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Potential for sexual conflict assessed via testosterone-mediated transcriptional changes in liver and muscle of a songbird

Mark P Peterson[1,*]; Kimberly A Rosvall[1]; Charlene A Taylor[1]; Jacqueline Ann Lopez[2,3]; Jeong-Hyeon Choi [2,4];Charles Ziegenfus[5]; Haixu Tang[2]; John K Colbourne[2,6]; and Ellen D Ketterson[1]

*Corresponding Author: petersm@juniata.edu
*Current Address: Pennsylvania State University, University Park, PA, USA, and Juniata College, Huntingdon, PA, USA.
[1]Dept. of Biology, Center for Integrative Study of Animal Behavior, Indiana University, Bloomington, IN, USA.
[2]Center for Genomics and Bioinformatics, Indiana University, Bloomington, IN.
[3]Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, USA
[4]Cancer Center, Department of Biostatistics, Georgia Health Sciences University, Augusta, GA, USA.
[5]Department of Mathematics, James Madison University, Harrisonburg, VA, USA.
[6]School of Biosciences, University of Birmingham, Birmingham, United Kingdom.

Summary

Males and females can be highly dimorphic in metabolism and physiology despite sharing nearly identical genomes, and males and females both respond phenotypically to elevated testosterone, a steroid hormone that alters gene expression. Only recently has it become possible to learn how a hormone like testosterone affects global gene expression in non-model systems, and whether it affects the same genes in males and females. To investigate the transcriptional mechanisms by which testosterone exerts its metabolic and physiological effects on the periphery, we compared gene expression by sex and in response to experimentally elevated testosterone in a well-studied bird species, the dark-eyed junco (*Junco hyemalis*). We identified 291 genes in the liver, and 658 in the pectoralis muscle that were differentially expressed between males and females. In addition, we identified 1,727 genes that were differentially expressed between testosterone-treated and control individuals in at least one tissue and sex. Testosterone-treatment altered the expression of only 128 genes in both males and females in the same tissue, and 847 genes were affected significantly differently by testosterone-treatment in the two sexes. These substantial differences in transcriptional response to testosterone suggest that males and females may employ different pathways when responding to elevated testosterone, despite the fact that many phenotypic effects of experimentally elevated testosterone are similar in the sexes. In contrast, of the 121 genes that were affected by testosterone-treatment in both sexes, 78% were regulated in the same direction (e.g. either higher or lower in testosterone-treated than control) in both males and females. Thus, it appears that testosterone acts through both unique and shared transcriptional pathways in males and females, suggesting multiple mechanisms by which sexual conflict can be mediated.

Introduction

Males and females often face divergent selective pressures due to inherent differences in reproductive strategy, and these differences can be reflected in life history traits, including reproductive effort, longevity, growth, and metabolism (Cox and Calsbeek, 2009). In several species, some life history traits (e.g., longevity and basal metabolic rate) appear to be at sub-optimum levels for each sex, and selection related to these phenotypes acts in opposite directions on males and females (Berg and Maklakov, 2012; Boratynski *et al.*, 2010). This suggests that sexually antagonistic selection has led to a phenotypic compromise (Bonduriansky and Chenoweth, 2009; Chapman *et al.*, 2003). Sexually dimorphic gene expression is thought to provide a solution to sexual conflict (van Doorn, 2009), given that males and females share nearly identical genomes (reviewed in Ellegren and Parsch, 2007). Sexually dimorphic patterns of gene expression are thought to account for many of the physiological differences between the sexes (Xu *et al.*, 2012). For example, sex differences in liver gene expression are substantial in rodents (Corton *et al.*, 2012), and explain several known sex differences in liver metabolism (Gatti *et al.*, 2010).

In many vertebrate species, androgens, such as testosterone (T), are one of the key regulators of sex differences in many aspects of adult phenotype, including growth and metabolism (Cox *et al.*, 2009; Woodward, 1993; Arnold *et al.*, 1997; Wikelski *et al.*, 1999). T plays a major role in directing the balance of energy expenditure (Marler and Moore, 1988), generally shifting energy away from metabolic processes of self-maintenance, such as immune function (Folstad and Karter, 1992), toward short-term reproductive efforts, such as courtship (Arnold, 1975; Wiley and Goldizen, 2003) and territory defense (Marler *et al.*, 1995). These phenotypic effects often occur in both males and females, but are likely to affect reproductive success differently in each sex (Ketterson *et al.*, 2005). Thus, there is likely to be conflict between the sexes over the optimal level of circulating T (Boratynski *et al.*, 2010; Mokkonen *et al.*, 2012).

Comparative studies have shown that endogenous levels of T are highly correlated between males and females among species, including in birds (Ketterson *et al.*, 2005; Moller *et al.*, 2005) and fish (Mank, 2007), raising the possibility that selection on circulating T levels in one sex may lead to a similar change in circulating T in the opposite sex. If, however, the sexes differ in their phenotypic and transcriptional response to circulating T, they may be able to reduce this conflict, and each sex may be better able to reach its own optimum phenotypic values (Rice, 1984). Behavior and physiology are known to be sensitive to experimentally elevated T, sometimes in both sexes, sometimes in only one.

For example, immune function is sensitive to experimental elevation of T in males of some species (Roberts *et al.*, 2004), but is responsive to T in females of only some, but not all, of these species (Ketterson *et al.*, 2005). The fact that phenotypic sensitivity to T varies between species and sexes strongly suggests evolutionary lability in the genes and phenotypes that respond to T.

To address the role of sexual dimorphism and T in mediating phenotypes via gene expression in a natural system, we studied gene expression in the liver and pectoralis of a wild songbird, the dark-eyed junco (*Junco hyemalis*) (Linnaeus, 1758). The dark-eyed junco is a mildly dimorphic North American sparrow (Nolan *et al.*, 2002) that has been the focus of ecological research for nearly a century (Rowan, 1925; Ketterson *et al.*, 2009; Miller, 1941), and recent genomic tools have expanded these studies (Peterson *et al.*, 2012). Sex differences and the phenotypic effects of experimentally elevated T have been studied extensively (Ketterson *et al.*, 2009; Ketterson *et al.*, 1991) providing a solid ecological foundation on which to interpret findings from genomic tools (Peterson *et al.*, 2012).

In particular, past research on free-living male and female juncos has detailed many phenotypic consequences of experimental T-treatments that maintain levels of T near the early breeding season peak for each sex (Ketterson *et al.*, 1992; Ketterson *et al.*, 1996; Ketterson *et al.*, 2005). Both male and female juncos respond phenotypically to experimentally elevated T by decreasing immune function (Casto *et al.*, 2001; Zysling *et al.*, 2006), and decreasing body mass (Clotfelter *et al.*, 2004; Ketterson *et al.*, 1991) along with a number of behavioral responses (reviewed in Ketterson *et al.*, 2005; Ketterson *et al.*, 2009). However, only males, and not females, increase their activity and home-range size in response to experimental T (Reichard and Ketterson, 2012; Lynn *et al.*, 2000; Chandler *et al.*, 1994). The net result of these and other phenotypic effects of T-treatment is an increase in reproductive fitness for males (Reed *et al.*, 2006) but a decrease in fitness for females (Gerlach and Ketterson, 2013), providing direct experimental support for the hypothesis that there is sexual conflict over optimal T levels in this species. As such, this is an ideal system in which to investigate the molecular mechanisms by which sexual conflict occurs and/or is resolved, by specifically asking whether the sexes diverge in the gene expression response to T-treatment.

Many sexually dimorphic and androgen-responsive phenotypes are mediated directly by changes in peripheral tissues such as liver and muscle. The liver plays a key role in whole-body metabolism, including gluconeogenesis, glycogenolysis, glycogen storage, amino acid synthesis, lipid synthesis and breakdown, and the production of insulin-like growth factor (Heubi, 1993; Miura *et al.*, 1992). Further, the liver is a key regulator of sexually dimorphic immune function – male mice are more susceptible to

liver infection than females are (Diodato *et al.*, 2001), and these differences are androgen-mediated (Mock and Nacy, 1988) through gene expression changes (Delic *et al.*, 2010). Sex differences in gene expression in liver can be substantial (Corton *et al.*, 2012), and are largely driven by activational effects of hormones (van Nas *et al.*, 2009). The physiological demands of flight are thought to have resulted in a larger liver in birds compared to mammals (Proctor, 1993), making hormonal influences of this organ particularly important in birds.

Similarly, muscle tissues are also often sensitive to T and play a primary role in mediating dimorphic behavior and physiology (Arnold *et al.*, 1997; Baur *et al.*, 2008; Fernando *et al.*, 2010). Gene expression appears to account for many sexually dimorphic muscle features in humans (Maher *et al.*, 2009; Welle *et al.*, 2008) and mice (Yang *et al.*, 2006). Androgen treatment leads to increases in strength and lean muscle mass (Hartgens and Kuipers, 2004), and these effects may be linked to T-mediated changes in gene expression (Montano *et al.*, 2007; Labrie *et al.*, 2005). Further, the effects of exercise on gene expression in muscle are sex-specific in humans (Liu *et al.*, 2010), suggesting that different transcriptional pathways may underlie some of the sex differences in muscle. The pectoralis muscle, which is the major avian flight muscle, accounts for approximately 20% of the mass of an individual bird (Marden, 1987). Androgen receptor is expressed in the pectoralis (Feng *et al.*, 2010), and T modifies the expression of at least two candidate genes related to muscle function in the pectoralis (Fuxjager *et al.*, 2012). Thus, the pectoralis provides an important, androgen sensitive tissue in which to investigate the sex-specific effects of hormones in the periphery.

We anticipated that many of the genes differentially expressed between sexes and in response to T-treatment in the liver and the pectoralis would have functions related to metabolism, muscle development, and immune function. Because many of the metabolic effects of T are similar in male and female juncos, we also predicted that many genes whose expression was altered in response to T-treatment in one sex would also be altered in the other sex. However, we also predicted that some genes would respond to T-treatment in one sex, but not the other, consistent with previous findings that (i) not all physiological effects of T are present in both sexes (Ketterson *et al.*, 2005) and (ii) the sexes respond differently to T-treatment at the level of gene transcription in the brain (Peterson *et al.*, 2013) providing a possible solution to the sexual conflict over T-levels observed in previous studies on free-living juncos (Gerlach and Ketterson, 2013; Reed *et al.*, 2006).

Materials and Methods

Animal collection and treatment

Adult dark-eyed juncos (14 male, 12 female) from near Mountain Lake Biological Station (Pembroke, VA; 37° 22′ 31″N, 80° 31′ 24″W) were captured, held in a semi-naturalist aviary, and treated as described in a previous study analyzing neural tissues (Peterson *et al.*, 2013). Briefly, T-treated individuals were implanted with silastic tubing filled with crystalline T (males: two 10 mm implants; females one 5 mm implant; Sigma-Aldrich, St. Louis, Missouri, USA), and control individuals were implanted with one 10 mm empty implant. These T implants result in levels of T near the physiological maximum in each sex (Ketterson *et al.*, 2005). Thus, while all animals had T levels above any threshold necessary to maintain reproductive physiology and behavior, animals given T implants had T levels that were at the high end of natural variation. Notably, this implant regiment has repeatedly been shown to affect many different phenotypes in male and female juncos, and experimental treatment with these T implants reveals that there is sexual conflict over T levels in this system – above average T levels are selectively advantageous for males and disadvantageous for females (see Gerlach and Ketterson, 2013; Ketterson *et al.*, 2009; and Reed *et al.*, 2006; summarized in Introduction).

We note that direct and indirect mechanisms of action and interaction with natural hormones are important to consider when evaluating our results for two reasons. First, our implants used T, which can be aromatized into estradiol, and thus, several of the effects described here may be mediated directly by estradiol after local conversion via aromatase (Herbst and Bhasin, 2004). These sex steroids may act directly on muscle tissue, and they also may directly alter activity, metabolism or other aspects of behavior and physiology that lead to indirect effects on gene expression in liver or muscle (Park *et al.*, 2012).

Furthermore, we used intact animals in breeding condition to ensure that seasonally variable aspects of behavior and physiology were characteristic of the breeding season, mimicking previous studies that have demonstrated sex differences in the phenotypic and fitness consequences of T in otherwise normal breeding birds. Importantly, the effects seen here likely reflect the mechanisms of action (i) in previous implant studies (e.g., Ketterson *et al.*, 1996; Gerlach and Ketterson, 2013; Reed *et al.*, 2006; Clotfelter *et al.*, 2004) as well as (ii) those that might occur in response to evolutionary increases in T levels (Ketterson *et al.*, 2009).

After 26 days of exposure to implants, individuals were euthanized by overdose of isoflurane, and tissues were collected rapidly (within 15 minutes) to ensure minimal RNA degradation (Cheviron *et al.*, 2011). Approximately two cubic centimeters from the tip of the right lobe of the liver and approximately one cubic centimeter from near the midline the pectoralis muscle were collected from all individuals. Other collected tissues remain available for future analyses. Sexes and treatments were balanced across day and time of sacrifice (between 0700 and 1230). All animal methods were reviewed and approved by the Institutional Animal Care and Use Committee at Indiana University – Bloomington (Protocol #09-037).

cDNA preparation and hybridization

Microarray experiments were conducted as described in (Peterson *et al.*, 2012; Peterson *et al.*, 2013) following (Lopez and Colbourne, 2011). RNA from liver and pectoralis was extracted in TRIzol following manufacturer directions (Invitrogen, Carlsbad, CA, USA). All extracted RNA assessed on Agilent Bioanalyzer (Santa Clara, CA, USA) and showed high quality: RNA integrity number (Schroeder *et al.*, 2006) scores ranged from 6.7-9.2. We then performed double strand cDNA synthesis with the Invitrogen SuperScript Double-Stranded cDNA Synthesis kit with labeled cDNA using 1 O.D CY-labeled random nonamer primer (either Cy3 or Cy5) and random hexamer primers and 100U Klenow fragment per 1ug ds-cDNA (following NimbleGen labeling protocols).

A full round robin design was used for each tissue (n = 6 per treatment group for each tissue). Each sample was tested once, and each treatment group was hybridized against each other group twice (once with each dye direction; Supplementary Figure 1). Thus, 15 μg of two labeled samples (one Cy3, one Cy5) were hybridized to each sub-array of a custom Nimblegen 12-plex microarray (Roche Nimblegen, Inc., Madison, WI) for the dark-eyed junco containing 100,635 features representing 33,545 contigs (assembled sequencing reads) in triplicate covering 22,765 isogroups (putative genes) based on transcriptome sequencing (Peterson *et al.*, 2012). Post-hybridization washing and scanning followed manufacturer's directions (Roche NimbleGen, Inc., Madison, WI). Axon GenePix 4200A scanner (Molecular Devices, Sunnyvale CA) with GenePix 6.0 software captured array images and NimbleScan 2.4 (Roche NimbleGen, Inc., Madison WI) was used to extract data. We then used the limma package (Smyth, 2005) in R (R Development Core Team, 2010) to process and normalize raw microarray data. Microarray data are available in the NCBI Gene Expression Ominubus repository (Accession number GSE41076).

Microarray analysis

Three comparisons for each tissue were made using limma (Smyth, 2005): control males vs. control females; control males vs. testosterone-treated males; control females vs. testosterone-treated females (n = 6 per treatment group for each tissue); and the interaction between testosterone and sex. Only contigs that were expressed in at least one of the compared treatment groups were analyzed (identified as described in Peterson *et al.*, 2012). Briefly, a gene was considered expressed if at least half of the individuals in a treatment group had expression scores greater than 97.5% of the random probes on the array.

In most isogroups, the $\log_2$ fold changes between treatment groups, along with the modified t-statistic and p-value, calculated in the limma package were used for calculations, statistics, and visualization. However, for isogroups represented by more than one contig (4,288 of 22,765 isogroups), we calculated the mean t-value of all contigs, and calculated significance on degrees of freedom equal to the total number of probes scored for the isogroup minus two. The median fold change from contigs was assigned to each isogroup. We used the R package qvalue (Storey, 2002) to calculate q-values using a global (across all eight contrasts) false discovery threshold of 0.05 (Benjamini and Hochberg, 1995). To further assess similarity in the effects of T-treatment between males and females, the direction of gene expression difference between comparisons was examined using a Fisher's exact test on genes that were differentially expressed between T-treated and control individuals in both sexes.

We then used topGO (Alexa and Rahnenfuhrer, 2010) with the weight algorithm (Alexa *et al.*, 2006) to identify the Gene Ontology (GO) terms (Ashburner *et al.*, 2000) that were significantly over-represented among the significantly differentially expressed genes in each comparison. Because we analyzed all three GO topologies, we used a Bonferroni corrected p-value cut-off of 0.0125. GO terms with fewer than five annotations were excluded from the analysis, and only terms with at least three genes in the significant gene set are reported.

Results

Sex differences

We identified significant differences in expression between control males and control females in both the liver and pectoralis. In the liver, 291 genes (of 12,206 expressed) were differentially expressed between control males and females (Figure 1a; Supplementary Table 1), including 218 that were more

highly expressed in males than in females and 73 that were more highly expressed in females than in males. Among these genes, 9 GO terms were significantly over-represented (Table 1).

In the pectoralis, 658 genes (of 11,465 expressed) were differentially expressed between control males and females (Figure 1b; Supplementary Table 1), including 450 that were more highly expressed in males than in females and 208 that were more highly expressed in females than in males. Among these genes, 18 GO terms were significantly over-represented (Table 2).

Among the genes differentially expressed between the sexes, 117 were significantly different in both liver and pectoralis (Figure 1c; Supplementary Table 1). Of these genes, 91 were higher in control males than control females in both tissues and 25 were higher in control females than control males in both tissues. Only one gene was differentially expressed by sex in opposite directions in the two tissues: *protein tyrosine phosphatase, receptor type C* was higher in control males than control females in the liver, but higher in control females than control males in the pectoralis. The general patterns of gene expression by sex were largely consistent between the two tissues. That is, genes that were more highly expressed in males than females in one tissue tended to be more highly expressed by males than females in the other tissue, and vice versa, more than expected by chance (Fisher's exact test, $p < 0.0001$).

Effect of T-treatment in females

In both liver and pectoralis, we identified significant differences in expression between control females and T-treated females. In the liver, 801 genes (of 12,064 expressed) were differentially expressed (Figure 2a; Supplementary Table 1) including 645 that were expressed at a higher level in T-treated females than controls and 156 that were expressed at a lower level in T-treated females than controls. Among these genes, 26 GO terms were over-represented (Table 3).

In the pectoralis, 402 genes (of 11,413 expressed) were differentially expressed between control females and T-treated females (Figure 2b; Supplementary Table 1), including 226 that were expressed at a higher level in T-treated females than controls and 174 that were expressed at a lower level in T-treated females than controls. Among these genes, 17 GO terms were over-represented (Table 4).

Among the genes differentially expressed between the T-treated and control females, 40 were significantly different in both liver and pectoralis (Figure 2c; Supplementary Table 1). Of these genes, 21 were higher in T-treated than control females in both tissues and 7 were lower in T-treated than

control females in both tissues; 12 genes were differentially expressed in opposite directions in both tissues. More genes were affected in the same direction (i.e., either higher or lower in T-treated than control females) in both tissues than expected by chance (Fisher's exact test, $p < 0.05$, demonstrating significant similarity in the direction of gene expression change in response to T-treatment in the two tissues in females.

Effect of T-treatment in males

In the liver, 283 genes (of 12,229 expressed) were differentially expressed between T-treated and control males (Figure 2d; Supplementary Table 1) including 99 that were expressed at a higher level in T-treated males than controls and 184 that were expressed at a lower level in T-treated males than controls. Among these genes, one GO term was over-represented: *acetylglucosaminyltransferase activity*.

In the pectoralis, 450 genes (of 11,282 expressed) were differentially expressed between control males and T-treated males (Figure 2e; Supplementary Table 1) including 148 that were expressed at a higher level in T-treated males than controls and 302 that were expressed at a lower level in T-treated males than controls. Among these genes, 8 GO terms were over-represented (Table 5).

Among the genes differentially expressed between the T-treated and control males, 21 were significantly different in both liver and pectoralis (Figure 2f; Supplementary Table 1). Of these genes, 6 were higher in T-treated than control males in both tissues and 10 were lower in T-treated than control males in both tissues; 5 genes were differentially expressed in opposite directions in the tissues. More genes were affected by T-treatment in the same direction (i.e., either higher or lower in T-treated than control males) in both tissues than expected by chance (Fisher's exact test; $p < 0.05$), suggesting similar changes in response to T-treatment in the two tissues in males.

Effect of T-treatment in both sexes

In both liver and pectoralis, some genes were differentially expressed between T-treated and control individuals of both sexes, though many genes were significantly differently affected in the two sexes (i.e. had a significant interaction effect). In the liver, 58 genes were differentially expressed in both sexes, representing only 5.6% of the 1,026 genes differentially expressed in at least one sex. There was a significant interaction between sex and the effect of T-treatment in the liver for 550 genes, including

366 (38%) of the genes that were significantly affected by T-treatment in only one sex (Figure 3a; Supplementary Table 1).

In the pectoralis, 68 genes were differentially expressed between T-treated and control individuals of both sexes, representing only 8.7% of the 784 genes that were differentially expressed in at least one sex. There was a significant interaction between sex and the effect of T-treatment in the pectoralis for 297 genes, including 189 (26%) of the genes that were only significantly affect by T-treatment in one sex (Figure 3b; Supplementary Table 1).

In the liver, the genes differentially expressed between T-treated and control individuals in both sexes include 28 that were expressed at a higher level in T-treated individuals than controls in both sexes, 11 that were expressed at a lower level in T-treated individuals than controls in both sexes, and 19 that were differentially expressed in opposite directions in the two sexes (Table 6). That is, 67% of genes differentially expressed by T-treatment in both sexes were differentially expressed in the same direction (i.e., either higher or lower in T-treated than control individuals in both sexes), more than expected by chance (Fisher's exact test, $p < 0.05$).

In the pecotralis, the genes differentially expressed between T-treated and control individuals in both sexes included 34 that were expressed at a higher level in T-treated individuals than controls in both sexes, 27 that were expressed at a lower level in T-treated individuals than controls in both sexes, and 7 that were differentially expressed in opposite directions in the two sexes (Table 6). That is, 90% of genes differentially expressed by T treatment in both sexes were differentially expressed in the same direction (i.e., either higher or lower in T-treated than control individuals in both sexes), more than expected by chance (Fisher's exact test, $p < 0.0001$).

Discussion

Using a microarray specific to the dark-eyed junco, we identified a large number of genes that were expressed differentially between males and females, and between T-treated and control individuals of each sex, in the liver and pectoralis. As predicted, many of the differentially expressed genes were functionally related to previously described phenotypic effects of T-treatment as well as known sexual dimorphisms. T-treatment tended to affect different genes in males and females; however, among the genes differentially expressed by T-treatment in both sexes, T-treatment affected most genes in the

same direction in males and females. This suggests that sexually dimorphic transcriptional responses to T may provide one solution to sexual conflict over circulating levels of T. Not only do these results provide a detailed view of the molecular mechanisms by which sexual conflict may be resolved, but they also lay a strong foundation for ecologically-relevant and evolutionarily-significant advances in our understanding of the mechanisms underlying life-history trade-offs and behavioral evolution in natural systems, such as the junco. Furthermore, by focusing on the liver and muscle, our findings point to the mechanisms by which sexual dimorphic peripheral responses to circulating hormones may play a role in sexual conflict and dimorphism, in addition to previously identified effects in the brain of juncos (Peterson *et al.*, 2013) and the sex-specific effects previously identified in rats (van Nas *et al.*, 2009; Yang *et al.*, 2006).

Sexually dimorphic gene expression

Similarly to previous studies on neural tissues in juncos (Peterson *et al.*, 2013) and multiple tissues in other species (reviewed in Ellegren and Parsch, 2007), we identified many genes that were expressed differentially between males and females. In the pectoralis, we identified 658 genes that were sexually dimorphic, and as predicted GO analysis revealed over-representation of terms related to muscle development, including *muscle system process* and both the *I band* and *A band* portions of the *sarcomere*. These genes were generally regulated in directions consistent with known sex differences in body-mass in the junco (Nolan *et al.*, 2002). For example, *titin*, a gene that regulates muscle elasticity (Itoh-Satoh *et al.*, 2002) is expressed at a higher level in control-males than females in the pectoralis. *SMAD-related protein 2* was more highly expressed in the pectoralis of control females than control males consistent with the known role of SMAD proteins in reducing cellular growth (Nakao *et al.*, 1997). Consistent with other studies comparing transcriptional patterns in skeletal muscle of males and females (Yang *et al.*, 2006; Roth *et al.*, 2002; Welle *et al.*, 2008), we found a large number of genes that differed in expression between the sexes, including several genes that were directly related to muscle development and growth.

We also identified 291 genes that showed significantly different expression between control males and control females in the liver, and several of the differentially expressed genes were related to known phenotypic differences between the sexes. For example, *Lipid phosphate phosphohydrolase 1*, a gene involved in glycerolipid synthesis and lipid uptake (Kai *et al.*, 1997), was expressed at a higher level in control males than females, consistent with sex differences in metabolic activity (Fernando *et al.*, 2010; Wikelski *et al.*, 1999). Further, the GO term *steroid binding* was over-represented among these genes.

For example, *hydroxysteroid dehydrogenase like 2* (*HSDL2*) was expressed more highly in the liver of control males than females. *HSDL2* plays a role in sterol binding (Dai *et al.*, 2003), and is marginally more highly expressed in the liver of females than males in mice (Gatti *et al.*, 2010). This suggests that the sexes might differ in their metabolism of sterol based compounds in the liver, but that this difference may vary between taxa.

Among genes differentially expressed between control males and control females in the pectoralis were several transcription factors. In both liver and pectoralis, *transcription factor III B 150* (*TFIIIB150*) was more highly expressed in control males than control females. *TFIIIB150* mediates transcription via RNA polymerase III (Schramm *et al.*, 2000), which is primarily involved in the expression of 5S rRNA, tRNA, and other small RNA's (Dieci *et al.*, 2007). In contrast, *Basic Transcription Factor 3* (*BTF3*) was expressed more in control females than males in the liver, and *activated RNA polymerase II transcriptional coactivator p15* (*P15*) was expressed more in control females than males in the pectoralis. Both *BTF3* (Zheng *et al.*, 1990) and *P15* (Kretzschmar *et al.*, 1994) activate expression via RNA polymerase II, which is the primary polymerase for the expression of protein coding genes (Sims *et al.*, 2004). These findings suggest that males and females may orchestrate gene expression differently, with males favoring expression of housekeeping type genes that may increase translation rates, and females favoring expression of protein coding genes. Transcription factors are among the genes that are differentially expressed by sex in human muscles (Roth *et al.*, 2002), and *BTF3* and *P15* are differentially expressed by sex in the liver and muscle of mice (Yang *et al.*, 2006). This suggests that transcription factors in general, and *BTF3* and *P15* in particular, may be involved in sexually dimorphic patterns of expression in many species.

Effect of T-treatment in females

Testosterone implants affected female gene expression in both tissues, and the effects were consistent with known phenotypic effects of T-treatment. For example, *Immunoglobulin A* (*IgA*) *heavy chain* had lower expression in the liver of T-treated females than controls, and *AF411388_1 basic,* a gene containing a conserved immunoglobulin region (Yoder *et al.*, 2002), was expressed at a lower level in the pectoralis of T-treated females than controls. Immunoglobulins play a major role in immune function (Litman *et al.*, 1993), so their lower expression in T-treated females is consistent with the known suppressive effect of T-treatment on immune function in female juncos (Zysling *et al.*, 2006).

Additionally, the GO term *growth* was over-represented among the genes differentially expressed in the pectoralis between T-treated and control females. Eleven of the seventeen genes annotated as *growth* were more highly expressed in T-treated females than controls, and the other six were expressed at a lower level in T-treated females than controls. Three of the genes that were expressed at a lower level are known repressors of growth (two representations of *Ankyrin repeat domain-containing protein 26*; Bera *et al.*, 2008) or transcription (*B-cell CLL/lymphoma 6 (zinc finger protein 51)*; Lemercier *et al.*, 2002). Both higher expression of growth promoting genes and lower expression of growth repressors are consistent with the role of elevated androgens in increasing muscular growth and maintenance (Woodward, 1993; Hartgens and Kuipers, 2004).

A number of the genes identified as differentially expressed between T-treated and control females were similar to those identified in studies of other organisms. The GO term *response to hormone stimulus* consists of genes identified as mediators of phenotypic effects of hormones in other species (Ashburner *et al.*, 2000), and was over represented among differentially expressed genes in female pectoralis. Among these genes, *Serotonin 1B receptor* was expressed at a higher level in T-treated than control females. *Serotonin 1B receptor* expression is up-regulated by mineralcorticoids in the aorta of rats (Banes and Watts, 2002); thus, expression of this serotonin receptor may be mediated indirectly by T-treatment through changes in other signaling molecules. *Serotonin 1B receptor* has a range of effects on both behavior and physiology (Donaldson *et al.*, 2013), though its role in skeletal muscle tissue is unclear. In addition, *carbonic anhydrase II*, a catalyst of the hydrolysis of carbon dioxide (Sterling *et al.*, 2001), was expressed at lower levels in the pectoralis of T-treated females than controls. Expression of a related gene, *carbonic anhydrase III*, is reduced by strength training in humans (Roth *et al.*, 2002), suggesting that the action of T-treatment may be related to changes in muscle activity. Expression of c*arbonic anhydrase II* is also reduced by exposure to estrogens in some tissues in rats (Caldarelli *et al.*, 2005), consistent with the view that some of the effects seen in our study may be mediated by conversion of T to estradiol. We anticipate that many of the genes we have identified play a role in mediating tissue-level responses to hormones in multiple species, patterns that will become clear in time.

Effect of T-treatment in males

We identified a large number of genes that were differentially expressed between T-treated and control males in both liver and pectoralis, and several of them are related to known phenotypic effects of T-treatment. For example, *heme oxygenase (decyclizing) 1* (*HMOX1*) was expressed at lower levels in the

liver of T-treated than control males. *HMOX1* is a key enzyme in the breakdown of heme (Platt and Nath, 1998), and has been implicated in the disruption of human glucose regulation (Bao *et al.*, 2012). Therefore, *HMOX1*'s lower expression in T-treated males is consistent with previous findings that T increases metabolism (Oppliger *et al.*, 2004; Fernando *et al.*, 2010), as well as other studies that have linked heme-related enzymes with activational effects of androgens (van Nas *et al.*, 2009). *Aldehyde oxidase 1* (*AOX1*) was also expressed at lower levels in the liver of T-treated males than controls. Aldehyde oxidases break down a number of metabolically active compounds (Hartmann *et al.*, 2012). So the lower expression of *AOX1* may indicate that T-treatment reduced catabolism in the liver, consistent with a previous study showing that castrated mice treated with androgens also showed significant changes in expression of a variety of metabolic genes in the liver (van Nas *et al.*, 2009).

Previous studies in humans (Michael *et al.*, 2005), rats (Wakley *et al.*, 1991) and chickens (Pederson *et al.*, 1999) have demonstrated that higher T reduces bone resorption, though several of the effects may be related to the conversion of T to estradiol (Oursler *et al.*, 1991). Consistent with these findings, we observed that *osteoclast inhibitory lectin*, which blocks the formation of bone-resorption osteoclasts (Hu *et al.*, 2004), was expressed at higher levels in T-treated than control males in both the liver and pectoralis.

Many of the effects of T-treatment that we have identified have the potential to play large, downstream roles, as evidenced by the over-representation of the GO term *rRNA metabolic process* among genes differentially expressed in the pectoralis. For example, *Serine/arginine-rich splicing factor 5* modulates the splice variant selection of many genes (Sebbag-Sznajder *et al.*, 2012) and thus plays a large role in cellular function. This gene was more highly expressed in both the liver and pectoralis of T-treated than control males. In the pectoralis, *MGC89063* was more highly expressed in T-treated males than controls, which is similar to what was found in the hypothalamus and medial amygdala of the junco (Peterson *et al.*, 2013). *MGC89063* is a transcription factor (Ashburner *et al.*, 2000; Hunter *et al.*, 2009), and the fact that *MGC89063* was more highly expressed in the hypothalamus, medial amygdala, and pectoralis (but not liver) of T-treated males than controls, and not differentially expressed by T-treatment in females, raises the possibility that *MGC89063* may play a tissue- and sex-specific role in meditating the effects of T-treatment via down-stream gene regulation. The specific down-stream effects of this gene remain unclear, but given its role in multiple target tissues, further investigation into the pleiotropic roles of *MGC89063* will likely provide novel insights into the integrated response to T-treatment. Continued focus on non-model organisms like the junco in these future studies may provide greater insight into the fitness consequences of genes like these.

Effect of T-treatment in both sexes

Many genes were differentially expressed in the liver and pectoralis between T-treated and control individuals of *both* sexes (63 genes in liver, and 70 genes in pectoralis). However this number represents only 5% of the genes differentially expressed by T-treatment in either sex, meaning 95% of genes that were affected by T were not significantly affected in both sexes. Further, in each tissue over a quarter of these genes were affected significantly differently in each sex (i.e. had a significant interaction effect), suggesting that many, though not all, of the genes identified in only one sex are truly only affected in that sex. This result, especially when combined with similar findings in neural tissue in juncos (Peterson *et al.*, 2013), lends some support to the hypothesis that T-treatment leads to transcriptional changes in largely different genes in the two sexes, and suggests a possible remedy to sexual conflict over T levels. On the other hand, among those genes that were differentially expressed in both males and females, most (78%) were differentially expressed in the same direction in both sexes. Collectively, these results suggest that there may be a core transcriptional response to T-treatment shared between the sexes, but this response is fine-tuned by sex-specific responses, which may reduce sexual conflict over circulating T levels.

Among the genes that were significantly differentially expressed between T-treated and control individuals in both sexes, several relate to the known effects of T-treatment on activity and metabolism (Wikelski *et al.*, 1999; Lynn *et al.*, 2000; Buchanan *et al.*, 2001). For example, in the liver and pectoralis of both sexes, *L-arginine:glycine amidinotransferase* was more highly expressed in T-treated individuals than controls. This gene encodes the enzyme for the rate-limiting step in creatine biosynthesis (Humm *et al.*, 1997), which in turn increases energy availability in muscle (Kraemer and Volek, 1999), and is also regulated by steroid hormones in rodents (Krisko and Walker, 1966). Therefore, greater expression of *L-arginine:glycine amidinotransferase* in T-treated individuals is consistent with steroid-induced increases in activity levels and metabolic rate. Similarly, *3-hydroxybutyrate dehydrogenase* (*3HBDH*) was expressed at a higher level in T-treated individuals in both sexes and both tissues. *3HBDH* catalyzes the reversible reaction between beta-hydroxybutyric acid and acetoacetate, a key step in the breakdown of fatty acids for energy (Bergmeyer *et al.*, 1967; Williamson *et al.*, 1962). Both male and female rats respond to androgen treatment with changes in the expression of fatty acid metabolizing genes as well (van Nas *et al.*, 2009). Together, the changes in the expression of these genes could be a major contributor to T-induced shifts in metabolism and activity in juncos (Chandler *et al.*, 1994; Lynn *et al.*, 2000) and other species (Wikelski *et al.*, 1999; Marler *et al.*, 1995). However, it remains possible that these changes in gene expression are indirect effects of T-

treatment, e.g. if T affects metabolism or activity via other routes and these genes respond in kind to altered metabolism or activity.

Several genes related to insulin signaling were differentially expressed between T-treated and control individuals of both sexes in the liver. *Insulin receptor substrate 4* (*IRS4*), for example, was expressed more highly in the livers of T-treated individuals than controls in both sexes. *IRS4* mediates the activity of a number of growth factors (e.g. Hinsby *et al.*, 2004), and lack of *IRS4* leads to a decrease in body size in knockout mice (Fantin *et al.*, 2000). Therefore, higher expression of *IRS4* in the liver of T-treated individuals than controls may mediate some of the previously reported metabolic and growth effects of T-treatment (Cox *et al.*, 2009; Wikelski *et al.*, 1999; Lynn *et al.*, 2000). In addition, *insulin-like growth factor 2 receptor* was more highly expressed in the liver of T-treated females than controls, and *insulin-like growth factor 1* was expressed at lower levels in the liver of T-treated males than controls. However, neither gene had a significant sex-by-treatment interaction term, suggesting that both genes may also have been regulated in the opposite sex, but below our limits of detection. Insulin like growth factors also mediate growth (Abuzzahab *et al.*, 2003; Petry *et al.*, 2005) and have been implicated in the expression of sexually selected traits (Emlen *et al.*, 2012), some of which are also meditated by androgens (Folstad and Karter, 1992).

Several genes related to the regulation of growth were differentially expressed in liver between T-treated and control individuals of each sex, though some of the specific genes affected by T-treatment differed between males and females. *Follistatin* was more highly expressed in the liver of T-treated individuals than controls in both sexes. *Follistatin* binds and inactivates members of the TGF-beta super family, including myostatin, such that increased *follistatin* is associated with increased muscle growth (Lee and McPherron, 2001). Further, *epidermal growth factor receptor* (*EGFR*) was more highly expressed in the liver of T-treated than control females and had a marginally significant sex-by-treatment interaction term (uncorrected $p = 0.04$), suggesting some sex-specific hormone regulation. Likewise, *opioid growth factor receptor* (*OGFr*) was less expressed in the liver of T-treated than control males and also had a significant sex-by-treatment interaction term. *EGFR* acts to increase cell proliferation and growth (Oda *et al.*, 2005), but *OGFr* acts to reduce growth (Zagon *et al.*, 2008). In human men, similar changes in the expression of growth-related genes, including *OGFr*, are observed in response to hormone manipulation, and the changes are believed to be related to lean muscle mass growth (Montano *et al.*, 2007). Thus, the expression changes seen in both male and female juncos are related to increased growth, but potentially via different transcriptional mechanisms.

Summary

In this study, we applied genomic tools to the dark-eyed junco, in order to identify ecologically relevant sex differences in gene expression and transcriptional responses to experimentally elevated testosterone. As predicted, many of the specific genes affected were associated with known physiological and metabolic effects of T-treatment, but the expression response to T-treatment was different in the two sexes: only 5% of regulated genes overlap in the two sexes. Interestingly, among genes that were differentially expressed between T-treated and control individuals in both sexes, most were differentially expressed in the same direction. Therefore, testosterone may be utilizing a shared core set of transcriptional paths in both sexes that are complemented and modified by sex specific transcriptional responses. Characterizing these effects in the periphery is particularly notable in light of the prevailing view in behavioral neuroendocrinology that many sex differences are mediated at the level of the brain. Our results detail some of the molecular mechanisms by which hormones have sex-specific activational effects in two important peripheral tissues. Whether these sex-specific mechanisms represent adaptive mechanistic responses to T is an open question that can be addressed by continued focus on natural species like the junco.

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Author Contributions

MPP, KAR, JHC, HT, JKC, and EDK contributed to the conception and design of the project. MPP, KAR, CAT, JAL, JHC, and CZ performed data collection or analysis. All authors contributed to the interpretation of results and the editing of the manuscript.

Figures

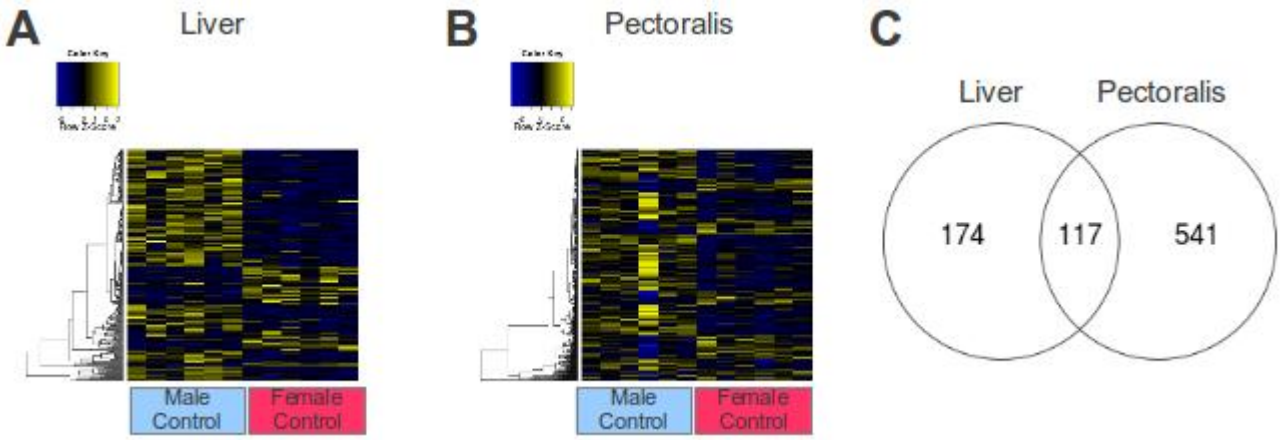

Figure 1. Sex differences in gene expression. Differences in gene expression between the sexes are represented by heat maps that show scaled individual expression scores for significantly differentially expressed genes in the liver (a) and pectoralis (b). Venn diagram shows the overlap in significant genes between the two tissues (c). Each column represents and individual, and each row a gene. Yellow represents high gene expression, blue represents low expression scaled to the levels of expression for each gene.

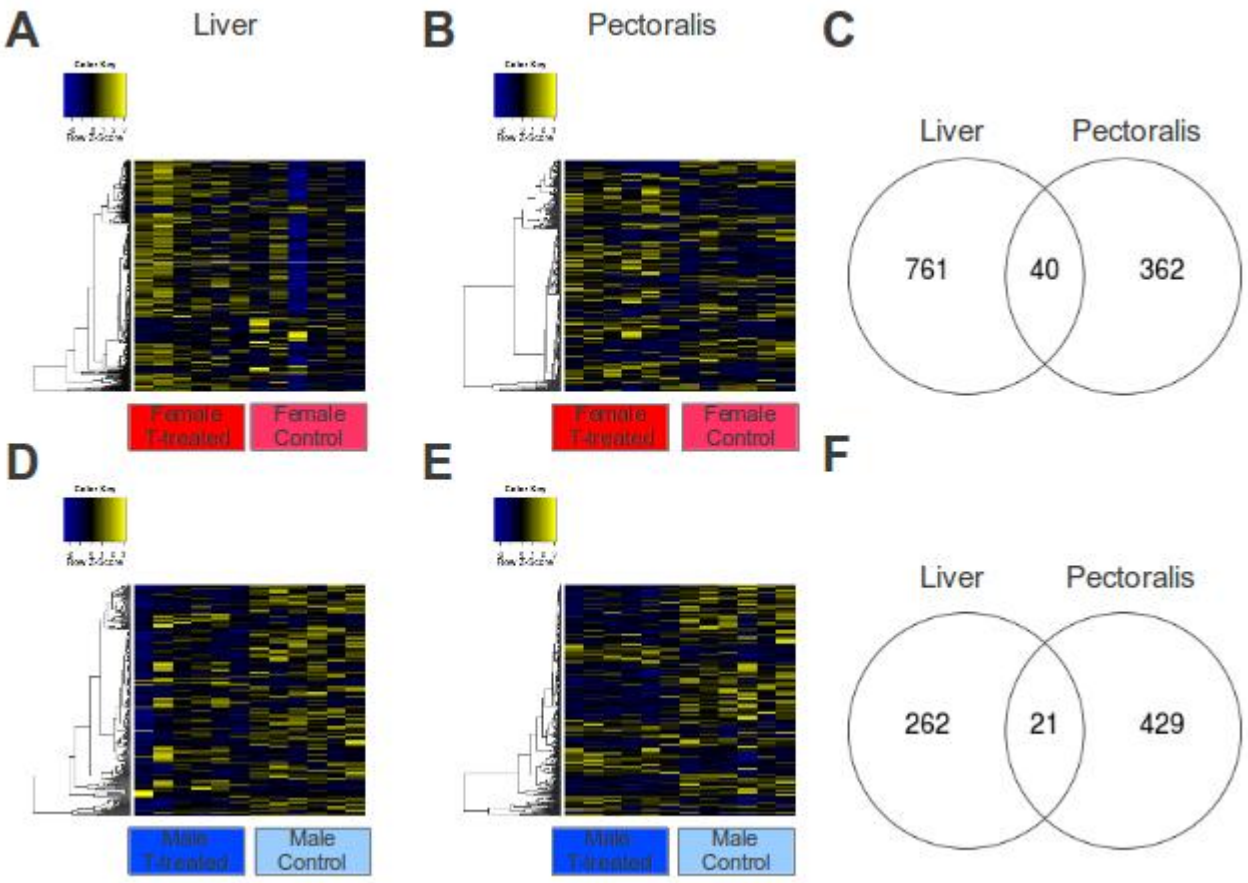

Figure 2. Gene expression in response to T-treatment in each sex. Differences in gene expression between T-treated and control individuals in both the liver (left column) and the pectoralis (middle column) in females (a-c) and in males (d-f). Heat maps show scaled individual expression scores for genes that were significantly differentially expressed between T-treated and control individuals in each sex (a,b,d,e). Venn diagrams (c,f) show the overlap of significant genes within each contrast between the tissues. See text and supplementary tables for more information. Each column represents and individual, and each row a gene. Yellow represents high gene expression, and blue represents low expression scaled to the levels of expression for each gene.

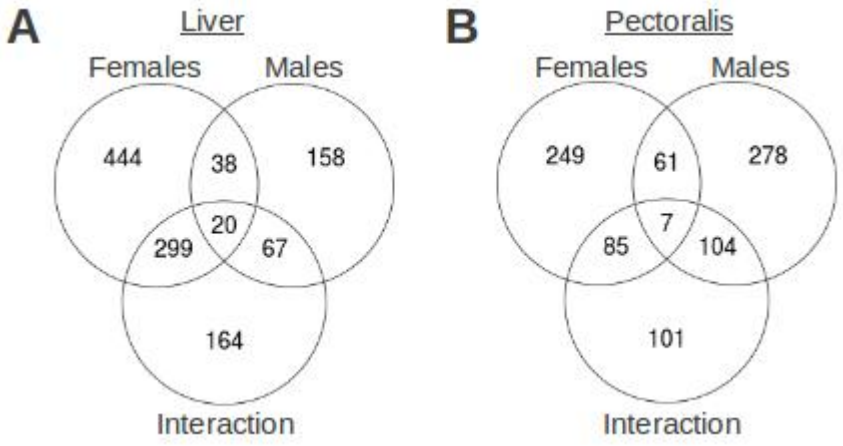

Figure 3. Comparing the effect of T-treatment in males and females. Venn diagrams for (A) liver and (B) Pectoralis showing the number of genes significantly differentially expressed between T-treated and control individuals in males and females, and those with a significant sex-by-treatment interaction effect.

Table 1. GO terms over-represented among genes differentially expressed in the liver between males and females.

GO ID	GO Description	Annotated Genes Expressed	Number Significantly DE	P value
GO:0002440	production of molecular mediator of immune response	26	4	0.0047
GO:0006672	ceramide metabolic process	7	3	0.0005
GO:0006892	post-Golgi vesicle-mediated transport	15	3	0.0059
GO:0007033	vacuole organization	28	5	0.0006
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	33	4	0.0093
GO:0044419	interspecies interaction between organisms	19	4	0.0038
GO:0046519	sphingoid metabolic process	14	3	0.0048
GO:0051259	protein oligomerization	111	8	0.0071
GO:0005496	steroid binding	17	3	0.0071

Table 2. GO terms over-represented among genes differentially expressed in the pectoralis between males and females.

GO ID	GO Description	Annotated Genes Expressed	Number Significantly DE	P value
GO:0000018	regulation of DNA recombination	9	4	0.0009
GO:0003007	heart morphogenesis	45	12	0.0000
GO:0007033	vacuole organization	24	7	0.0002
GO:0009306	protein secretion	16	4	0.0095
GO:0014866	skeletal myofibril assembly	15	9	0.0000
GO:0031929	TOR signaling cascade	9	3	0.0106
GO:0048585	negative regulation of response to stimulus	13	4	0.0042
GO:0048738	cardiac muscle tissue development	23	11	0.0000
GO:0051046	regulation of secretion	27	5	0.0105
GO:0051095	regulation of helicase activity	5	3	0.0015
GO:0051899	membrane depolarization	6	3	0.0029
GO:0004866	endopeptidase inhibitor activity	31	6	0.0057
GO:0004896	cytokine receptor activity	5	3	0.0015
GO:0032135	DNA insertion or deletion binding	5	3	0.0015
GO:0030017	sarcomere	92	17	0.0027
GO:0031672	A band	23	9	0.0000
GO:0031674	I band	62	12	0.0001
GO:0032300	mismatch repair complex	5	3	0.0013

Table 3. GO terms over-represented among genes differentially expressed in the liver between T-treated and control females.

GO ID	GO Description	Annotated Genes Expressed	Number Significantly DE	P value
GO:0000087	M phase of mitotic cell cycle	144	19	0.0048
GO:0001707	mesoderm formation	5	3	0.0033
GO:0001708	cell fate specification	5	3	0.0033
GO:0006275	regulation of DNA replication	7	3	0.0102
GO:0006874	cellular calcium ion homeostasis	47	9	0.0075
GO:0006892	post-Golgi vesicle-mediated transport	15	5	0.0030

GO ID	GO Description	Annotated Genes Expressed	Number Significantly DE	P value
GO:0006999	nuclear pore organization	5	3	0.0033
GO:0008105	asymmetric protein localization	11	4	0.0057
GO:0009057	macromolecule catabolic process	271	38	0.0014
GO:0010518	positive regulation of phospholipase activity	9	4	0.0024
GO:0016197	endosomal transport	27	6	0.0105
GO:0016477	cell migration	172	22	0.0077
GO:0019751	polyol metabolic process	24	7	0.0011
GO:0035195	gene silencing by miRNA	12	4	0.0080
GO:0043171	peptide catabolic process	6	3	0.0062
GO:0051603	proteolysis involved in cellular protein catabolic process	184	24	0.0006
GO:0005275	amine transmembrane transporter activity	25	7	0.0015
GO:0008017	microtubule binding	13	5	0.0015
GO:0016712	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	6	3	0.0064
GO:0016769	transferase activity, transferring nitrogenous groups	16	5	0.0044
GO:0016790	thiolester hydrolase activity	53	11	0.0012
GO:0019787	small conjugating protein ligase activity	135	22	0.0002
GO:0042562	hormone binding	10	4	0.0040
GO:0005874	microtubule	18	6	0.0011
GO:0009925	basal plasma membrane	9	4	0.0024
GO:0031231	intrinsic to peroxisomal membrane	6	3	0.0061

552

553 **Table 4. GO terms over-represented among genes differentially expressed in the pectoralis**

554 **between T-treated and control females.**

GO ID	GO Description	Annotated Genes Expressed	Number Significantly DE	P value
GO:0006665	sphingolipid metabolic process	28	4	0.0109
GO:0008406	gonad development	27	5	0.0014
GO:0010038	response to metal ion	94	8	0.0093

GO ID	GO Description	Annotated Genes Expressed	Number Significantly DE	P value
GO:0035265	organ growth	10	3	0.0032
GO:0048511	rhythmic process	29	4	0.0123
GO:0048545	response to steroid hormone stimulus	89	8	0.0067
GO:0055088	lipid homeostasis	14	3	0.0087
GO:0004091	carboxylesterase activity	23	4	0.0044
GO:0005267	potassium channel activity	17	5	0.0001
GO:0008017	microtubule binding	15	3	0.0091
GO:0015179	L-amino acid transmembrane transporter activity	5	3	0.0003
GO:0016298	lipase activity	26	5	0.0009
GO:0005874	microtubule	17	3	0.0117
GO:0005887	integral to plasma membrane	48	5	0.0115
GO:0009925	basal plasma membrane	12	3	0.0042
GO:0031012	extracellular matrix	84	10	0.0005
GO:0031461	cullin-RING ubiquitin ligase complex	20	4	0.0022

Table 5. GO terms over-represented among genes differentially expressed in the pectoralis between T-treated and control males.

GO ID	GO Description	Annotated Genes Expressed	Number Significantly DE	P value
GO:0009066	aspartate family amino acid metabolic process	11	3	0.0044
GO:0009895	negative regulation of catabolic process	11	3	0.0044
GO:0016072	rRNA metabolic process	13	3	0.0072
GO:0022904	respiratory electron transport chain	29	5	0.0020
GO:0030301	cholesterol transport	13	3	0.0072
GO:0005342	organic acid transmembrane transporter activity	23	4	0.0050
GO:0016829	lyase activity	72	8	0.0016
GO:0048037	cofactor binding	63	7	0.0032

Table 6. Comparing gene expression in response to T-treatment in males and females. Genes that were significantly differentially expressed between T-treated and control individuals in both sexes within liver or pectoralis. These genes represent less than 10% of the genes differentially expressed in at least one sex. See text and supplementary tables for more information.

Liver				**Pectoralis**		
	Lower in T-treated than control females	Higher in T-treated than control females			Lower in T-treated than control females	Higher in T-treated than control females
Higher in T-treated than control males	9	28		Higher in T-treated than control males	5	34
Lower in T-treated than control males	11	10		Lower in T-treated than control males	27	2

Supplementary Materials

Supplementary Figure 1. Hybridization design for microarray experiments.

Supplementary Table 1. Genes significantly differentially expressed.

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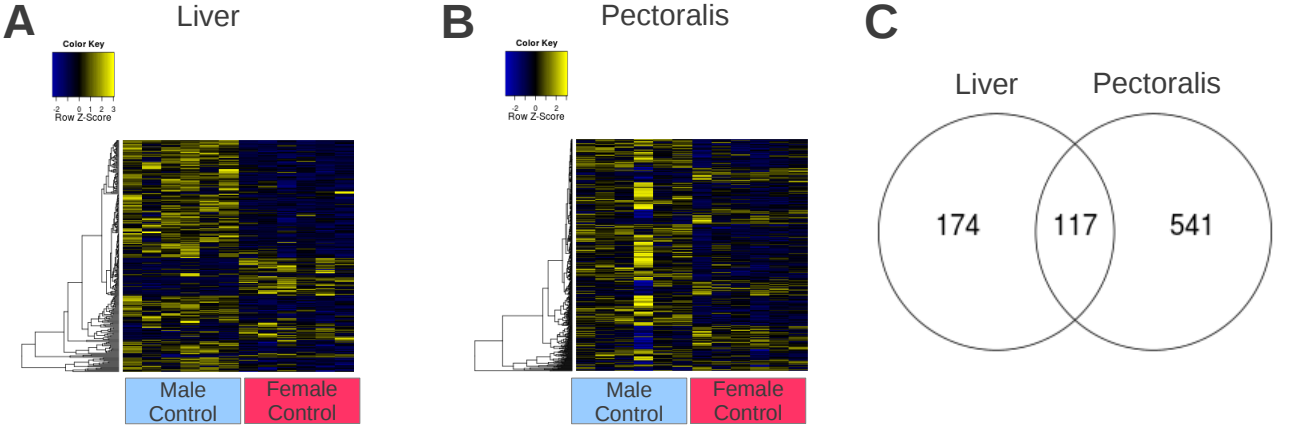
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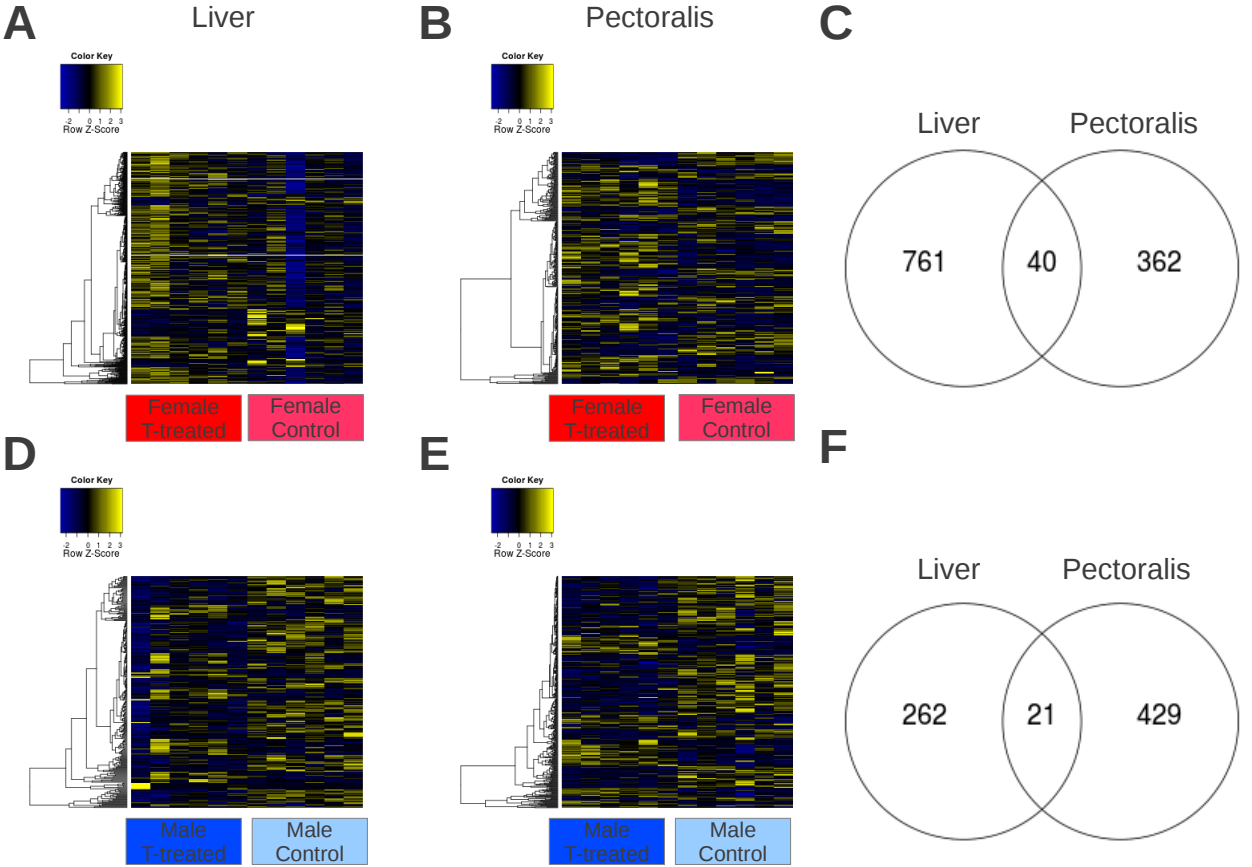
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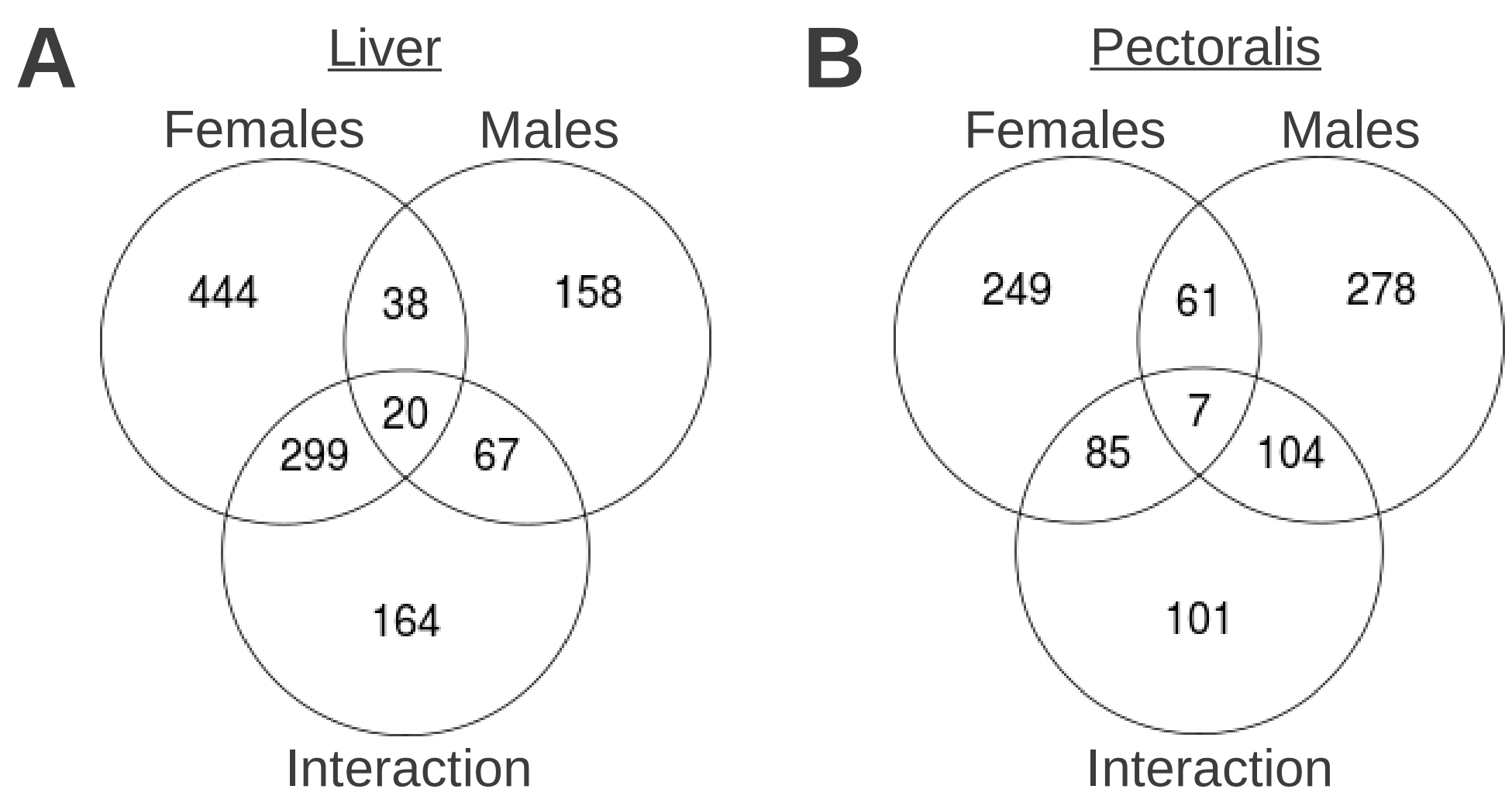
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