates represent significant hits where one query sequence matches one subject gene and *vice versa*. 
### Table 3. Over-represented GO terms for individual tissue types from Fisher’s Exact tests (FDR ≤ 0.5) in Blast2Go.

| Tissue      | GO term                                      | FDR       | # Sequences |
|-------------|----------------------------------------------|-----------|-------------|
| Liver       | ATP binding                                  | 5.31E-24  | 184         |
|             | zinc ion binding                             | 5.93E-20  | 154         |
|             | transcription factor complex                 | 3.91E-19  | 148         |
|             | electron carrier activity                    | 8.53E-18  | 251         |
|             | structural constituent of ribosome           | 5.51E-15  | 117         |
|             | soluble fraction                             | 2.35E-12  | 97          |
|             | microsome                                    | 1.53E-10  | 83          |
|             | protein homodimerization activity            | 2.75E-10  | 81          |
|             | oxygen binding                               | 1.97E-09  | 93          |
|             | perinuclear region of cytoplasm              | 9.92E-09  | 69          |
|             | GTP binding                                  | 7.64E-08  | 62          |
|             | GTPase activity                              | 2.82E-05  | 42          |
|             | ubiquitin-protein ligase activity            | 2.82E-05  | 42          |
|             | NADH dehydrogenase (ubiquinone) activity     | 5.01E-05  | 40          |
|             | drug binding                                 | 6.65E-05  | 39          |
|             | sequence-specific DNA binding                | 6.65E-05  | 39          |
|             | double-stranded DNA binding                  | 8.90E-05  | 38          |
|             | mitochondrial respiratory chain complex I    | 1.18E-04  | 37          |
|             | transcription coactivator activity           | 1.18E-04  | 37          |
|             | catalytic step 2 spliceosome                 | 1.58E-04  | 36          |
| Brain       | protein complex                              | 1.27E-06  | 569         |
|             | plasma membrane                              | 4.30E-02  | 567         |
|             | signal transduction                          | 2.15E-39  | 525         |
|             | cytosol                                      | 1.79E-08  | 411         |
|             | cell differentiation                         | 5.07E-28  | 372         |
|             | anatomical structure morphogenesis           | 1.89E-30  | 291         |
|             | cell death                                   | 1.78E-06  | 247         |
|             | cell-cell signaling                          | 2.79E-61  | 232         |
|             | ion transport                                | 3.12E-17  | 209         |
|             | cytoplasmic membrane-bounded vesicle         | 1.33E-22  | 197         |
|             | golgi apparatus                              | 1.51E-10  | 168         |
|             | cytoskeleton organization                    | 9.13E-13  | 145         |
|             | cellular homeostasis                         | 9.82E-16  | 134         |
|             | behavior                                     | 6.72E-28  | 133         |
|             | calcium ion binding                          | 7.69E-13  | 109         |
|             | actin binding                                | 3.54E-15  | 93          |
|             | response to abiotic stimulus                 | 4.97E-08  | 88          |
|             | protein kinase activity                      | 1.61E-03  | 77          |
|             | ion channel activity                         | 5.21E-17  | 62          |
|             | motor activity                               | 8.38E-06  | 48          |
| Gonads      | nucleic acid binding                         | 1.87E-08  | 1101        |
|             | nuclear chromosome                           | 9.86E-06  | 119         |
|             | reproduction                                 | 1.92E-06  | 680         |
|             | RNA binding                                  | 6.70E-04  | 637         |
|             | viral reproduction                           | 1.74E-02  | 339         |

GO terms have been reduced to their most specific terms. Only common GO terms over represented for one tissue compared to the other two tissues are shown. The top 20 terms are shown, see Table S2 for full list of GO annotations.
Table 4. Candidate loci exhibiting $p_N/p_S > 1$.

| Sequence name      | $p_N/p_S$ | Gene name                        | Gene function                                                                 |
|--------------------|-----------|----------------------------------|-------------------------------------------------------------------------------|
| HP_contig01773     | 1.01      | Translocation protein SEC62      | Post-translational protein translocation into the endoplasmic reticulum; plasma membrane protein |
| HP_contig02632     | 1.05      | 39S ribosomal protein L51        | Part of mitochondrial ribosomal large subunit (39S); involved in protein translation |
| HP_contig02656     | 1.07      | Histone H1-like protein in spermatids 1 | Transcriptional regulation and / or chromatin remodeling through DNA binding during spermatogenesis |
| HP_contig01699*    | 1.07      | Complement factor B               | Circulates in the blood; functions in the alternative pathway of the complement system during innate immune responses |
| HP_contig01778     | 1.12      | PHD finger protein 8              | Removal of methyl groups from histones                                           |
| HP_contig03615     | 1.12      | Aldo-keto reductase family 1, member C12 | Scaffold protein that connects regulatory elements with plasma membrane proteins; regulation of ion transport |
| HP_contig01919     | 1.18      |                                | Endoplasmic reticulum plasma membrane protein; may regulate ER mediated signaling |
| HP_contig03408     | 1.62      | Orm1-like 3                      | Metabolic process; mitochondrial inner membrane protein                        |
| HP_contig00870     | 1.74      | Camello-like 1                   | Metabolic process; testosterone 7a-hydroxylase activity                        |
| HP_contig01783     | 1.89      | Cytochrome P450 2A15             |                                                                                   |
| CP_contig00473     | 1.23      | Fibrinogen alpha chain           | Glycoprotein circulating in the blood; functions in blood coagulation and part of the most abundant component of blood clots |
| RR_contig01212*    | 1.26      | Isoform cra_a                    | Uncharacterized cellular membrane protein                                         |
| CP_contig01204     | 1.55      | Solute carrier organic anion transporter family member 1A5 | Membrane protein; transports hormones; facilitates intestinal absorption of bile acids and renal uptake of indoxyl sulfate |
| NYBG_contig00118*  | 1.76      | Orf 2                            | Contains reverse transcriptase domain                                            |
| CP_contig00256     | 1.76      | Serine protease inhibitor a3c     | Bind to proteases and inhibit proteolysis; often involved in blood coagulation and inflammation |
| CP_contig00748     | 1.97      | Alpha-1-acid glycoprotein 1       | Transport protein in the blood stream; binds and distributes synthetic drugs throughout body; modulates innate immune response |
| RR_contig00157     | 6.50      | Complement Factor H-like          | Regulates activation of the alternative complement pathway in the innate immune response |

* = Gene contained $p_N/p_S > 1$ in two independent population pairwise comparisons
Supporting Information

Figure S1. Frequency distribution of depth of coverage (reads / contig). (a) The Newbler cDNA assembly. Red line indicates median coverage = 4.9 reads, Interquartile range (IQR) = 4.1. (b) The Newbler genomic assembly, median = 4.7 reads, IQR = 4.6. (c) The Cap3 assembly, median = 5.0 reads, IQR = 7.0.

Figure S2. Distribution of species with the most top-hit BLASTX results in Blast2Go using the Newbler cDNA assembly as the query.

Table S1. Sequencing and assembly statistics for Newbler cDNA transcriptome assembly by tissue type and 454 sequencing plate.

Table S2. Full list of over represented GO terms for all tissue pairwise comparisons from Fisher’s Exact Test (FDR ≤ 0.5). (a) Liver. (b) Brain. (c) Gonads.

Table S3. Candidate loci with $p_N/p_S$ between 0.5 and 1.