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Comparative Transcriptomics of Seasonal Phenotypic Flexibility in Two North American Songbirds

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Synopsis Phenotypic flexibility allows organisms to reversibly alter their phenotypes to match the changing demands of seasonal environments. Because phenotypic flexibility is mediated, at least in part, by changes in gene regulation, comparative transcriptomic studies can provide insights into the mechanistic underpinnings of seasonal phenotypic flexibility, and the extent to which regulatory responses to changing seasons are conserved across species. To begin to address these questions, we sampled individuals of two resident North American songbird species, American goldfinch (*Spinus tristis*) and black-capped chickadee (*Poecile atricapillus*) in summer and winter to measure seasonal variation in pectoralis transcriptomic profiles and to identify conserved and species-specific elements of these seasonal profiles. We found that very few genes exhibited divergent responses to changes in season between species, and instead, a core set of over 1200 genes responded to season concordantly in both species. Moreover, several key metabolic pathways, regulatory networks, and gene functional classes were commonly recruited to induce seasonal phenotypic shifts in these species. The seasonal transcriptomic responses mirror winter increases in pectoralis mass and cellular metabolic intensity documented in previous studies of both species, suggesting that these seasonal phenotypic responses are due in part to changes in gene expression. Despite growing evidence of muscle nonshivering thermogenesis (NST) in young precocial birds, we did not find strong evidence of upregulation of genes putatively involved in NST during winter in either species, suggesting that seasonal modification of muscular NST is not a prominent contributor to winter increases in thermogenic capacity for adult passerine birds. Together, these results provide the first comprehensive overview of potential common regulatory mechanisms underlying seasonally flexible phenotypes in wild, free-ranging birds.

Introduction

Seasonally variable environments promote the evolution of flexible phenotypes, thereby permitting fine-tuned matching of phenotypes, including physiology, to changing environmental demands (Pigliucci 2001; Piersma and van Gils 2011). Thermoregulatory performance in vertebrate animals is a flexible trait that varies predictably in response to environmental demands, such as cold temperatures, for both endotherms and ectotherms (Angilletta 2009; Swanson 2010). Ectothermic vertebrates typically show metabolic compensation to temperature in variable thermal environments, mediated through adjustments in key enzyme activities (e.g., an increase of muscular citrate synthase activity in the cold) and other physiological changes (Seddon and Prosser 1997; Seebacher 2005; White et al. 2012). These adjustments partially or completely compensate for temperature effects on metabolic rates, so acclimation maintains metabolic function across a range of environmental temperatures (Kent et al. 1988; Donelson and Munday 2012). Phenotypic flexibility in ectotherm physiological capacities may vary seasonally, so that temperature compensation occurs only during some seasons (e.g., Basson and Clusella-Trullas 2015). Whereas phenotypic flexibility in ectotherms acts to maintain metabolic performance under variable thermal conditions, endotherms increase metabolic capacity in response to increases in energy demands for activity or thermoregulation

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(Lovegrove 2005; Weibel and Hoppeler 2005; Swanson 2010). As a consequence, climatic variation in winter-active endotherms increases energy demands and tends to elevate, rather than maintain, metabolic capacities. Thus endotherms, especially small endotherms, with their high surface area to volume ratios and consequent high rates of heat loss, show a fundamentally different pattern of metabolic flexibility in response to variable climates than ectotherms (Gaston et al. 2009; Bozinovic et al. 2011; Fristoe et al. 2015).

For small birds, winter or cold temperatures result in increments of maximum cold-induced metabolic rate (summit metabolism, Msum) (Swanson 2010). In turn, Msum is positively correlated both with cold tolerance (Swanson and Liknes 2006) and with overwinter survival (Petit et al. 2017) in small birds. The mechanistic underpinnings of such increases in Msum may include increases in pectoralis muscle and/or heart masses (e.g., Liknes and Swanson 2011a; Petit and Vezina 2014; Swanson et al. 2014a), cellular metabolic intensity and/or lipid catabolic capacity (e.g., Marsh and Dawson 1982; Yacoe and Dawson 1983; Liknes and Swanson 2011b; Peña-Villalobos et al. 2014; Zheng et al. 2014a, 2014b), and oxygen transport (O'Connor 1996; Petit et al. 2017), but these underlying mechanisms and their specific contributions to seasonal or cold-induced changes in Msum are not fully understood. Indeed, consistent changes in these pathways with winter or cold-acclimation do not occur for all species or for all winters (or cold-exposure treatments) (Swanson 2010; Swanson et al. 2014a; Barceló et al. 2017). Moreover, additional pathways, such as oxidative balance and immunity, may also vary between summer and winter in birds (Owen-Ashley and Wingfield 2007; Raja-aho et al. 2012), but whether such seasonal variation occurs regularly in these pathways is not well known.

Among the most consistent contributors to the winter (or cold-acclimation) phenotype in small birds are increases in pectoralis muscle and heart masses and increases in lipid transport and catabolic capacities (Marsh and Dawson 1989; Swanson 2010). Regulation of gene and protein expression in these and other pathways in response to changing seasons or temperatures is a likely mechanism for generating flexible phenotypic responses at the organismal level. Such regulation allows the gene products mechanistically responsible for flexible phenotypes to be upregulated or downregulated to match changing environmental conditions (Ayroles et al. 2009; Cheviron et al. 2014; Stager et al. 2015). Several recent studies have investigated seasonal or cold-induced regulation of gene and protein expression in muscle remodeling and lipid transport pathways. Among the prime candidates for regulation of seasonal or cold-induced muscle remodeling in birds is myostatin, an autocrine/paracrine inhibitor of muscle growth (Lee 2004), and its metalloproteinase activators TLL-1 and TLL-2. In general, seasonal or cold-induced increases in Msum are accompanied by increments of pectoralis muscle and heart masses, which in turn are associated with reductions in mRNA or protein expression of myostatin and/or the TLLs (Swanson et al. 2009; Swanson et al. 2014a; Zhang et al. 2015a). However, these relationships are not completely consistent across all species or all conditions differing in energy demands (Swanson et al. 2014b; Stager et al. 2015; Zhang et al. 2015a, 2017). Likewise, gene and protein expression of fat transporters and fat catabolism pathways also tends to be positively correlated with Msum or increasing thermoregulatory demands, but expression patterns in these pathways are not always positively correlated for all species or for all steps in the pathways (Stager et al. 2015; Zhang et al. 2015b, 2015c). Muscle remodeling and lipid transport and catabolism pathways, however, almost certainly only partially account for seasonal variation in Msum and cold tolerance in small birds inhabiting cold winter climates and contributions of additional metabolic pathways to seasonal variation at the organismal level are largely unknown.

Transcriptomic studies have the potential to identify additional key pathways important to seasonal phenotypic flexibility, but the only study examining transcriptome-wide patterns of gene expression in response to cold-acclimation in small birds of which we are aware is that of Stager et al. (2015), which measured transcriptomic profiles of captive dark-eyed juncos (*Junco hyemalis*) that were sampled during the winter and exposed for six weeks to controlled photoperiod-temperature treatments. Only cold-exposure increased Msum in these birds (Swanson et al. 2014b), but both temperature and photoperiod modified transcriptional profiles of the pectoralis muscle (Stager et al. 2015). Enhanced thermogenic performance in cold-acclimated birds was accompanied by upregulation of genes involved in muscle hypertrophy, angiogenesis, lipid transport and oxidation, and catabolic enzyme activities (Stager et al. 2015). Concerted expression across tricarboxylic acid and oxidative phosphorylation pathways was upregulated in cold-acclimated juncos on short days, consistent with winter increases in cellular aerobic capacity in winter birds, but expression across fatty acid metabolism pathways increased on

long days, perhaps associated with development of migratory disposition (Stager et al. 2015). These changes in expression suggest prominent roles for muscle remodeling and fuel delivery and catabolism pathways in the winter phenotype, but no studies have examined transcriptomic profiles in seasonally acclimatized wild birds from cold winter climates to determine if similar adjustments of gene expression also occur in response seasonal conditions in the natural environment.

The goal of the present study was to measure seasonal variation in transcriptomic profiles in wild-caught individuals of two species of year-round resident birds from the cold winter climate of South Dakota, black-capped chickadee (*Poecile atricapillus*) and American goldfinch (*Spinus tristis*), to identify conserved and species-specific transcriptomic responses to winter. Both of these species show substantial winter increments of Msum at this location (Cooper and Swanson 1994; Liknes et al. 2002; Swanson and Liknes 2006), and also show seasonal variation pectoralis muscle and heart masses (Liknes and Swanson 2011a; Petit and Vezina 2014; Swanson et al. 2014a) and in the myostatin and fat transport and catabolism pathways (Liknes and Swanson 2011b; Swanson et al. 2014a; Zhang et al. 2015b). Chickadees also show increases in blood oxygen transport and oxidative defense in winter or with experimentally elevated energy demands (Petit and Vezina 2014). These two species should, therefore, serve as good models for investigating seasonal variation in the transcriptome in response to cold winter climates, and should help elucidate conserved and variable patterns of gene expression associated with seasonal phenotypes of small passerine birds.

Materials and methods

Sampling

We captured black-capped chickadees and American goldfinches by mist net before 1000 CST in summer (June–August; $n = 10$ for both species) and winter (December–February; $n = 7$ for both species) from wild populations near Vermillion, Clay County, South Dakota, USA (approximately 42°47′N, 97°W). All goldfinches captured in both seasons were adults (aged by skull ossification) and sex ratios were 3 males:4 females in winter and 4 males:6 females in summer. Of the 10 summer chickadees, four were independent hatch-year birds with incompletely ossified skulls (captured in mid to late July). These hatch-year birds were likely 1.5–2 months old, as chickadees begin nesting in South Dakota in mid-April (Drilling et al. 2016), and did not differ significantly in body or muscle masses from adult birds at the same season. We did not sex chickadees, as sexing birds by plumage is not possible. After capture we transported birds back to the laboratory and euthanized them by cervical dislocation within 2 h of capture. We quickly excised pectoralis muscles on ice and weighed them to the nearest 0.1 mg, before removing approximately 0.1 g samples and placing them into 2 mL of RNAlater (Ambion, Grand Island, NY, USA) in microcentrifuge tubes. Samples in RNAlater were flash frozen in liquid nitrogen and stored frozen at −80 °C until subsequent analyses. We thawed these samples once, with sub-samples removed from RNA later for qRT-PCR analyses in Swanson et al. (2014a) and Zhang et al. (2015a), before re-freezing and storing them at −80 °C. Subsequently, we shipped frozen samples in RNAlater on dry ice to the University of Illinois for transcriptome analysis. We collected birds on appropriate state (10–2, 11–7, 12–2) and federal (MB758442) scientific collecting permits. All procedures in the current study were approved by the University of South Dakota Institutional Animal Care and Use Committee (Protocol No. 79-01-11-14C).

RNA-Seq library preparation, sequencing, and read mapping

To investigate seasonal changes in gene expression, we performed high-throughput RNA sequencing of pectoral muscle transciptomes in each of the sampled individuals (RNA-Seq; Wang et al. 2009). We extracted total RNA from approximately 25 mg of pectoral muscle using TRI Reagent (Sigma–Aldrich, St. Louis, MO, USA) following the manufacturer protocol, and used the extracted RNA for preparation of Illumina sequencing libraries using the TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA). The resulting libraries were sequenced as 100 nt single-end reads on the Illumina HiSeq 2500 platform. Up to 10 individuals were multiplexed within a single sequencing lane and all of the pooled individuals were indexed with unique Illumina index adaptor barcodes to allow for downstream identification of individuals. Individuals from each species and season were arbitrarily assigned to one of three sequencing lanes to avoid batch effects resulting from separate sequencing runs. Prior to downstream analysis, we performed a series of filtering steps to remove low quality reads and base calls. First, we removed any reads with an average Phred quality score of <30 from each individual library, and then we trimmed low quality bases from the

resulting filtered read set using the *Trim Sequences* function in CLC Genomics Workbench v6 (Trimming Settings: Trim Using Quality Scores, Limit = 0.05). Finally, we scanned each read for adaptor sequences also using the *Trim Adaptors* function, and if detected, to trim the adaptors from the sequencing reads. This sequencing and filtering strategy produced an average of ~16.5 million reads/individual and 17 million reads/individual for *S. tristis* and *P. atricapillus*, respectively (Supplementary Table S1). Raw sequencing reads have been deposited in NCBI short read archive (accession No. PRJNA414588).

Because genome sequences are not publically available for either of our study species, we mapped cleaned and filtered reads to the zebra finch (*Taeniopygia guttata*) genome (assembly taeGut3.2.4), which represents the highest quality passerine genome that is currently available. Our two study species, American goldfinch and black-capped chickadee, diverged from zebra finch roughly 34 mya (95% CI = 21–47 mya) and 42 mya (95% CI = 36–51 mya), respectively (Hedges et al. 2015). These divergence times fall within the range of divergences of other studies that have successfully used heterospecific references for read mapping. For example, Velotta et al. (2016) quantitatively compared estimates of differential gene expression in deer mice (*Peromyscus maniculatus*) obtained by mapping to a heterospecific (*Mus musculus*) reference to those obtained by mapping to a conspecific reference and found that while ~10% more reads mapped to the conspecific reference, the estimates of differential expression ($\log_2$ fold change) were highly correlated between the two mappings ($R^2 = 0.95$, $P < 0.0001$). *Mus musculus* and *P. maniculatus* diverged approximately 28.3 mya (CI = 27.9–37.4) (Hedges et al. 2015), suggesting that inferences of differential gene expression are largely unaffected choice of reference over this span of evolutionary divergence. Qualitatively similar results have been reported in studies of primates that span a greater range of divergence time (up to 100 mya) (Hornett and Wheat 2012).

Read mapping was performed using the core mapper in CLC Genomics Workbench with the following mapping parameters: minimum length fraction = 0.9, minimum similarity fraction = 0.8, and maximum number of hits per read = 10. We computed transcript abundance using the number of reads that mapped uniquely to each gene using CLC Genomics Workbench. In total, sequence reads were mapped to 16,038 unique genes. However, since genes with low read counts are subject to increased measurement error, we removed those with less than an average of five reads/individual (Robinson and Smyth 2007). This filtering strategy increases the statistical power to detect differential expression of well-sampled (i.e., high coverage) genes across seasons because it reduces the total number of independent tests that are performed. A total of 11,309 genes were retained in both species after filtering for low coverage.

Data analysis

We took two approaches to identify transcriptomic differences between individuals that were sampled across seasons, and to compare the seasonal transcriptomic responses between species. First, we used a generalized linear model (GLM) approach to differentiate sets of genes that responded similarly to changes in season in both species from those that exhibited species-specific responses. Second, we used weighted gene co-expression network analyses (WGCNA—Langfelder and Horvath 2008) to identify groups of genes that exhibited correlated expression patterns and thus represent putative transcriptional modules of co-regulated genes. We then statistically summarized the expression of these modules and tested for differential expression of entire modules across seasons within species. These two approaches provide different, but complementary perspectives on the transcriptomic responses to changing seasons. The gene-centered approach allowed for the identification of specific genes that differed in expression across seasons to test for differential expression of candidate genes and pathways, whereas the module-centered approach allowed for *de novo* construction of correlation networks to examine how the expression of suites of correlated genes changed in concert across seasons.

Testing for differential gene expression

We used a GLM to identify genes exhibiting significant main effects of species, season, and their interaction using the program edgeR. In this framework, genes exhibiting a significant main effect of season without an interaction represent conserved seasonal transcriptomic responses between species, whereas those that exhibit significant interactions represent species-specific responses. Those that exhibit significant main effects of species without an interaction represent constitutive differences in expression between the two species and are not of primary interest to this study. Prior to GLM analysis, we normalized read counts for each individual using the function calcNormFactors, and we estimated model dispersion

for individual genes using the function estimateTagwiseDisp. Following these normalization steps, we fit the GLM to each gene using the function glmFit and tested for significant main effects and their interactions using likelihood ratio tests in edgeR with the function glmLRT. We controlled for multiple tests by enforcing a genome-wide false discovery rate (FDR) of 0.1. Following the GLM analysis, we performed Fisher's exact tests as *post hoc* tests to determine whether genes with significant main effects of season exhibited significant and concordant shifts in gene expression in both species. Exact tests were also performed in edgeR using the exactTest function. Finally, in this gene-centered analysis, we also tested for concerted differential expression of genes in particular pathways that are hypothesized to contribute to seasonal phenotypic flexibility in thermogenic capacity in birds. For these analyses, we focused on genes in several key pathways, which included core metabolic pathways (fatty-acid metabolism, glycerolipid metabolism, glycolysis, pyruvate metabolism, citric acid cycle, oxidative phosphorylation), and genes involved in nonshivering thermogenesis (NST; sarcolipin, *Sln*; ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2, *ATP2A2*; avian uncoupling protein, *avUCP*; and adenine nucleotide translocase, *avANT*). We constructed these lists of candidate genes using *T. guttata* annotations and pathway maps available in the KEGG database (Kanehisa and Goto 2000), which were downloaded using CytoKEGG (v.0.0.5) (Stager et al. 2015). We tested for concerted changes in gene expression (consistent up or down-regulation of genes within a pathway) using binomial tests of $\log_2$ fold-change estimates of genes that were significantly differentially expressed (exact test $q < 0.1$) across seasons in each species. Concerted expression was identified at a binomial test significance level $P \leq 0.05$.

Module definition, tests of differential expression, and assessment of module conservation

In addition to the gene-centered analyses described above, we also took a network-based approach. This analysis allowed us to first determine which groups of genes exhibited highly correlated patterns of expression within species, and then to test for changes in the expression of these correlated groups of genes across seasons. We used weighted gene co-expression network analysis (WGCNA—Langfelder and Horvath 2008) to define these suites of correlated genes. Prior to WGCNA analysis, the raw read counts for each individual were normalized by the total library size for each individual using the functions calcNormFactors and cpm to produce counts per million (cpm) values for downstream analysis. Network construction and module detection were performed using the blockwiseModules function in WGCNA with default parameters, and these structures were defined for each species independently. We used Pearson correlation to define a correlation matrix for all pairwise comparisons of expressed genes, and then we created an adjacency matrix by raising the correlation matrix to a soft thresholding power (β). The β value was chosen to approximate a scale-free network topology, which favors strong correlations over weak correlations in the network definition. We chose a β value of 16 for both species as this value represented the asymptote in the relationship between scale-free topology model fit and the threshold power. Once the transcriptional modules were defined, we summarized the expression of each module using principal components analysis implemented in the blockwiseModules function in WGCNA. Because transcriptional modules are, by definition, groups of genes that exhibit highly correlated gene expression patterns, scores along the first principal component axis (PC1) represent overall expression of the entire module. We identified modules that significantly differed in expression across seasons using *t*-tests of module PC1 scores in R, and we corrected for multiple comparisons using a Bonferroni correction. Finally, we tested whether seasonally responsive modules were significantly conserved across species using the function modulePresevation in WGCNA (Langfelder et al. 2011) with 1000 random permutations and the American goldfinch modules set as the reference network. Modules with $Z_{summary}$ scores > 2 were considered significantly conserved between species (Langfelder et al. 2011).

Functional annotation and enrichment analyses

We used a series of annotation tools to test for functional enrichment among genes that were differentially expressed across seasons within each species. First, we used db2db (Mudunuri et al. 2009) to convert the Gene IDs associated with the zebra finch genome to zebra finch ENSEMBL IDs, and to extract gene ontology (GO) information and gene symbols. Next, we tested for enrichment of GO terms in three separate gene lists that resulted from the gene-centered differential expression analysis (GLM $q \leq 0.1$): (1) genes that exhibited a significant main effect of species without an interaction; (2) genes that exhibited a significant main effect of season

without an interaction; and (3) genes that exhibited significant species× season interactions. Functional enrichment tests were performed using gProfiler (Reimand et al. 2016), and we controlled for multiple tests using the g:SCS algorithm implemented in gProfiler and significance threshold of $\alpha \leq 0.05$.

Results

Mapping success

In total, 50.5% of chickadee reads mapped uniquely to the *T. guttata* genome (Table 1). For goldfinches, mapping success was higher, with 53.5% of reads mapping uniquely (Table 1). Although significantly more reads were mapped for goldfinches (Student's *t*-test; $t = 13.3$, $df = 32$, $P < 0.0001$), this difference in mapping success did not seem to lead to systematic downward bias in expression estimates for chickadees as just over half (54%) of the detected genes had higher normalized read counts in chickadees compared with goldfinches. These results indicate that sequence similarity between each of our study species and *T. guttata* is sufficient for read mapping and expression estimation, consistent with previous studies (Hornett and Wheat 2012; Stager et al. 2015). Visual inspection of MA-plots and the correlation between normalized winter and summer read counts for each species suggested that the normalization procedures did not introduce any systematic basis into our gene expression estimates (Supplementary Figs. S1 and S2).

Differential gene expression across seasons

After normalization and filtering, we detected the expression of 11,309 genes in a total of 34 birds (10 summer and 7 winter for both species). Multidimensional scaling (MDS) analyses of the normalized read count data revealed strong separation of individuals according to both species identity (axis 1 of MDS plot; Fig. 1) and season sampled (axis 2; Fig. 1). The winter and summer samples exhibited much greater seasonal separation for chickadees than goldfinches (Fig. 1), suggesting a stronger transcriptional response to changes in season.

Consistent with the MDS results, our GLM analyses revealed large-scale baseline differences in gene expression between the two species. A total of 7568 genes exhibited significant main effects of species without a season× species interaction (Supplementary Table S1). A total of 1279 genes exhibited significant main effects of season without an interaction (Supplementary Table S2), and 10 genes exhibited significant species×season interactions (Supplementary Table S3). *Post hoc* exact tests revealed that the transcriptomic response to changing seasons was more pronounced in chickadees than in goldfinches, which is also consistent with the MDS results. Of the 1279 seasonally responsive genes, 744 were significantly differentially expressed across seasons in chickadees alone (exact test FDR < 0.1), whereas 151 were significant in goldfinches alone (exact test FDR < 0.1). A total 247 seasonally responsive were differentially expressed across season in both species (exact test FDR < 0.1). As expected from the GLM results, the direction of seasonal gene expression change in the seasonally responsive genes (i.e., up- vs. down-regulated) was significantly correlated in both species ($r^2 = 0.78$, $P = 2.2 \times 10^{-16}$) (Fig. 2), but the seasonal shifts in expression were generally more subtle in goldfinches than in chickadees. This subset of genes exhibiting concordant seasonal changes in expression likely represents a conserved set of genes that underlie flexible responses of muscle phenotypes and physiology to changing seasons (Supplementary Table S2), however, as a whole these genes seem to be more responsive to seasonal changes in chickadees (Fig. 3).

Functional annotation and enrichment analysis of differentially expressed genes

We used a suite of functional annotation and gene enrichment analysis tools to determine the molecular functions of the set of conserved seasonally responsive genes (i.e., the subset of genes with significant main effects of season without an interaction; Supplementary Table S2). Neither the subset of genes with significant main effects of species nor the subset of genes exhibiting significant interactions was significantly enriched for biological processes GO terms. These results likely stem from the sheer size of the two gene lists. The genes with species effects represent over half of the sequenced transcriptome, making it difficult to differentiate this list from a random selection of the transcriptome, and only 10 genes exhibited interactions, limiting power to detect significant functional enrichment. Within the conserved set of seasonally responsive genes, the list of genes that were up-regulated in the winter was significantly enriched for genes that participate in lipid metabolism and other metabolic processes (e.g., GO: 0034440, GO: 191564, GO: 0015980, GO: 009752, GO: 006520) (Supplementary Table S4). The list of genes that were down-regulated in winter was significantly enriched for genes that participate in cellular signaling (e.g., GO: 0007154, GO:0007267), cytokine production (GO:0001816), and blood vessel

Table 1 Mapping success

Species	Total reads	Total mapped reads	Total mapped %	Number of unique mapped reads	Unique mapped %
Poecile atricapillus	17.0 (5.4)	9.3 (3.1)	54.3 (0.8)	8.6 (2.9)	50.5 (0.8)
Spinus tristis	16.5 (2.9)	9.4 (1.6)	57.0 (0.7)	8.8 (1.5)	53.5 (0.5)

Note: Read counts are expressed in millions of reads and values in parentheses are standard deviations.

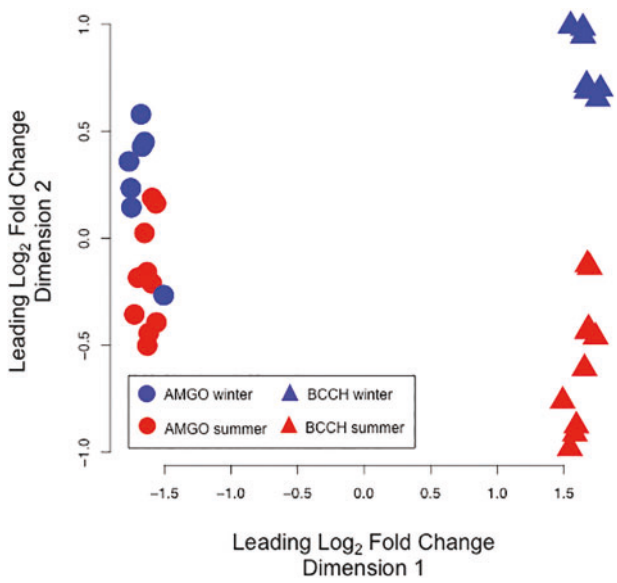

Fig. 1 Transcriptomic signatures of pectoral muscle separated samples by species and season. Multi-dimensional scaling (MDS) plot of normalized (cpm) read counts. *Poecile atricapillus* (BCCH) samples are depicted as triangles, and *Spinus tristis* (AMGO) samples are depicted as circles. Winter and summer samples for both species are blue and red, respectively.

development (e.g., GO:0001568, GO:0001525, GO:1901342, GO:0001569) among others (Supplementary Table S5).

Differential expression of candidate genes and pathways

In addition to the functional enrichment analyses described above, we also tested for concerted changes in gene expression across several candidate metabolic pathways (Table 2). Enrichment of GO terms related to core metabolic pathways (fatty-acid oxidation, glycerolipid metabolism, the citric acid cycle, and oxidative phosphorylation) and upregulation of genes that encode metabolic enzymes suggests upregulation of these pathways in winter (Table 2). For chickadees, several core metabolic pathways (fatty-acid oxidation, glycerolipd metabolism, the citric acid cycle, and oxidative phosphorylation) exhibited concerted patterns of upregulation in winter (Table 2). Only glycerolipid metabolism exhibited significant patterns of upregulation in winter goldfinches, consistent with the overall reduction in differentially expressed genes in this species. We found little evidence for differential expression of genes involved in NST across seasons in both species. Taken together, the concerted upregulation of genes that encode key enzymes within metabolic pathways associated with lipid catabolism and aerobic ATP production suggests a general upregulation of cellular metabolic intensity in winter chickadees, and winter increases in lipid catabolic capacities in both species, but little evidence that either species utilizes winter increases in NST in skeletal muscle.

Network structure and conservation

We used WGCNA to: (1) identify suites of co-expressed genes within each species; (2) determine which suites of co-expressed genes were differentially expressed across seasons within species; and (3) determine the extent to which transcriptional modules are conserved across species. For goldfinches, we identified a total of 17 modules of co-expressed genes that ranged in size from 1587 to 56 genes (mean = 301.3 genes). Of these 17 modules, 5 exhibited differential expression across season ($P \leq 0.05$), 3 of which remained significant after Bonferroni correction ($P \leq 0.0029$) (Supplementary Table S5). These three seasonally responsive modules contained a total of 1721 genes, 92% of which were members of a single large module (AmGo_M1; 1587 genes; Supplementary Table S5). Although fewer modules were identified for chickadees, a greater proportion exhibited differential expression across seasons. We identified 12 modules in chickadees that ranged in size from 5606 to 40 genes (mean = 656.2 genes), but nearly half (5) of these modules exhibited significant differential expression across seasons ($P \leq 0.05$), 3 of which remained significant after Bonferroni correction ($P \leq 0.0042$) (Supplementary Table S6). These seasonally responsive modules contained a total 6655 genes, and as with goldfinches, the majority (84.2%) of these genes were contained in a single very large module (BcCh_M1; 5606 genes; Supplementary Table S6). There was substantial overlap among the genes that comprised the differentially expressed modules in both species. Specifically, 73.1% of the genes in the goldfinch

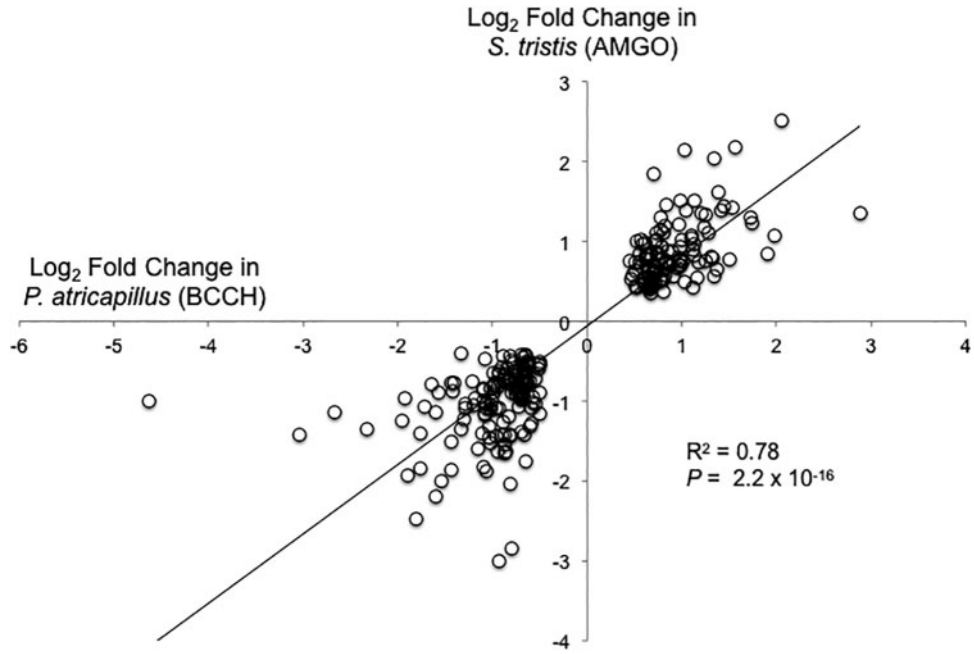

Fig. 2 Expression values are highly correlated among the subset of genes ($n = 247$ genes) that exhibited differential expression across seasons in both species. Positive $\log_2$ fold change values indicate genes that are up-regulated in winter samples, whereas negative values indicate down-regulation in winter.

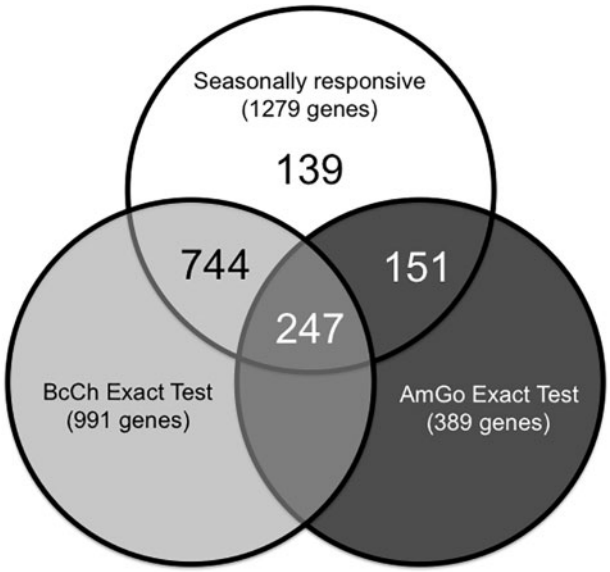

Fig. 3 The transcriptomic response to changes in season was more pronounced for *P. atricapillus* (BCCH) than *S. tristis* (AMGO). Venn diagram summarizing the degree of overlap in the results of the GLM analysis and the *post hoc* exact tests. The white circle represents the 1279 genes that comprise the set of conserved seasonally responsive genes, which were defined as those that exhibited significant main effects of season without an interaction in the GLM analysis. The light and dark gray circles represent the number of the seasonally responsive genes in *post hoc* pairwise exact tests in BCCH and AMGO, respectively.

seasonally responsive modules were also found in the modules that responded to season in chickadees. However, much of this overlap may be due to the sheer size of module BcCh_M1, which contained nearly half of the measured transcripts. Finally, permutation analyses revealed a high degree of preservation in module membership between species. Of the 17 modules identified in goldfinches, 15 (88.2%) were significantly conserved in chickadees ($Z_{summary} > 2$), including all three of the seasonally responsive modules (Table 3). Similarly, 11 of 12 modules (92%) defined in chickadees were significantly conserved in goldfinches ($Z_{summary} > 2$), and this includes all three of the modules that were seasonally responsive in chickadees (Table 3). Taken together, these results suggest considerable similarity in the correlational structure of the pectoralis transcriptome in both species, and that similar suites of co-expressed gene respond to changes in season in both species as well (Supplementary Tables S5 and S6).

Discussion

A major outstanding question in the study of organismal responses to seasonality is the degree to which the mechanisms that underlie physiological responses to changing seasons are conserved across species (Swanson 2010; Williams et al. 2017). Transcriptomic analysis provides a means to address this question from a gene regulatory perspective, allowing both qualitative and quantitative comparison of seasonal transcriptomic changes among species. From this perspective, many unanswered questions remain.

Table 2 Summary of candidate pathway

		Poecile atricapillus				*Spinus tristis*			
Pathway	**Number of genes in pathway**	**Proportion DE (%)**	**Upregulated**	**Downregulated**	***P***	**Proportion DE (%)**	**Upregulated**	**Downregulated**	***P***
Citric acid cycle	22	27.3	**6**	**0**	**0.031**	0.0	–	–	–
Fatty acid metabolism	43	46.5	**18**	**2**	**0.0004***	7.0	2	1	1
Glycerolipid metabolism	38	34.2	**11**	**2**	**0.0023**	21.1	**8**	**0**	**0.0078**
Glycerophospholipid metabolism	91	16.5	11	4	0.1185	0.0	–	–	–
Glycolysis	44	20.5	5	4	1	6.8	3	0	0.25
Oxidative phosphorylation	255	25.1	**59**	**5**	**<0.00001***	3.5	3	6	0.5
Pyruvate metabolism	31	32.3	8	2	0.11	12.9	3	1	0.625

Note: The presented *P*-values are for binomial tests to evaluate the presence of concerted expression across the pathway. Significant values are in bold.

Table 3 Summary of module preservation analysis of seasonally responsive transcriptional modules

Module	Number of genes	$Z_{summary}$
AmGo_M1	1587	26.1
AmGo_M2	77	4.98
AmGo_M3	57	3.82
BcCh_M1	5606	8.52
BcCh_M2	1008	26.23
BcCh_M3	40	4.47

Notes: Modules prefixed with AmGo were defined and seasonally responsive in goldfinches (*S. tristis*), whereas those prefixed BcCh are defined and seasonally responsive in chickadees (*P. atricapillus*). $Z_{summary}$ scores > 2 indicate significant module conservation between species.

For instance, at what level of biological organization does conservation in gene regulatory responses emerge; do species alter the regulation of the same genes, or different genes in the same functional category or biochemical pathway? At what evolutionary distance does each level of conservation (e.g., genes, pathways, functional categories) begin to break down? To begin to answer these questions, we examined transcriptomic differences in the pectoral muscles of two resident North American songbird species sampled during winter and summer. We found that very few genes exhibited divergent responses to changes in season between species, and instead, a core set of over 1200 genes responded to season concordantly in both species. Moreover, the correlational structure of the pectoral muscle transcriptome was largely conserved between species, and there was considerable overlap in the genes that comprised seasonally-responsive transcriptional modules in both species. Together, these results begin to define the core transcriptomic responses that underlie phenotypic flexibility in pectoral muscle form and function in response to changing seasons in passerine birds.

Patterns of regulatory conservation

We took several complementary analytical approaches to test for conservation in the transcriptomic response to changing seasons. In the first series of analyses, we focused on gene-specific responses, performing standard differential expression analyses. These gene-centric analyses revealed a number of salient results. First, and perhaps most striking, was the overall greater intensity of the transcriptomic response in chickadees compared with goldfinches. Chickadees sampled in different seasons exhibited greater separation in MDS analysis compared with goldfinches (Fig. 1), and

correspondingly, differential expression analyses revealed over twice as many differentially expressed genes across seasons in chickadees (Table 4). *Post hoc* analysis of the subset of conserved seasonally responsive genes revealed that only a minority was differentially expressed across seasons in both species. Specifically, of the 1279 genes that exhibited significant main effects of season in the GLM analysis, 989 were differentially expressed across seasons in chickadees, whereas only 398 were differentially expression in goldfinches. In the second series of analyses, we focused on specific candidate pathways, testing for evidence of concerted and parallel up- or down-regulation of genes that encode enzymes in core metabolic pathways. For chickadees, four of these candidate pathways were up-regulated in winter individuals (fatty-acid oxidation, glycerolipd metabolism, the citric acid cycle, and oxidative phosphorylation), while only one (glycerolipd metabolism) showed a parallel response in goldfinches. In the third series of analyses, we used coexpression network analyses (WGCNA) to define the correlational structure of the pectoral muscle transcriptome and to identify suites of co-expressed genes (i.e., transcriptional modules) in each species. We then tested for the seasonal differential expression of the transcriptional modules, and examined similarity in the differentially expressed modules between species. These analyses revealed that the transcriptional modules that responded to season were very similar in their gene compositions between species. Such a pattern of increasing conservation of expression patterns from individual genes to functional pathways is consistent with previous studies on these two species, where both species show similar responses to winter at the organismal level (i.e., increases in Msum and cold tolerance), but different responses for individual components within pathways controlling muscle remodeling, cellular aerobic capacity and lipid transport, and metabolism (Marsh and Dawson 1989; Cooper and Swanson 1994; Liknes et al. 2002; Liknes and Swanson 2011a, 2011b; Swanson et al. 2014a; Zhang et al. 2015b).

Because some (4 of 10) of the summer chickadee samples consisted of fully-grown, hatch-year birds, some of the seasonal differences in gene expression between the two species might be due to inclusion of hatch-year chickadees in the cross-species comparisons. However, hatch-year chickadees in this study did not differ significantly in body mass or in masses of pectoralis, supracoracoideus, and leg muscles or heart (all $P > 0.257$, data not shown), so the body composition of hatch-year and after hatch-year birds was similar. In addition, fully grown hatch-year birds often show similar thermogenic performance to adult birds (e.g., Liknes and Swanson 1996; Swanson and Dean 1999). Thus, it seems unlikely that age differences between species contributed greatly to seasonal differences in gene expression related to metabolism and thermogenic performance.

Table 4 Summary of the *post hoc* analysis of genes that comprise the set of conserved seasonally responsive genes, defined as those that exhibited significant main effects of season without an interaction in the GLM analysis

Species	Total	Up-regulated in winter	Down-regulated in winter
Poecile atricapillus	991	607 (61.3%)	384 (38.7%)
Spinus tristis	398	202 (50.8%)	196 (49.2%)

Note: Presented are the total numbers of genes that were significantly differentially expressed in *post hoc* Fisher's exact tests.

Species differences in diets or seasonal variation in diets might also impact the gene expression patterns between species and across seasons. Diet varies among seasons for both species, with animal matter in the diet generally higher in summer than in winter for both species. However, in general, chickadees are much more insectivorous than goldfinches, with approximately 80–90% of the summer diet and 50% of the winter diet composed of animal matter (Foote et al. 2010). Goldfinches are largely granivorous year-round, taking insects in summer only occasionally (McGraw and Middleton 2009). These diet differences might partially explain differential patterns of gene expression between the species, especially for gene pathways related to nutrient processing.

Regulatory mechanisms of seasonal phenotypic flexibility

Like many winter active, homeothermic endotherms, both chickadees and goldfinches are known to increase their thermogenic capacities during the winter (Cooper and Swanson 1994; Liknes et al. 2002; Swanson and Liknes 2006). As the pectoralis is the primary thermogenic organ in birds, these seasonal regulatory changes likely contribute to changes in pectoral muscle form and function that support these winter increases in Msum (Swanson et al. 2009, 2014a; Stager et al. 2015; Zhang et al. 2015a). Previous work has identified a number of physiological changes in pectoral muscle that accompany winter increases in Msum in small passerines, including increases in pectoral mass (Liknes and Swanson 2011a; Petit et al. 2017; Swanson et al. 2014a; Barceló et al. 2017), tissue vascularization and oxygen delivery (O'Connor 1996; Petit and Vezina 2014; Stager et al. 2015), and cellular lipid

catabolic and aerobic capacities (e.g., Marsh and Dawson 1982; Yacoe and Dawson 1983; Liknes and Swanson 2011b; Peña-Villalobos et al. 2014; Zheng et al. 2014a, 2014b). Genes that participate in many of these pathways were shown to be differentially regulated in response to temperature and photoperiod cues in captive dark-eyed juncos (Stager et al. 2015), and here we found signatures of differential regulation for many of the same genes and pathways in wild, free-ranging birds (Table 2). Our study corroborates these earlier studies, underscoring the importance of these key physiological processes in muscle remodeling to support phenotypic flexibility in avian thermogenic capacity, but it also extends these studies in several ways. First, the comprehensive extent of our transcriptomic datasets allowed for the examination of differential expression among genes across entire pathways, a level of analysis that was not possible in previous studies of wild birds that focused on candidate genes. Second, our WGCNA analyses allowed us to identify co-expression modules that contained previously identified candidate loci. These co-expression analyses revealed networks of genes with correlated expression patterns within which candidate genes were embedded. Together these analyses provide novel insights in the regulatory basis of pectoral muscle remodeling in response to changing seasons in passerines.

Changes in pectoral muscle mass are among the most prominent and consistent aspects of seasonal phenotypic flexibility in passerine birds (Swanson 2010). Previous studies have revealed that winter increases in pectoralis mass tend to be associated with reduced expression of myostatin and its activators *TLL-1* and *TLL-2* (Swanson et al. 2009, 2014a; Zhang et al. 2015a). Although myostatin was not among the differentially expressed genes recovered in the gene-level analysis in either species, myostatin was a member of module BcCh_M1, a very large module that was differentially expressed across seasons in chickadees. Similarly, genes that participate in BMP-signaling (*TOB1* and *BMPR2*) were among the conserved seasonally responsive gene set (Supplementary Table S2). The BMP-signaling cascade is known to be a key regulator of muscle growth in adult mice (Sartori et al. 2013, 2014), but to our knowledge, has not been investigated as regulator of muscle hypertrophy in seasonally acclimatized birds. Together these results help to flesh out the suite of genes that regulate seasonal muscle growth in passerine birds.

Similarly, increases in the capacities to catabolize lipid fuels and increases in cellular aerobic capacities are also common features of the winter phenotype in small birds (Marsh and Dawson 1989; Swanson 2010). Indeed, both chickadees and goldfinches tend to exhibit increases in the activities of key metabolic enzymes [e.g., β-hydroxyacyl Co-A dehydrogenase (HOAD), PFK] in the pectoralis during winter (Marsh and Dawson 1982; Yacoe and Dawson 1983; Liknes and Swanson 2011b; Swanson et al. 2014a; Zhang et al. 2015b), although these patterns are not entirely consistent across studies. Here, we found evidence of gene regulatory changes that may contribute to metabolic shifts that can support increased cellular aerobic capacity in winter-acclimatized individuals. The conserved seasonally responsive gene set was enriched for genes whose products encode enzymes that participate in carbohydrate metabolism and glycolysis (Supplementary Table S4), and these genes tended to be upregulated in winter. Moreover, focused analysis of core metabolic pathways revealed concerted upregulation of genes that encode enzymes in several core metabolic pathways, including fatty-acid oxidation, glycerolipid metabolism, the citric acid cycle, and oxidative phosphorylation in winter chickadees, and concerted upregulation of the glycerolipid metabolism pathway in winter goldfinches (Table 2). Together, these results suggest that seasonal changes in lipid catabolic and aerobic capacities documented in previous studies are underlain at least in part by changes in gene regulation, and provide more fine scale resolution on specific portions of core metabolic pathways that are differentially regulated in each species.

Fat is the dominant substrate during shivering in birds (Vaillancourt et al. 2005), so elevated use of lipids might be expected to decrease rates of carbohydrate use during high rates of shivering (Marsh and Dawson 1989). Indeed, enhanced sparing of pectoralis muscle glycogen during cold exposure is a common element of winter acclimatization in small birds (Marsh and Dawson 1982; Yacoe and Dawson 1983; Marsh et al. 1990), suggesting an increased reliance on lipids relative to carbohydrates to fuel high rates of winter thermogenesis. Consistent with this idea, pectoralis β-oxidation capacity, as measured by HOAD activity, is often upregulated in winter relative to summer for birds wintering in cold climates (Marsh and Dawson 1982; Yacoe and Dawson 1983; Carey et al. 1989; Liknes and Swanson 2011b). However, such an increment in HOAD activity is not always observed for all species under all cold-exposure conditions (O'Connor 1995; Liknes and Swanson 2011b; Zhang et al. 2015b). The winter increase in reliance on lipids to fuel shivering also is not necessarily accomplished by a seasonal

reduction in glycolytic capacity, as the activities of glycolytic enzymes may also be elevated in winter relative to summer, although usually not to the same degree as HOAD activity (Marsh and Dawson 1982; Yacoe and Dawson 1983; Marsh et al. 1990; O'Connor 1995). Moreover, plasma glucose turnover during cold exposure sometimes is reduced under cold stress in winter relative to summer in small birds (Marsh and Dawson 1982), but this is often not the case (Yacoe and Dawson 1983; Marsh et al. 1984). In contrast, Stager et al. (2015) documented downregulation of glycolytic gene expression along with upregulation of lipid catabolism gene expression in cold-acclimated juncos, which is generally consistent with the data on goldfinches and chickadees in the present study. The differing conclusions drawn from the single enzyme and transcriptomic studies are also consistent with the idea that pathway-level changes identified by transcriptomic studies may not always be evident in studies that focus on single key regulatory enzymes or single candidate genes.

Birds lack brown adipose tissue (Mezentseva et al. 2008), but evidence for the occurrence of muscular NST in birds, particularly in precocial chicks, continues to accumulate (e.g., Teulier et al. 2010, 2014). The mechanistic basis for avian muscular NST is not known with certainty, but could conceivably involve uncoupling of oxidative phosphorylation from ATP production, with avUCP or adenine nucleotide translocator (avANT) as potential candidates promoting uncoupling (Toyomizu et al. 2002), or futile cycling of intramyocyte calcium pumps (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, ATP2A2) regulated by sarcolipin (Bal et al. 2012; Newman et al. 2013). Skeletal muscle avUCP and avANT are often upregulated by cold-acclimation in young precocial birds (Raimbault et al. 2001; Toyomizu et al. 2002; Rey et al. 2010; Teulier et al. 2010; Gasparino et al. 2015), although whether this is associated with NST or with oxidative balance in uncertain (Emre et al. 2007). However, we found little evidence for seasonal changes in expression for any genes putatively associated with NST for either goldfinches or chickadees. Thus, the data in the present study do not support seasonal modification of muscular NST as a prominent contributor to increased thermogenic capacity in the winter phenotype for adult passerine birds.

Conclusions

Phenotypic flexibility allows organisms to reversibly alter their phenotypes to match the demands of temporally fluctuating selective pressures in seasonal environments. While the adaptive value of properly matching phenotypes to prevailing environmental conditions is clear, the physiological and regulatory mechanisms that underlie seasonal changes in phenotype are not. Because phenotypic flexibility is mediated, at least in part, by changes in gene regulation, comparative transcriptomic analyses can provide insights into the mechanistic underpinnings of seasonal phenotypic flexibility, and the extent to which regulatory responses to changing seasons are conserved across species. In this study, we identified a core set of over 1200 genes that responded to season concordantly in both species. These conserved expression patterns suggest that common metabolic pathways, regulatory networks, and gene functional classes are recruited to induce seasonal changes in muscle phenotype, fuel provision, and metabolic intensity in both species. Importantly, many of the regulatory changes we observed mirror physiological and morphological changes that have been documented in previous studies, suggesting that seasonal remodeling of pectoral muscle phenotype is due in part to changes in gene expression. Future studies that expand the phylogenetic, temporal, and systems breadth of comparison will be able to better define conserved regulatory and physiological responses to changing seasons, and providing needed insight into the mechanistic basis of seasonal phenotypic flexibility (Williams et al. 2017). In addition, gene and protein expression studies of candidate genes relevant to cold acclimation or seasonal acclimatization in birds often provide different results (e.g., Swanson et al. 2014a; Zhang et al. 2015b), so future studies examining post-transcriptional regulation of key pathways and modules will also help better define mechanistic bases for seasonal phenotypic flexibility.

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Supplementary data

Supplementary data are available at *ICB* online.

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