5 Developmental signature of the ageing-related transcriptional profile in a seasonal butterfly

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Abstract

Ageing is a by-product of natural selection shaping the life history of organisms to ensure maximal reproductive output in balance with optimal lifespan. To understand the public, evolutionarily shared mechanisms of ageing, it is crucial to understand the genetic regulation of lifespan in relation to adaptation in the relevant evolutionary environment. We use the butterfly *Bicyclus anynana* to study transcriptional patterns associated with seasonal developmental plasticity of adult life history. In response to seasonal temperatures during development, larvae develop into either fast reproducing but relatively short-lived wet season adults, or long-lived dry season adults that delay reproduction. The plasticity in life histories is assumed to be regulated by alternative genetic programs, activated by environmental cues. Using custom-designed microarrays, we probed the transcriptional profile of young and old butterflies developed in dry or wet season conditions, and observed substantial ageing-related expression changes. Approximately half of all gene expression changes were sex-specific, with females up-regulating stress response ►►

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►► genes and down-regulating reproduction-related genes with age. In dry season adults, age-related expression changes were abrogated compared to the wet season morph. In particular, they lacked the age-related up-regulation of immune genes and the down-regulation of reproduction genes that we observed in wet season butterflies, likely contributing to their long-lived phenotype. Only a small number of genes showed seasonal expression bias independent of age, with several of these seasonally imprinted genes being related to Insulin signalling. The redeployment of this highly conserved nutrient-sensing pathway in the specific ecological circumstances of *B. anynana* illustrates the versatility of hormonal systems that can play additional roles in different life stages or environments.

Introduction

Mutational studies in model organisms have greatly enhanced our understanding of the molecular genetic mechanisms regulating in lifespan. Of particular importance has been the discovery that mutations in genes of the Insulin signalling pathway, a conserved nutrient sensing pathway, increase lifespan in worms (Kenyon *et al.* 1993), fruit flies (Tatar *et al.* 2001), and mice (Holzenberger *et al.* 2003; Selman *et al.* 2008). Identifying the molecular mechanisms by which reduced Insulin signalling affects lifespan in such a variety of animals is one of the major aims in the contemporary field of ageing (Fontana *et al.* 2010; Partridge & Gems 2006). At the same time, nutritional manipulation studies in laboratory animals have revealed extensive plasticity in lifespan. Dietary restriction (DR) has been shown to substantially increase adult lifespan across a wide range of animals, usually accompanied by a decrease in reproductive output (Fontana *et al.* 2010; Mair & Dillin 2008). This has been interpreted in the context of life history theory as a reallocation of limiting adult resources towards organismal processes enhancing survival under adverse conditions (e.g. Tatar *et al.* 2003). Although Insulin signalling does play a role in DR-mediated lifespan extension, the regulatory links are not as straightforward as initially hypothesised, and several other pathways are involved as well (Fontana *et al.* 2010; Kenyon 2005). The accumulating experimental evidence for the DR response has contributed to the more general notion that lifespan and life histories are highly malleable (Fielenbach & Antebi 2008). The underlying assumption is that the plasticity in life histories is regulated by alternative genetic programs, activated by environmental cues. The search for these genetic programs has become another major aim in ageing studies (Fielenbach & Antebi 2008; Tatar *et al.* 2003).

Most studies aimed at elucidating molecular pathways involved in plastic life history responses to the environment (e.g. DR) have done so using the traditional laboratory model organisms *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus* (Fielenbach & Antebi 2008; Swindell 2012; Tatar 2011). Transcriptomic approaches, probing transcriptional responses at the whole-genome level, have proven particularly powerful in shedding light on genetic programs responsible for environment-induced life history variation (Pletcher *et al.* 2005). In *D. melanogaster*, artificial selection and experimental evolution approaches have been used to identify novel genes underlying natural variation in ageing (e.g. (Doroszuk *et al.* 2012; Remolina *et al.* 2012). However, the difficulty with these model organisms is that relatively little is known about their natural life history in the wild. The link to the natural ecology is essential, because it allows testing whether any plastic responses are adaptive, and thus whether they evolved as a result of natural selection (van den Heuvel *et al.* 2013). Using organisms with a well-studied ecology makes it possible to establish the evolutionary relevance of these environmental responses. Meanwhile, many instances of adaptive developmental plasticity in life history have been described in the ecological and evolutionary literature (see Beldade *et al.* 2011; Simpson *et al.* 2011). Even so, the genetic programs regulating environment-specific expression of life history phenotypes have traditionally rarely been studied in these organisms. Interestingly, on-going advances in sequencing technology have opened up possibilities for a detailed

molecular characterisation of these ecological models of developmental plasticity (Aubin-Horth & Renn 2009), but so far few studies have explicitly focused on ageing plasticity. Notable exceptions include transcriptional changes underlying differences in ageing in the honey bee *Apis mellifera* (Bull *et al.* 2012; Corona *et al.* 2005) and in the parasitic nematode *Strongyloides ratti* (Thompson *et al.* 2009).

The butterfly *Bicyclus anynana* has emerged as a laboratory model for developmental plasticity of wing pattern (e.g. Brakefield *et al.* 1998), life history (e.g. Pijpe *et al.* 2007) and behaviour (e.g. Prudic *et al.* 2011), fuelled by extensive knowledge of the natural ecological situation where these plastic responses presumably evolved (Brakefield & Zwaan 2011). Young adults developed in wet season conditions show increased mass allocation to the abdomen as well as higher rates of early egg laying, whereas dry season adults start their life with a higher body fat percentage (Brakefield & Zwaan 2011). It has been shown that both developmental plasticity and adult acclimation contribute to the seasonal adaptation (Brakefield *et al.* 2007; De Jong *et al.* 2013). For example, females developed in dry season conditions but switched to wet season conditions as young adults are able to increase, after an acclimation period, their initially low egg laying rates (Fischer *et al.* 2003). Manipulating the developmental environment separately from the adult environment provides the opportunity to uncover molecular mechanisms underpinning developmental imprint on adult life history without the confounding effects of the prevailing adult environment (Brakefield *et al.* 2007; Zwaan 2003).

There is some knowledge on the hormonal mechanisms linking environmental variation with the induction of alternative developmental pathways (e.g. Chapters 2 and 3 of this thesis). However, the more downstream molecular machinery bringing about the seasonal phenotypes is still largely a black box. Here, we combine the extensive ecological knowledge of this species with the power of high-throughput gene expression profiling. Genomic studies in this species have until now largely focused on wing pattern development (e.g. Beldade *et al.* 2006; Beldade *et al.* 2009; Conceição *et al.* 2011, but see De Jong *et al.* 2013; Pijpe *et al.* 2011) and no study thus far has examined expression across the whole genome. We use custom designed microarrays (P. Beldade, unpubl. data) to analyse whole-genome expression profiles in adult males and females of different ages, developed under alternative seasonal conditions. We focus on gene expression in the abdomen, the body part containing the reproductive organs and the fat body, an important signalling tissue (Klowden 2007). In addition, relative abdomen size has been used previously as a proxy for early life reproductive investment, and has been causally linked to season-specific Ecdysteroid signalling during the pupal stage (see Chapters 2 and 3).

The first goal of this study was to characterise the transcriptional signature of ageing for a species not traditionally used as a model in ageing studies but for which there is extensive evolutionary and ecological knowledge. Furthermore, we explicitly addressed sex-specificity in the transcriptional response to ageing. Although sex-specific trade-offs, selective pressures and strategies are pervasive in life history evolution, ageing studies often focus on a single sex females only, while studies that include both sexes show differential effects in males and females (e.g. Maklakov *et al.* 2008). The second goal was to characterise

the transcriptional profile underlying developmental plasticity of the adult life history. In recent years, several prime examples of adaptive phenotypic plasticity have been subjected to whole-genome expression analysis, e.g. beetle horn dimorphism (Snell-Rood *et al.* 2011), reproductive division of labour in ants (e.g. Ometto *et al.* 2011) and honey bees (e.g. Grozinger *et al.* 2007), alternative mating strategies in salmon (Aubin-Horth *et al.* 2005), wing polyphenism in aphids (Brisson *et al.* 2007), and phase polyphenism in locusts (Badisco *et al.* 2011). Our focus here was on the extent to which juvenile seasonal conditions leave a transcriptional signature throughout adult life, even when those conditions are no longer experienced. Genes showing such a lifelong imprint of juvenile conditions could be effector genes underlying the adult phenotypic differences, directly regulated by the pre-adult hormonal cascades known to be involved in developmental plasticity (see Chapters 2 and 3 in this thesis). Alternatively, such genes could have a more regulatory nature, acting as a developmental 'gatekeeper' between the hormonal cascades and the downstream effector genes (Brakefield *et al.* 2005). Finally, we compared the transcriptional response to ageing among cohorts reared under the alternative conditions. This allowed us to assess which transcriptional changes contribute to the alternative seasonal life histories, including lifespan.

Materials and methods

Experimental design, animal rearing and sampling

We employed a full factorial design to examine the effects of age, sex, and seasonal developmental condition on the abdominal expression profile of adult *Bicyclus anynana* butterflies. We reared parallel cohorts of larvae at two different temperatures representing the alternative seasonal environments, transferred the freshly eclosed adults to a common environment, and sampled their abdomens at three different ages to probe whole-genome expression profiles using microarrays. We used the *B. anynana* outbred laboratory stock population reared under standard conditions (Brakefield *et al.* 2009) to obtain a genetically diverse pool of wild type eggs. Larvae hatched at 23°C and were randomly divided over two environmental climate chambers (Sanyo Versatile Environmental Test Chamber model MLR-351H): one at the dry season temperature of 20°C and one at the wet season temperature of 25°C (N = 200 per temperature per sex). All larvae were fed with young maize plants and kept at 70% relative humidity and a 12h:12h L:D photoperiod. After eclosion, adults were kept in single-sex cages at their developmental temperature for approximately 24 hours. Subsequently, adults from both temperature conditions were transferred to a single large climate room kept at the wet season temperature of 25°C. Here, females and males from the same developmental temperature were brought together into mating cages where they were kept for 72 hours. Other experiments at 27°C (M. Saastamoinen pers. comm.) and 23°C (V. Oostra unpubl. data) indicated that 95% of females will have mated after 72 h with males. After mating, females and males were separated into single-sex cages, with a maximum of 11 adults per cage. Of the total of 800 one day old larvae that started the experiment (N = 200), 514 eclosed successfully as adults and entered the next phase of

the experiment (N = 121 to 132 per sex per developmental temperature). To account for potential micro-environmental influences on life history and gene expression, we rotated cages throughout the climate chamber on a biweekly basis. Throughout their life, adult females had access to fresh maize leaves for oviposition and all adults were fed moist banana *ad libitum*.

We sampled single adults of three different ages for gene expression profiling, using demographic age rather than chronological age in order to be able to compare adults of different sex or developmental history within the same age class (*cf.* Doroszuk *et al.* 2012). We chose three time points representing young, old and very old butterflies and sampled them when the cohort survival was at 90, 50 or 20%, respectively (see Fig 1a and 1b for actual survival curves and sampling points). We monitored mortality separately for each cohort having the same combination of sex and developmental temperature condition. The day at which mortality of a particular cohort (of the same sex and developmental history) reached either 90, 50 or 20%, we sampled a total of ten randomly selected adult butterflies

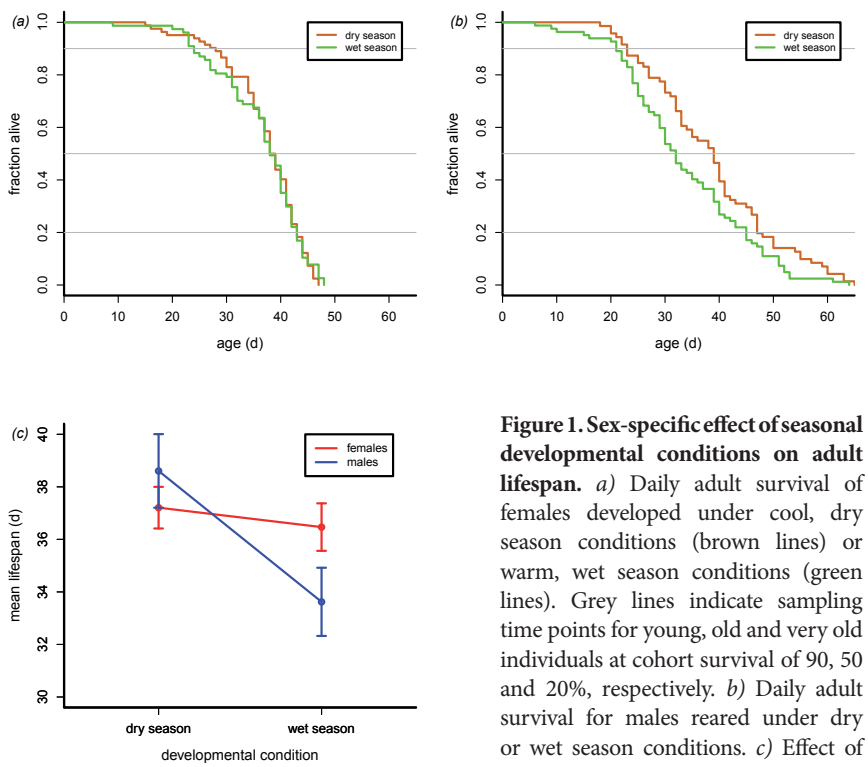

Figure 1. Sex-specific effect of seasonal developmental conditions on adult lifespan. *a)* Daily adult survival of females developed under cool, dry season conditions (brown lines) or warm, wet season conditions (green lines). Grey lines indicate sampling time points for young, old and very old individuals at cohort survival of 90, 50 and 20%, respectively. *b)* Daily adult survival for males reared under dry or wet season conditions. *c)* Effect of seasonal developmental condition on mean adult lifespan for females (red) and males (blue).

from that cohort for RNA isolation, ensuring that we sampled individuals from all different cages. Butterfly sampling and processing followed Pijpe *et al.* (2011). Briefly, we flash-froze the live butterfly in liquid N_2, cut off the abdomen with micro-scissors into a micro-tube kept in liquid N_2, and stored the sample at -80°C until further processing. Samples were taken at the same time of day (+/- one hour), at the end of the dark period of the diurnal cycle. For each cohort, we measured lifespan by monitoring daily survival of all butterflies that were not sampled for expression analysis, providing *ad libitum* food and water until all butterflies had died.

RNA isolation and cDNA synthesis and amplification

We used the Nucleospin 96 RNA kit (Machery-Nagel, Germany) to extract total RNA from 120 whole butterfly abdomens (2 developmental temperatures x 3 time points x 2 sexes x 10 biological replicates). We homogenised the abdomens in 350 µl RA1 lysis buffer (with 1% v:v β-mercaptoethanol), using glass beads in a 96 wells plate TissueLyser II (Qiagen) at 25 Hz for 2 x 2.5 minutes. We included a filtering step (Machery-Nagel) prior to binding of homogenate to the silica membrane, and we incubated the RNA on column with DNase for 15 min. We eluted each RNA sample with 100 µl H_20 and measured concentration and purity with an ND1000 spectrophotometer (NanoDrop), and assessed quality by visually inspecting fragment size distribution of each sample run on a 1.1% agarose gel. Yields ranged from 3.8-24.5 µg per abdomen, and we excluded six RNA samples of low purity ($OD_{260:280}$ < 1.9 or $OD_{260:230}$ < 1.3) or showing indications of degradation, and stored the remaining samples at -80°C.

We synthesised and amplified single-stranded cDNA from 96 RNA samples (N = 8 per experimental group) randomly selected from the 114 high quality samples, using the Applause 3'-Amp System (NuGEN) following manufacturer's recommendations. After amplification, we purified the cDNA using the MinElute Reaction Cleanup Kit (Qiagen) with a final elution volume of 15 µl. All cDNA samples were of sufficient concentration and purity (corrected $OD_{260:280}$ > 1.8), with yields ranging 2.4-6.5 µg, as measured spectrophotometrically. We stored the cDNA at -20°C prior to shipment on dry ice. All cDNA samples passed additional quality control performed at Roche Nimblegen using gas chromatography (Agilent Bioanalyzer).

Microarrays: cDNA labelling, hybridization and slide scanning

To measure gene expression profiles, we used Custom Gene Expression 4x72K Arrays (Roche Nimblegen), designed previously from *ca.* 100,000 expressed sequence tags (ESTs) assembled into 17,154 contigs and singletons (Beldade *et al.* 2009), hereafter called transcripts or genes. These single-colour oligonucleotide microarrays have 1-6 60mer probes per transcript, totalling 72,000 probes, and are printed in groups of four on each slide. Labelling, microarray hybridization, scanning and image extraction of the cDNA samples was performed in-house at Roche Nimblegen (Reykjavik, Iceland). All 96 cDNA samples (N = 8 per experimental group) were processed in this way.

Microarray data analysis

Quality control, data normalization and exploratory analysis

We performed quality control of the raw data by visually inspecting scanned array images as well as boxplots, density plots and MA plots of raw probe-level intensity values. The latter were computed by comparing each array against an artificial array constructed by taking the per-probe average across all 96 arrays. Based on this quality control, two samples were excluded from subsequent analysis. Probe-level data of the remaining arrays were summarised across probes targeting the same transcript using the median polish summarization algorithm (Irizarry *et al.* 2003). We employed quantile and scale normalization on the gene-level intensity data, and compared these and the non-normalised data using again MA plots, density plots and boxplots. We decided to continue further analysis with the quantile normalised data, as the distributions across arrays were most similar for these data. This data exploration was performed in the R/Bioconductor environment (R Development Core Team 2010) using the package *limma* (Smyth 2005). To reduce dimensionality of our data and gain some preliminary insight into the variance structure, we performed a principal components analysis (PCA) on the normalised expression data, and plotted various PCs against one another and against the fixed predictor variables (sex, developmental temperature and age). To simplify subsequent analyses, we chose to focus, for this investigation, on the young and old adults only, and excluded the very old adults (sampled at 20% cohort survival) from differential expression analyses. This reduced the total data set to 62 arrays (2 developmental temperatures x 2 time points x 2 sexes x 8 biological replicates, minus 2 samples of lesser quality).

Differential expression analysis

In order to statistically test the effects of age and developmental temperature on the female and male gene expression profiles, and to identify genes differentially expressed in a particular comparison, we performed Analyses of Variance (ANOVAs) on normalised expression data using the package MAANOVA (Parmigiani *et al.* 2003) in R/Bioconductor. We first fitted linear models with treatment group (combination of sex, developmental temperature, and age) as only fixed effect and no random effects. We then constructed a set of ten contrasts to test specific hypotheses regarding the effect of particular conditions on expression (Table 1), excluding the very old adults (samples at 20% cohort survival). Pair-wise *t*-tests were performed with an Empirical Bayes test with 2000 permutations. We corrected for multiple testing by setting the False Discovery Rate at 5% using the jsFDR / qvalue method (Storey 2002). We thus obtained, for each contrast of interest, a set of up- and down-regulated genes that could then be compared between contrasts. For the purpose of plotting mean expression of various gene sets as a function of age (Figures 2 to 5), we standardised expression values of each gene by applying a standard normal transformation. This yielded expression values comparable across genes, that were then averaged within gene sets of interest

Table 1. Contrasts and data subsets used in statistical analyses to test specific hypotheses regarding the effects of age, seasonal developmental condition, and sex on gene expression.

contrast	data subset
young *vs*. old [1]	all data
young *vs*. old	females
young *vs*. old	males
DSF *vs*. WSF [2]	all data
DSF *vs*. WSF	females
DSF *vs*. WSF	males
young *vs*. old	females DSF
young *vs*. old	females WSF
young *vs*. old	males DSF
young *vs*. old	males WSF

[1] Young adults were sampled when the fraction of individuals still alive in that cohort was 90%, old adults were sampled at 50% survival.
[2] DSF: dry season form, adults reared in cool, dry season conditions; WSF: wet season form, adults reared in warm, wet season conditions.

Re-annotation of B. anynana EST assembly and Gene Enrichment Analyses

For a meaningful biological interpretation of groups of genes significantly up or down-regulated in a particular context, we analysed the larger gene sets using the Gene Ontology (GO) framework (Ashburner *et al.* 2000). To do this, we first functionally annotated the 17,154 transcripts represented on the microarray that were assembled from *ca.* 100,000 sequenced ESTs (Beldade *et al.* 2009). We used the analysis tool Blast2Go (Götz *et al.* 2008) to perform parallel BLASTX searches of each transcript's DNA sequence against NCBI's non-redundant ("nr") protein database containing annotated proteins across all organisms (http://blast.ncbi.nlm.nih.gov/), using standard parameters. This resulted in 7,545 sequences (44% of total) with a significant BLASTX hit (Expect value < 10^{-5}) to a listed protein. The best hits were to proteins from the butterflies *Danaus plexippus* and *Heliconius melpomene*, the moth *Bombyx mori*, and the beetle *Tribolium castaneum*. These hits were then used to map biological processes, cellular components or molecular functions in the GO hierarchy to each *B. anynana* gene. Finally, to further augment the annotation, we used InterProScan (within the Blast2Go software environment) on each gene sequence to obtain additional GO terms based on protein domain and motif information (see Zdobnov & Apweiler 2001), which were then merged to the GO terms already retrieved. Not all proteins in the list are associated with one or more GO terms, but for 4,576 genes (27% of total) we were ultimately able to provide some level of annotation. The majority (*ca.* 59%) of these genes could be associated with only three or less GO terms (of any level), with the remainder associated with four or more terms. This yielded a total of 18,521 annotations, an average of 4 annotations per transcript. To identify GO terms overrepresented within a particular gene

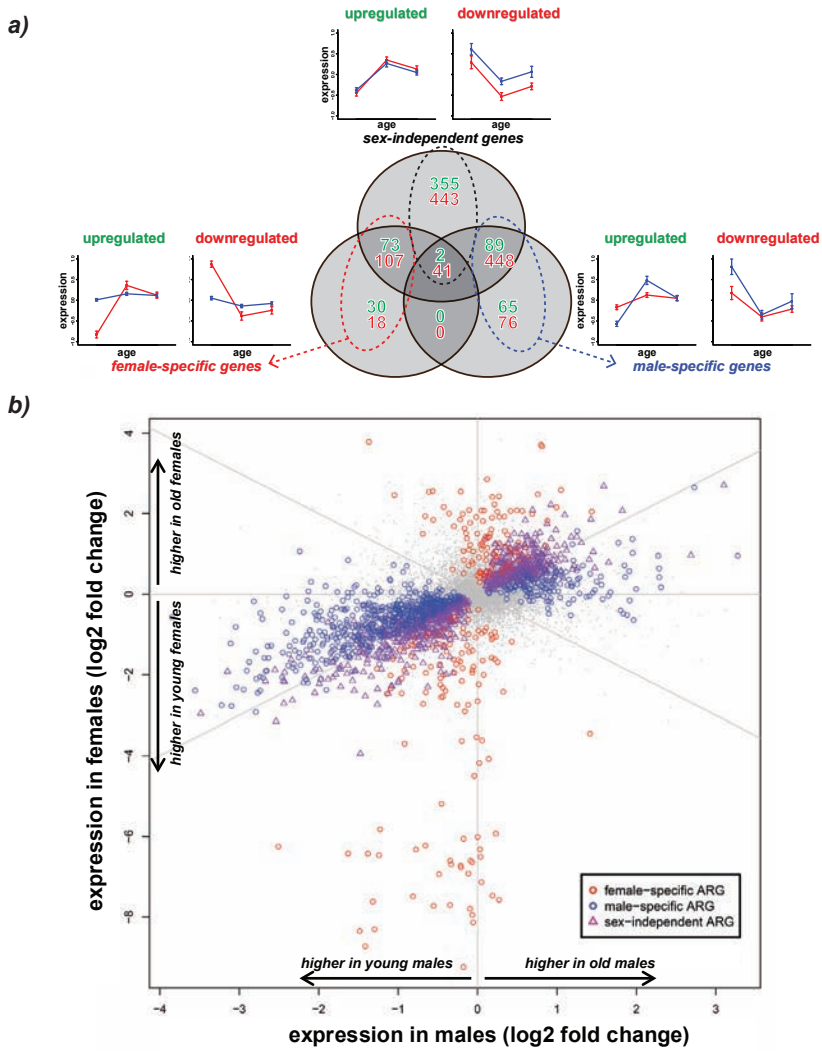

Figure 2. Sex-specificity in transcriptional response to ageing. *a)* Venn diagram showing the groups of genes significantly up- and down-regulated with age in a pooled-sex (top), a female-only (left) and a male-only (right) analysis, as well as overlap between these groups. The six smaller plots show mean standardised expression (+/- S.E.) as a function of age (young, old and very old) for the sex-independent, female-specific and male-specific ageing-related genes, plotted for females (red) and males (blue) separately (see Methods on how expression was standardised). *b)* Fold change for all genes in expression of young versus old females plotted as a function of fold change of young versus old males, with positive values indicating genes up-regulated with age and negative values indicating genes down-regulated with age. Each data point represents one gene on the array. Sex-independent, female-specific, and male-specific ageing-related genes (ARG) are indicated by purple triangles, red circles and blue circles, respectively, whereas genes not significantly differentially expressed with age are indicated with grey dots. *c)* Gene Ontology (GO) terms significantly overrepresented (Fisher exact test $p < 0.05$) among the sex-independent, female-specific and male-specific ARG, separately for up and down-regulated genes. * C: Cellular Component; F: Molecular Function; P: Biological Process ►►

induction	sex-specificity	GO term	GO category*	GO ID	Fisher exact test p	# genes in test set	# genes in reference set
up	sex-independent	calcium ion binding	F	GO:0005509	0.004	5	73
up	sex-independent	lipid particle	C	GO:0005811	0.048	4	95
down	sex-independent	transcription factor activity	F	GO:0003700	0.000	7	54
down	sex-independent	DNA metabolic process	P	GO:0006259	0.002	12	181
down	sex-independent	nuclease activity	F	GO:0004518	0.010	6	74
down	sex-independent	signal transducer activity	F	GO:0004871	0.033	6	98
down	sex-independent	response to endogenous stimulus	P	GO:0009719	0.034	3	28
down	sex-independent	DNA binding	F	GO:0003677	0.043	10	223
down	sex-independent	cell recognition	P	GO:0008037	0.046	2	13
up	female-specific	response to stress	P	GO:0006950	0.001	7	264
up	female-specific	calcium ion binding	F	GO:0005509	0.011	3	75
down	female-specific	external encapsulating structure	C	GO:0030312	0.000	13	4
down	female-specific	reproduction	P	GO:0000003	0.000	13	163
down	female-specific	cell differentiation	P	GO:0030154	0.000	14	227
down	female-specific	anatomical structure morphogenesis	P	GO:0009653	0.000	13	235
down	female-specific	structural molecule activity	F	GO:0005198	0.000	16	416
down	female-specific	multicellular organismal development	P	GO:0007275	0.000	15	422
down	female-specific	calcium ion binding	F	GO:0005509	0.028	3	75
up	male-specific	cell-cell signaling	P	GO:0007267	0.018	2	49
up	male-specific	protein complex	C	GO:0043234	0.025	6	573
up	male-specific	transport	P	GO:0006810	0.025	6	577
up	male-specific	nucleolus	C	GO:0005730	0.026	2	59
up	male-specific	organelle organization	P	GO:0006996	0.041	4	322
down	male-specific	extracellular region	C	GO:0005576	0.003	10	131
down	male-specific	DNA metabolic process	P	GO:0006259	0.009	11	182
down	male-specific	carbohydrate binding	F	GO:0030246	0.012	6	70
down	male-specific	nutrient reservoir activity	F	GO:0045735	0.012	2	5
down	male-specific	carbohydrate metabolic process	P	GO:0005975	0.022	10	182
down	male-specific	receptor activity	F	GO:0004872	0.025	6	84
down	male-specific	antioxidant activity	F	GO:0016209	0.045	3	29
down	male-specific	peptidase activity	F	GO:0008233	0.048	10	209

set, we used Fisher's exact test implemented in Blast2Go. This test compares the frequency of occurrence of a particular GO term between the gene set of interest (test set) and the total set of annotated *B. anynana* genes (the reference set). Blast2Go uses evidence code weights and reduces the set of overrepresented terms to include only the most specific terms. We only included GO terms with $p < 0.05$. Smaller gene sets, not amenable to GO term enrichment analysis, were annotated in more detail. In addition to using BLASTX against the "nr" database within Blast2Go, we also used BLASTX to compare each gene sequence against the set of annotated proteins expressed in *Drosophila melanogaster* (a subset of the "nr" protein database), and against the *Heliconius melpomene* predicted protein set (The Heliconius Genome Consortium 2012) genome assembly v1.1, primaryScaffs_Protein). For *D. melanogaster* proteins with a significant hit to any of our *B. anynana* genes of interest, we extracted GO annotation information from FlyBase (McQuilton *et al.* 2012; http://www.flybase.org/). In all BLASTX analyses, we excluded hits with an Expect value higher than 10^{-5}.

Lifespan data analysis

We analysed lifespan of the individuals not sampled for expression analysis (N = 77 to 88 per sex per temperature) by fitting a generalised linear model (GLM) for age at death, with a gamma distribution and a log link function, and with developmental temperature, sex and their interaction as fixed variables. Subsequently we analysed lifespan for each sex separately, fitting a GLM with developmental temperature as the sole predictor variable. All analyses were performed in R.

Results

Effect of seasonal developmental temperature on lifespan

Adult lifespan was lower in butterflies that developed as larvae in warm, wet season conditions compared to those developed in cool, dry season conditions (GLM $F_{1,311} = 6.41$, $p = 0.012$ for effect of developmental temperature). However, females and males were not equally affected by seasonal temperature (Fig. 1c; GLM $F_{1,311} = 3.63$, $p = 0.058$ for interaction effect). Analysing the sexes separately revealed that seasonal temperature significantly affected lifespan in males (Fig. 1b; GLM $F_{1,151} = 6.69$, $p = 0.010$) but had no effect on female lifespan (Fig. 1a; GLM $F_{1,158} = 0.38$, $p = 0.538$). Thus, males lived a shorter time on average if they developed in wet season conditions, but for females this was not the case.

Principal components analysis (PCA) of transcription profile

Pooling gene expression data from all 94 microarray samples (2 developmental temperatures x 3 time points x 2 sexes x 8 biological replicates minus 2 samples of lesser quality), the PCA revealed that the vast majority of expression variation was associated with the differences between the sexes. Principal Component (PC) 1 strongly separated females from males, and accounted for 43% of total variation. In contrast, PC2 only accounted for 4% of variation. This PC separated young adults from old and very old ones, both for males

and females. Seasonal developmental condition was not unambiguously associated with any particular PC, indicating a relatively small contribution of this treatment to overall expression variation.

Effect of ageing on gene expression

In the first differential expression analysis, we pooled data from both sexes and seasonal conditions to probe the overall effect of age on the transcription profile, comparing young with old and excluding very old individuals (first contrast in Table 1). We identified 1558 transcripts whose expression was significantly affected by age, representing 10% of all genes on the array. One third (519) of these genes showed increased expression with age and 1039 genes were down-regulated in old individuals. Gene Ontology (GO) analysis of the up-regulated genes revealed significant enrichment for calcium ion binding, and, to a lesser extent, response to abiotic stimulus and response to stress (Table 2). Genes in these categories included *troponin c 25d*, *annexin x*, *heat shock protein*, *catalase*, *superoxide dismutase* and *lebocin-like protein*. The 1039 genes down-regulated with age were significantly enriched for 15 GO categories, of which the most highly overrepresented were external encapsulating structure, DNA metabolic process, anatomical structure morphogenesis and reproduction (Table 2). Genes in these categories included *endonuclease-reverse transcriptase*, *DNA ligase* and *hormone receptor 3*, as well a large number of chorion proteins. This latter group suggests that an important part of the transcriptional response to ageing is driven by sex-specific changes.

Sex-specificity of transcriptional response to ageing

The strong effects on expression of certain sex-specific genes (e.g. chorion proteins) likely indicate that many of the patterns found in the initial pooled-sex analysis are driven by responses in a single sex. In order to gain more detailed insight into the sex-specificity of the transcriptional response to ageing, we analysed females and males separately (second and third contrasts in Table 1) and then compared sets of up and down-regulated genes among the female-only, male-only and the initial pooled-sex analyses (see Fig. 2a for a detailed break-down of numbers of genes as well as their expression levels in each group). This comparison yielded three groups of genes:

i) *Sex-independent ageing-related genes*. This group included ageing-related genes identified in the pooled-sex analysis but not represented in the female-only or in the male-only analyses. Their expression was affected by age in the same direction in both sexes but this was only significant when pooling samples, not when analysing each sex separately, indicating that the age-related expression changes were subtle for these genes. In addition, this group also included genes identified both in the female-only and male-only analyses, indicating a strong and similar expression response to age in both sexes.

ii) *Female-specific ageing-related genes*. These genes were identified as differentially expressed between young and old females, but showed no significant age effect in males.

iii) *Male-specific ageing-related genes*. These genes were identified as significantly affected by age in the male-only analysis, but showed no significant effect in females.

Table 2. Gene Ontology (GO) terms significantly overrepresented (Fisher exact test p < 0.05) among the 1558 transcript significantly up or down-regulated with age (pooled across sexes and seasonal conditions).

induction	GO term	GO category[1]	GO ID	Fisher exact test p	# genes in test set	# genes in reference set
up	calcium ion binding	F	GO:0005509	1.62E-04	8	78
up	response to abiotic stimulus	P	GO:0009628	0.018807	5	76
up	response to stress	P	GO:0006950	0.048074	10	271
down	external encapsulating structure	C	GO:0030312	4.59E-18	15	17
down	DNA metabolic process	P	GO:0006259	3.70E-04	22	193
down	anatomical structure morphogenesis	P	GO:0009653	4.16E-04	26	248
down	reproduction	P	GO:0000003	0.004265	18	176
down	cell differentiation	P	GO:0030154	0.0066	22	241
down	structural molecule activity	F	GO:0005198	0.008626	34	432
down	multicellular organismal development	P	GO:0007275	0.017256	33	437
down	electron carrier activity	F	GO:0009055	0.017756	10	90
down	receptor activity	F	GO:0004872	0.017756	10	90
down	antioxidant activity	F	GO:0016209	0.023401	5	32
down	extracellular region	C	GO:0005576	0.031033	13	141
down	transcription factor activity	F	GO:0003700	0.037806	7	61
down	carbohydrate metabolic process	P	GO:0005975	0.040832	16	192
down	carbohydrate binding	F	GO:0030246	0.042746	8	76
down	nutrient reservoir activity	F	GO:0045735	0.04773	2	7

[1] C: Cellular Component; F: Molecular Function; P: Biological Process

We identified 841 *sex-independent ageing-related genes*, of which 357 were more highly expressed in old adults and 484 showed decreased expression with age. Their expression patterns were highly similar between the sexes (Fig. 2a). GO analysis showed that the up-regulated genes were slightly enriched for calcium ion binding and lipid particle, including genes such as *annexin*, *cadherin-like protein*, *zinc finger protein noc-like* and *ribosomal protein p1*. The down-regulated genes were most highly enriched for transcription factor activity and DNA metabolic process, and these categories included genes such as *transcription factor dp-1*, *hormone receptor 3*, *endonuclease-reverse transcriptase* and *pol-like protein* (Fig. 2c).

We identified 228 *female-specific ageing-related genes* of which 103 genes were up-regulated, and 125 genes down-regulated. These genes were not significantly affected by age in males (Fig. 2a). The up-regulated female-specific genes were enriched for response to

stress, and, to a lesser extent, for calcium ion binding. The first category included genes such as *catalase*, the antimicrobial peptides *defensin-like protein precursor*, *lebocin-like protein*, and several heat shock proteins. The second category was also enriched in the sex-independent up-regulated gene set (see above), but consisted of different genes in the female-specific up-regulated gene set, such as *fibulin* and *troponin c 25d*. The female-specific down-regulated genes were highly enriched for external encapsulating structure, reproduction, cell differentiation and anatomical structure morphogenesis, including almost exclusively chorion proteins. There was one GO category that was slightly enriched among the down-regulated genes (calcium ion binding), due to *troponin c*. Paradoxically, this same category was also enriched among the up-regulated genes in the female-specific and sex-independent gene sets, which also included *troponin*-like genes. It thus seems that some genes go down with age while others associated with the same biological process go up, either in females only or in both sexes, although the limited number of genes and subsequent relatively high p values (0.11 and 0.28) may preclude definite conclusions (Fig. 2c).

Finally, we found 678 *male-specific ageing-related genes*, of which 154 showed increased expression with age and 524 were down-regulated. In females, expression of these genes was not significantly affected by age (Fig. 2a). A number of GO categories were slightly overrepresented among the male-specific up-regulated genes including cell-cell signalling and protein complex with genes such as *creb-binding protein* and *28s ribosomal protein mitochondrial-like*. The down-regulated male-specific genes were enriched for GO categories extracellular region, carbohydrate binding, and nutrient reservoir activity including the genes *fibroin p25*, *chondroitin proteoglycan-2*, *methionine rich storage protein* and *chitinase*. An additional GO category overrepresented among the down-regulated male-specific genes was DNA metabolic process, including *DNA ligase iv* and several endonuclease-reverse transcriptases. This category was also overrepresented in the sex-independent down-regulated gene set, which also included several (but other) endonuclease-reverse transcriptases (Fig. 2c).

An overview of all enriched GO categories among the sex-independent, female-specific and male-specific age-regulated genes is presented in the table in Fig. 2c.

Strikingly, the number of genes significantly down-regulated with age was much higher for the male-specific (524) than for the female-specific (125) genes, even though the sexes were sampled at sex-specific demographic age. This was also the case when only considering the genes that were solely identified in the single-sex, but not in the pooled-sex analysis: for males there were 76 such genes but for females only 18 (Fig. 2a). Among the genes up-regulated with age, there were also more male-specific (154) than female-specific (103) ageing-related genes, but this difference was less pronounced than among the down-regulated genes. Thus, it appears that males show a stronger overall transcriptional response to ageing than females, particularly among the down-regulated genes.

Among the 103 female-specific ageing-related genes that were up-regulated with age (red circles in Fig. 2b), 72 also showed increased expression with age in males (though not significantly so), and among the 125 female-specific down-regulated genes, 95 also showed decreased expression in males. Likewise, among the 154 male-specific up-regulated genes

(blue circles in Fig. 2b), 122 also showed increased expression with age in females while among the 524 male-specific down-regulated genes, 488 also showed decreased expression in females. At a False Discovery Rate (FDR) of 5%, we found no genes that were significantly down-regulated with age in females and up-regulated in males, or *vice versa*. Consistent with this, genes that showed increased expression with age in males also tended to show increased expression in females, and genes showing decreased expression with age in males also generally showed decreased expression in females (Fig. 2b). Relaxing significance thresholds and comparing the sign of fold change across all 17,154 genes irrespective of p value, the number of genes showing age-related expression changes in the same direction in males and females (both up or both down) was higher (9,515) than those genes showing age-related expression changes in opposite directions (6,240). Fold change in expression with age between males and females could also be compared more formally for all 17,154 genes, using a linear regression of female fold change on male fold change. This confirmed that part of the age-related expression changes were similar between the sexes, with a slope of 0.39 significantly deviating from zero ($F_{1,15753} = 1829$, $p < 10^{-10}$), but age-related expression variation in one sex only explained a limited amount of age-related expression variation in the other sex ($R^2 = 0.104$).

Together, these results indicate that age-related expression changes are to some extent similar in females and males. In some cases, ageing-related genes identified in the single-sex analysis (i.e. only significantly regulated with age in one sex) do respond in the same direction in the other sex. However, these responses are usually much weaker in magnitude, and not statistically significant. Thus, while the direction of age-related expression changes is often similar between the sexes, the magnitude of these changes is much more sex-specific.

Season-biased expression

We analysed the effect of developmental temperature on gene expression irrespective of age, first pooling the sexes and subsequently for each sex separately. A total of 21 genes showed evidence of a significant imprint of seasonal rearing temperature on adult expression (Fig. 3a, Table 3).

In the pooled-sex analysis, one gene (S4679) was highly overexpressed in wet season adults, and it showed no significant similarity to any known protein, including any of the predicted *H. melpomene* genes. The six frame translations of this transcript revealed two open reading frames of 120 and 108 amino acids in length, approximately. However, neither of these open reading frames showed significant similarity to any known protein, and no putative conserved domains could be identified in the 6 frame translations of this gene. This might suggest that this sequence represents a long non-coding RNA rather than a protein-coding gene (Ponting *et al.* 2009). Alternatively, it might be a transcribed pseudo gene (*cf.* Pei *et al.* 2012).

Two genes were more highly expressed in dry (cold) versus wet (warm) season males and females. One (C7872) was most similar to *HM00015* (CBH09252) in *H. melpomene* and to *CG30373* in *Drosophila melanogaster*, a protein with no described GO associations. The other dry season-biased gene (S723) showed no similarity to any known protein.

Considering only females, three genes were up-regulated in adults reared under high, wet season temperatures, of which two showed significant similarity to other known proteins. The first one (C1437) was most similar to *hypothetical protein KGM_12805* in the monarch butterfly *Danaus plexippus* (EHJ76115) and to *CG12398* in *D. melanogaster*, associated with GO terms 'chorion' and glucose dehydrogenase activity. The second gene up-regulated in wet season females (S8341) showed the highest similarity *to hypothetical protein KGM_15522* in *D. plexippus* (EHJ79277), and to *pudgy* in *D. melanogaster*. The latter protein is involved in several fat metabolic processes, and in the negative regulation of insulin receptor signalling pathway (Xu *et al.* 2012). It also showed high similarity to *Luciferin 4-monooxygenase* in the harvester ant *Camponotus floridanus* (EFN72607) and to *luciferase* in the beetle *Pyrophorus plagiophthalamus* (AAQ11720). The third wet season-biased gene in females (S5587) was not similar to any known protein. Seven genes were down-regulated in females reared under wet season conditions (i.e. up-regulated in dry season), of which only two showed a significant similarity to any known protein. The first one (S2729) was most similar to *conventional protein kinase C* in *D. plexippus* (EHJ63450), and to *protein C kinase 53E* in *D. melanogaster*, involved in protein phosphorylation (Wang *et al.* 2012). The other dry season-biased gene (S7643) had highest similarity to *glycosyl hydrolase family 31 protein D. plexippus* (EHJ74126) and to *target of brain insulin* in *D. melanogaster*, associated with alpha-glucosidase activity (Buch *et al.* 2008).

In males, six genes showed significant wet season-biased expression, of which one had (S8414) no significant similarity to any known protein. Two genes had a significant hit to a *D. melanogaster* protein, allowing functional annotation using GO. One (C1963) was most similar to *rhodopsin 5*, a G-protein coupled photoreceptor sensitive to UV-A. It also showed significant similarity to *blue-sensitive visual pigment* in the butterfly *Dryas iulia* (ADN96745). The other (C6910) was most similar to the *D. melanogaster* protein *farnesyl pyrophosphate synthase*, associated with dimethylallyltransferase activity and isoprenoid biosynthetic process, among others. It also had a significant hit to *dimethylallyltransferase* in the moth *Agrotis ipsilon* (CAA08918). The fourth gene up-regulated in adult males developed in warm, wet season conditions (C1568) had no significant *D. melanogaster* hit, and was most similar to *hypothetical protein KGM_06477* in *D. plexippus* (EHJ76078). It also showed significant (10^{-97}) similarity to *takeout/Juvenile hormone binding protein-like protein* in the swallowtail butterfly *Papilio xuthus* (BAM18104). In *D. melanogaster*, *takeout* is associated with the GO Biological Processes adult feeding behaviour, behavioural response to starvation, circadian rhythm and male courtship behaviour. The fifth wet season-biased gene (C8112) was most similar to *alcohol dehydrogenase* in *D. plexippus* (EHJ65259), but showed no significant similarity to any *D. melanogaster* gene. The sixth and final gene up-regulated in adult males from the wet season (S691) again had no *D. melanogaster* hit, but did show significant similarity to *hypothetical protein KGM_07310* in *D. plexippus* (EHJ67666). More revealingly, 11 of the 15 next best BLASTX matches (10^{-68} to 10^{-26}) were (similar to) zinc finger proteins, for example *similar to zinc finger protein* in the beetle *Tribolium castaneum* (XP_001812645) and *zinc finger protein* in the mosquito *Aedes aegypti* (XP_001650281).

Table 3. Genes differentially expressed in adults reared under wet season conditions compared to those reared under dry season conditions, pooled across age classes. See also Figure 3.

gene name	seasonal bias [1]	sex	fold change [2]	best hit accession [3]	best hit organism [3]	% ID [3]	best *H. melponene* hit [4]	% ID [4]	best *D. melanogaster* hit [5]	% ID [5]	*D. melanogaster* GO associations [6]
S4679	wet	both	113.49	-	-	-	-	-	-	-	
C7872	dry	both	-3.95	CBH09252	*Heliconius melpomene*	89	HMEL000015-PA	76	CG30373	50	-
S723	dry	both	-6.47	-	-	-	-	-	-	-	
C1437	wet	females	3.03	EHJ76115	*Danaus plexippus*	87	HMEL003259-PA	47	CG12398	47	alcohol metabolic process choline dehydrogenase activity chorion flavin adenine dinucleotide binding glucose dehydrogenase activity oxidation-reduction process
S5587	wet	females	1.20	-	-	-	-	-	-	-	
S8341	wet	females	3.30	EHJ79277	*Danaus plexippus*	81	HMEL002123-PA	67	pudgy (CG9009)	43	acyl-CoA biosynthetic process fatty acid catabolic process long-chain fatty acid-CoA ligase activity negative regulation of insulin receptor signalling pathway triglyceride catabolic process
S7643	dry	females	-13.54	EHJ74126	*Danaus plexippus*	77	HMEL008000-PA	58	target of brain insulin (CG11909)	58	alpha-glucosidase activity carbohydrate metabolic process glucosidase II complex
S4071	dry	females	-4.90	-	-	-	-	-	-	-	
S4072	dry	females	-2.91	-	-	-	-	-	-	-	
S639	dry	females	-4.60	-	-	-	-	-	-	-	

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Table 3. Genes differentially expressed in adults reared under wet season conditions compared to those reared under dry season conditions, pooled across age classes. See also Figure 3. (continued)

gene name	seasonal bias [1]	sex	fold change [2]	best hit accession [3]	best hit organism [3]	% ID [3]	best *H. melponene* hit [4]	% ID [4]	best *D. melanogaster* hit [5]	% ID [5]	*D. melanogaster* GO associations [6]
S2729	dry	females	-3.32	EHJ63450	*Danaus plexippus*	80	HMEL012616-PA	79	protein C kinase 53E (CG6622)	83	ATP binding calcium-dependent protein kinase C activity diacylglycerol binding intracellular signal transduction protein kinase C activity protein phosphorylation protein serine/threonine kinase activity regulation of hemocyte proliferation zinc ion binding
S8613	dry	females	-2.91	-	-	-	-	-	-	-	
S8792	dry	females	-8.67	-	-	-	-	-	-	-	
C1568	wet	males	5.46	EHJ76078	*Danaus plexippus*	88	HMEL015741-PA	58	-	-	
C1963	wet	males	2.22	ADN96745	*Dryas iulia*	95	HMEL010706-PA	89	rhodopsin 5 (CG5279)	57	absorption of visible light G-protein coupled photoreceptor activity G-protein coupled receptor signalling pathway integral to membrane phototransduction response to light stimulus rhabdomere sensory perception of sound UV-A, blue light phototransduction visual perception

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Table 3. Genes differentially expressed in adults reared under wet season conditions compared to those reared under dry season conditions, pooled across age classes. See also Figure 3. (continued)

gene name	seasonal bias [1]	sex	fold change [2]	best hit accession [3]	best hit organism [3]	% ID [3]	best *H. melponene* hit [4]	% ID [4]	best *D. melanogaster* hit [5]	% ID [5]	*D. melanogaster* GO associations [6]
C6910	wet	males	3.70	CAA08918	*Agrotis ipsilon*	92	HMEL017961-PA	49	farnesyl pyrophosphate synthase (CG12389)	57	dimethylallyltranstransferase activity farnesyl diphosphate biosynthetic process geranyltranstransferase activity germ cell migration isoprenoid biosynthetic process wing disc development
C8112	wet	males	4.22	EHJ65259	*Danaus plexippus*	91	HMEL013743-PA	60	-	-	
S691	wet	males	2.92	EHJ67666	*Danaus plexippus*	96	HMEL005858-PA	94	-	-	
S8414	wet	males	2.28	-	-	-	-	-	-	-	
C1578	dry	males	-2.72	BAB39502	*Dendrolimus spectabilis*	42	-	-	-	-	
C3987	dry	males	-1.43	-	-	-	-	-	-	-	

1. Dry season-biased genes are overexpressed in adults reared at low, dry season temperatures; wet season-biased genes are overexpressed in adults reared at high, wet season temperatures.
2. Expression in wet season relative to dry season (positive values), or expression in dry season relative to wet season (negative values).
3. From BLASTX similarity search against NCBI's non-redundant (nr) protein database, maximum E-value = 10^{-5}.
4. From BLASTX similarity search against *Heliconius melpomene* protein database (The Heliconius Genome Consortium 2012).
5. From BLASTX similarity search against *D. melanogaster* subset of NCBI's non-redundant (nr) protein database.
6. Retrieved from FlyBase.

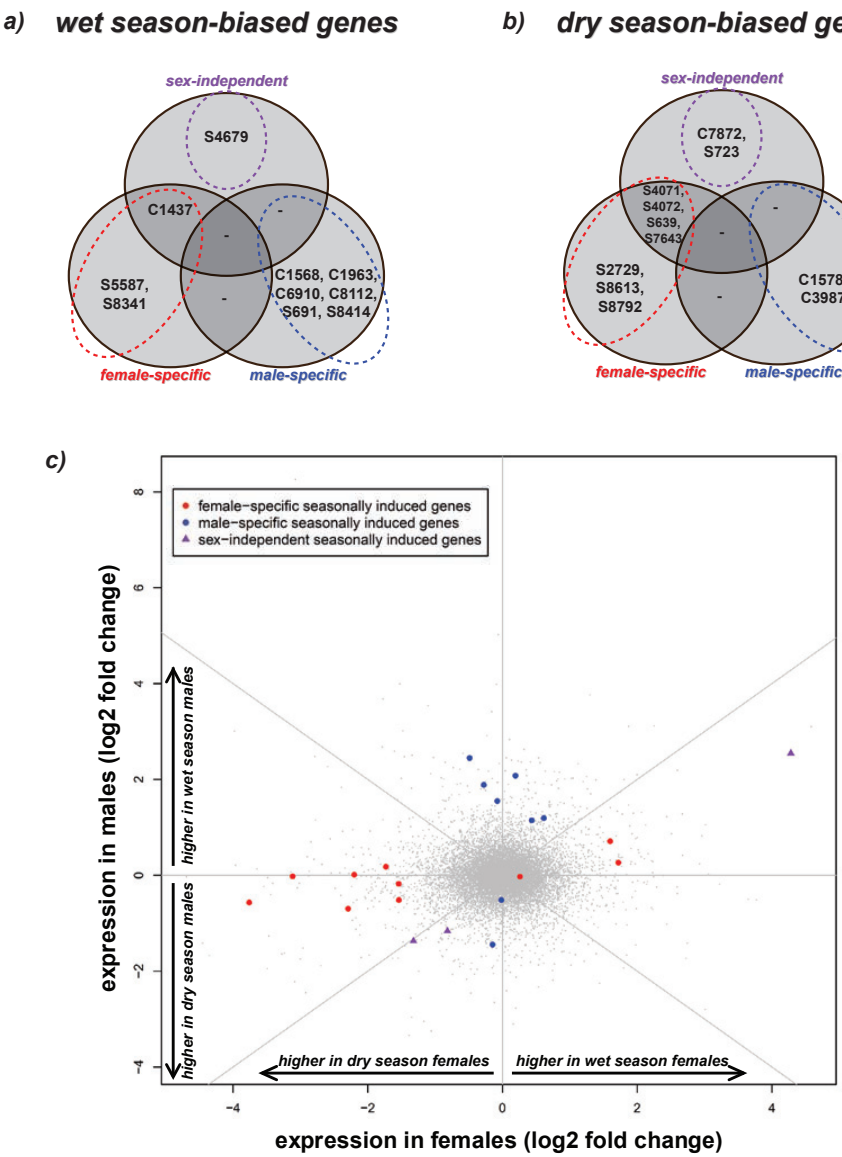

Figure 3. Season-biased gene expression in female and male adults. *a)* Venn diagram showing genes overexpressed in adults developed in warm, wet season conditions, in a pooled-sex (top), a female-only (left) and a male-only (right) analysis, as well as overlap between these groups. Names of genes correspond to those in Table 3. *b)* Venn diagram showing genes overexpressed in adults developed in cool, dry season conditions. *c)* Season-biased gene expression in males (fold change) plotted as a function of season-biased gene expression in females, with positive values indicating genes up-regulated in adults reared under wet season conditions and negative values indicating genes up-regulated in adults reared under dry season conditions. Sex-independent, female-specific, and male-specific genes are indicated by purple triangles, red circles and blue circles, respectively, whereas genes not significantly differentially expressed between seasons are indicated with grey dots.

Two genes showed dry season-biased expression in males. The first one (C1578) was most similar to *fibroin 25* in the pine moth *Dendrolimus spectabilis* (BAB39502), and showed no similarity to any *D. melanogaster* gene. The other gene (C3987) was not similar to any annotated protein, nor to any predicted *H. melpomene* gene. All season-biased genes including annotations are listed in Table 3.

Effect of seasonal condition on transcriptional response to ageing

To analyse whether developmental conditions, in addition to affecting adult gene expression levels, might influence age-related changes in adult gene expression, we compared young with old adults for the seasonal morphs separately, and compared these analyses to the earlier, pooled analysis. This yielded two sets of genes: 1) genes whose expression was significantly affected by age in adults developed as larvae in cool, dry season conditions but not in those reared in warm, wet season conditions (dry season-specific ageing-related genes), and 2) genes that were only affected by age in wet, but not dry season conditions (wet season-specific ageing-related genes). As these sets of season-specific ageing-related were relatively small, we examined the genes on an individual basis, focusing on those genes that showed significant similarity to other known and annotated proteins. Given the extensive sex-specificity observed previously, we did this for each sex separately (Figures 4 and 5 for females and males, respectively).

In females, we identified 23 dry season-specific ageing-related genes of which ten showed increased expression with age while 13 genes were down-regulated in older individuals (Fig. 4). The up-regulated genes included three genes that showed significant similarity to known proteins: *luciferase*, *zinc finger protein 782* and *nessun dorma*. Among the down-regulated genes, only two showed high similarity to any annotated protein. The first had a hit to *pacifastin-related serine protease inhibitor precursor*, and the second one to *low-density lipoprotein receptor*, involved in the endocytic uptake of circulating lipoproteins.

A total of 83 genes showed wet season-specific age-related expression changes, with 31 genes showing up-regulation with age and 52 decreasing expression with age. Among the up-regulated genes, twenty showed significant similarity to annotated proteins. A large fraction of these were associated with immunity, including *Hemolin*, an immunoglobulin expressed during oogenesis, as well as several antimicrobial peptides such as *defensin-like protein precursor* and two *gallerimycin-like proteins*. In addition, we found two genes that were both most similar to *Prophenoloxidase-activating proteinase 3* (in *D. plexippus*). Comparing it to *D. melanogaster*, the first gave a hit to *Melanization Protease 1*, associated with the melanisation defence response, while the second had its best fruit fly hit to *Serine protease 7*, also involved in the melanisation defence response. Finally, two genes showed a significant hit to *ejaculatory bulb protein III* in *D. melanogaster*, which is associated with response to viruses, among others. The other genes up-regulated with age specifically in wet season-reared females were more varied and included *heat shock protein 70* (associated with determination of adult lifespan), *neutral lipase* (associated with lipid metabolism), *muscle-specific protein 300* (associated with locomotion) and *deoxyribonuclease II* (associated with DNA degradation).

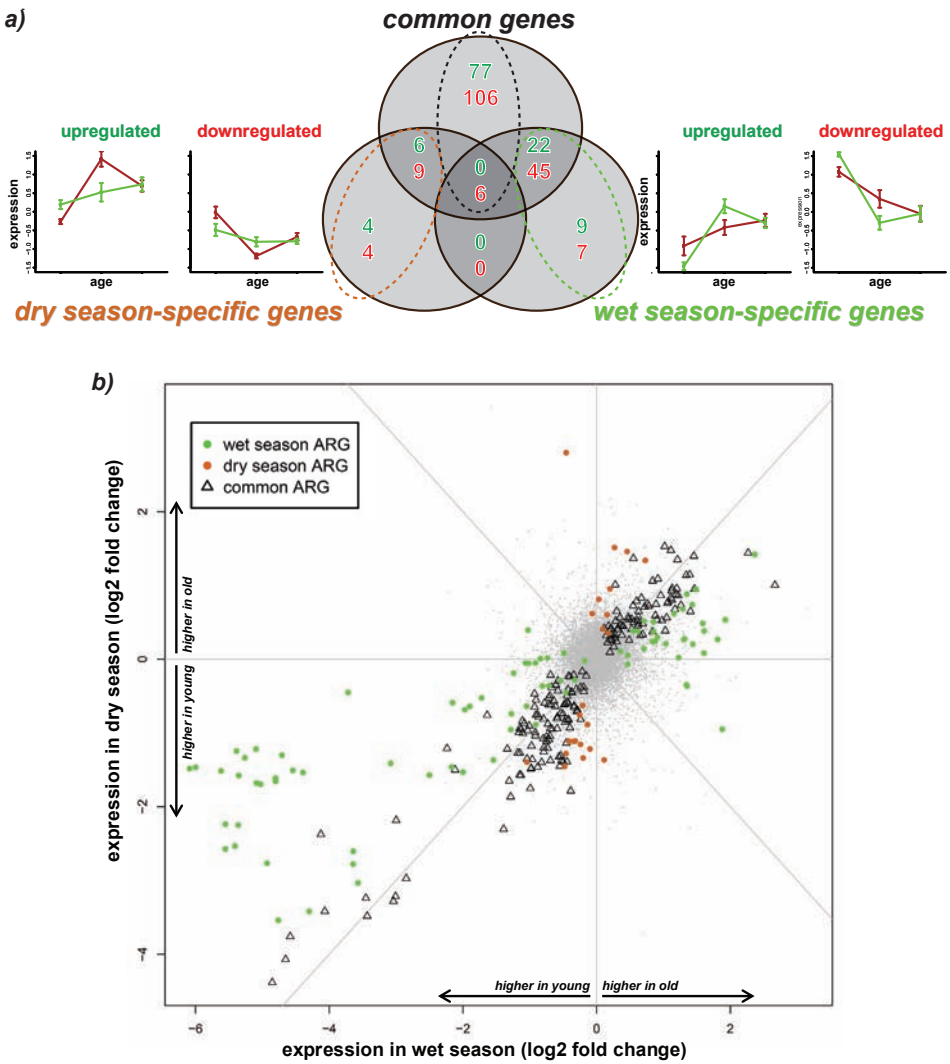

Figure 4. Effect of seasonal condition on transcriptional response to ageing in females. *a)* Venn diagram showing the groups of genes significantly up- and down-regulated with age in an analysis pooling all females (top), for females reared in dry season conditions (left), or for females reared in wet season conditions (right), as well as overlap between these groups. The four smaller plots show mean standardised expression (+/- S.E.) as a function of age (young, old and very old) for the dry season-specific (left) and wet season-specific (right) ageing-related genes, plotted for dry season-reared females (brown) and wet season-reared females (green) separately. *b)* Fold change in gene expression of young versus old females reared in dry season conditions plotted as a function of fold change of young versus old females reared in wet season conditions, with positive values indicating genes up-regulated with age and negative values indicating genes down-regulated with age. Common, dry season-specific, and wet season-specific ageing-related genes (ARG) are indicated by black triangles, brown circles and green circles, respectively, whereas genes not significantly differentially expressed with age are indicated with grey dots. Annotation of genes in each group can be found in Supplementary Table 1.

Of the genes that showed decreased expression at old age specifically in wet season-reared females, 35 showed a significant similarity to other known proteins. Ten of these were *chorion proteins* or *chorion precursor proteins*. In addition, two genes with decreased expression in old wet season-reared females had significant BLASTX hits to *eukaryotic translation initiation factor theta subunit*, involved in initiation of protein translation. Two other genes down-regulated were similar to *zinc finger proteins*, associated with nucleic acid binding and potentially involved in regulation of transcription. Four genes showed significant similarity to carboxylesterase: one gene was most similar to *antennal esterase cxe18*, one to *carboxylesterase*, and two to *carboxyl choline esterase cce016b* as well as to the *D. melanogaster* gene *α-Esterase-7*. In addition to carboxylesterase activity, the latter gene is also associated with lipid storage and determination of adult lifespan. Among the genes in the down-regulated gene set, we also found two genes with significant similarity to *aldehyde dehydrogenase* and *aldehyde oxidase*, respectively, and both involved in oxidation-reduction process. One gene showed a hit to *sugar transporter*, associated with monosaccharide transmembrane transporter activity. For three of the down-regulated genes, the best BLASTX hit were similar (but not identical) to the best BLASTX hit of genes up-regulated with age in wet season-reared females. This was the case for *serine protease*, *seminal fluid protein hacp044*, and *glucose dehydrogenase*.

In males, there were 31 genes that showed significant age-related expression changes in adults reared in dry, but not wet season conditions, of which 14 increased their expression with age while 17 showed down-regulation with age (Fig. 5). Only two of the up-regulated genes showed any significant similarity to known proteins in other species, but for neither was any annotation information available. In contrast, for eight of the 17 down-regulated genes a significant similarity to known proteins was found, mostly in *D. plexippus*. These included the genes *hemolymph proteinase 16*, *sugar transporter*, *amino acid transporter*, *tubulin beta-2 chain-like* and *sulfide quinone reductase* as well as *D. melanogaster* genes *ndl* (involved in Toll signalling) and *rk* (associated with G-protein coupled receptor activity).

A markedly higher number of genes (282) were specifically affected by age in wet season-reared males. Expression increased with age for 31 of these wet-season specific genes, while 251 genes were down-regulated. The up-regulated genes that showed a significant BLASTX hit included the genes *prophenoloxidase*, *nadh:ubiquinone dehydrogenase*, *nadh dehydrogenase* (*ND23* in *D. melanogaster*), *cyclic-nucleotide-gated cation channel*, *anaphase-promoting complex subunit (mr* in *D. melanogaster)*, and *28s ribosomal protein mitochondrial-like (mRpS30* in *D. melanogaster)*. Among the 251 down-regulated genes, 123 showed a significant similarity to other known proteins. These genes showed enrichment (at $p < 0.05$) for only two GO terms. The first process, DNA metabolic process, included five endonuclease *reverse transcriptases* and *gag-pol polyprotein*, most similar to *pol* in *D. melanogaster*, a gene that has been characterised as a transposable element (Kaminker *et al.* 2002). The second process, carbohydrate metabolic process, included genes with significant hits to *beta-glucosidase*, *chitinase*, *mucin related 89F*, and *chondroitin proteoglycan-2*. The total number of genes associated with these two GO terms was only 12, contributing to the relatively high p values for enrichment ($p = 0.026$) and giving limited

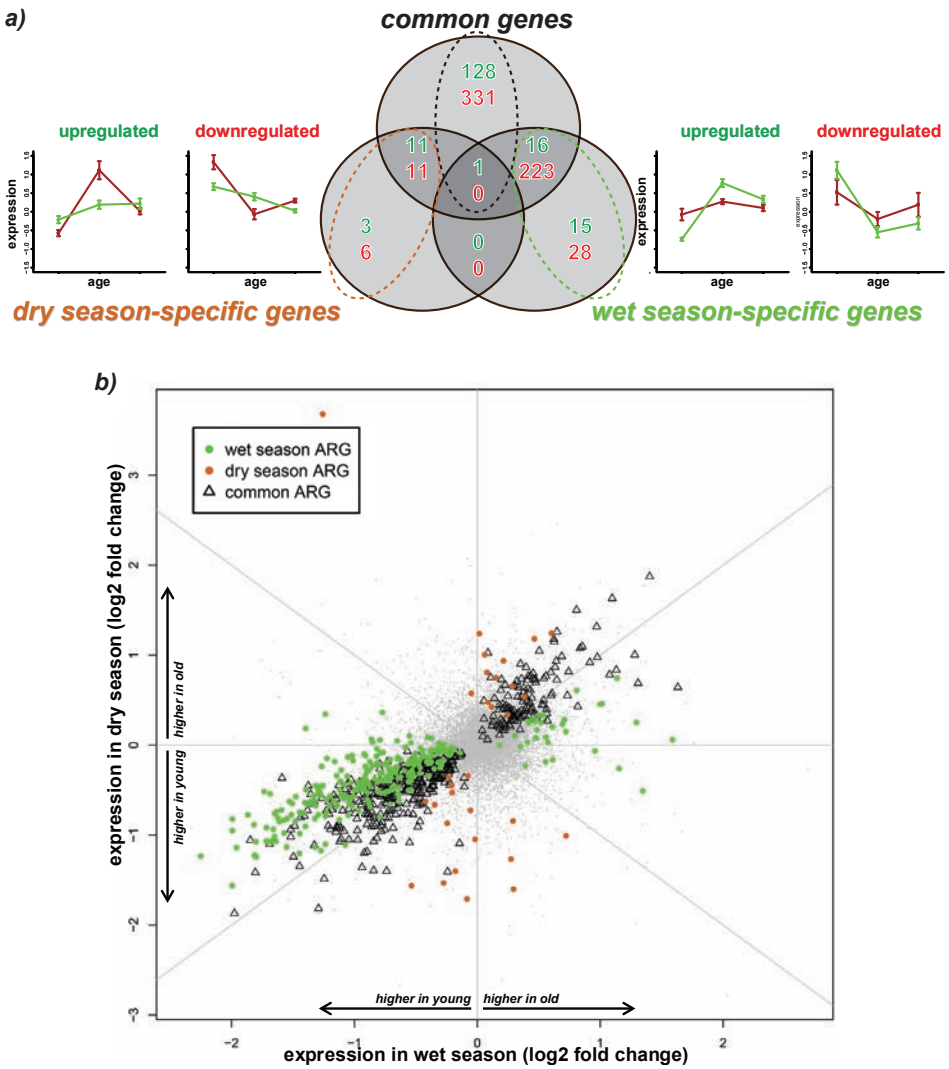

Figure 5. Effect of seasonal condition on transcriptional response to ageing in males. *a)* Venn diagram showing the groups of genes significantly up- and down-regulated with age in an analysis pooling all males (top), for males reared in dry season conditions (left), or for males reared in wet season conditions (right), as well as overlap between these groups. The four smaller plots show mean standardised expression (+/- S.E.) as a function of age (young, old and very old) for the dry season-specific (left) and wet season-specific (right) ageing-related genes, plotted for dry season-reared males (brown) and wet season-reared males (green) separately. *b)* Fold change in gene expression of young versus old males reared in dry season conditions plotted as a function of fold change of young versus old males reared in wet season conditions, with positive values indicating genes up-regulated with age and negative values indicating genes down-regulated with age. Common, dry season-specific, and wet season-specific ageing-related genes (ARG) are indicated by black triangles, brown circles and green circles, respectively, whereas genes not significantly differentially expressed with age are indicated with grey dots. Annotation of genes in each group can be found in Supplementary Table 2.

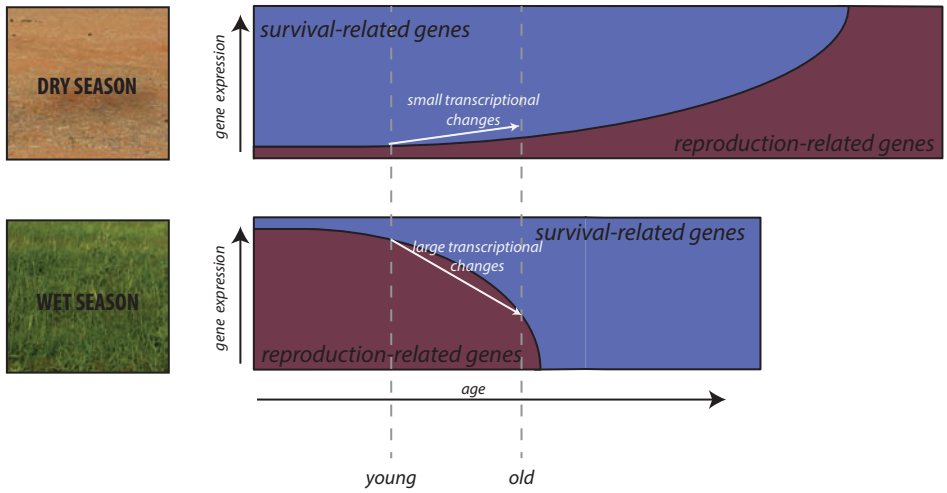

Figure 6. Graphical representation of season-specificity in age-related transcriptional changes. In this model, individuals reared in the wet season start their life as adults with high expression of reproduction-related genes (indicated in blue), which show a relatively rapid down-regulation with age. In contrast, individuals reared in the dry season start their life with high expression of survival-related genes (indicated in purple), which only slowly change with age. This would explain the results in our experiment, where wet season-reared individuals showed much more age-related transcriptional changes than those reared in the dry season (Fig. 4 and 5).

insight into the biological processes represented in this group. We therefore examined the set of wet season-specific genes down-regulated with age in males at an individual basis, focusing on those genes that showed significant similarity to other known and relatively well-annotated proteins. In this gene set we observed two genes, *catalase* and *oxidase peroxidase* associated with response to oxidative stress and the oxidation reduction process. One wet season-specific gene, *lysozyme B*, is associated with the antimicrobial humoural response. We also identified two histone genes, *histone h1* and *histone h2a*, nucleosome proteins of which the latter is also involved in response to DNA damage. A number of genes was related to Ecdysone signalling or metabolism, including *disembodied* (encoding a Cytochrome p450 enzyme), *Ecdysone-dependent gene 78E* and *Ecdysone-dependent gene 84A*. *Hormone receptor* 3 codes for a putative nuclear hormone receptor that contains a zinc finger DNA binding domain and ligand binding domain of nuclear receptors, and has some similarity to Ecdysone receptor and other nuclear receptors. A different Cytochrome P450 enzyme, *Cyp6a2*, was also identified in this gene set, as were two genes showing significant similarity to *juvenile hormone binding protein*. Three other genes potentially related to reproduction were also among the wet season-specific ageing-related genes down-regulated in males. The first one was the gene identified as *beta-glucosidase*, but it was also highly similar to *seminal fluid protein* in *H. melpomene*. The other one had a significant BLASTX hit to *male sterility domain-containing protein* as well as to *Fatty acyl-CoA reductase*. The

third down-regulated gene potentially related to reproduction had *yellow* as its highest *D. melanogaster* hit, a gene associated with male courtship and mating behaviour. Finally, one of the genes down-regulated with age among wet season-reared males was highly similar to *sterol o-acyltransferase* and to *midway* in *D. melanogaster*, which is involved in the regulation of lipid storage.

A complete list of all season-specific ageing-related genes including their annotation can be found in Supplementary Tables 1 and 2 for females and males, respectively.

Discussion

The condition-dependent expression of alternative phenotypes from the same genotype ultimately results from on transcriptional regulation (Beldade *et al.* 2011). Here, we applied the power of high-throughput gene expression profiling to *Bicyclus anynana*, a species for which there is extensive ecological knowledge. We used custom-designed microarrays to study how ageing and developmental plasticity of life history contribute and interact to affect the expression profile. All adults were kept in the same wet season conditions, and differed only in the seasonal environment they experienced as larvae. A myriad of genes was affected by age, with pervasive sex-specificity in the transcriptional response. The seasonal morphs showed relatively modest differences in their age-related expression changes, with the long-lived dry season morph lacking some of the transcriptional changes observed in the wet season morph. Independent of age, a small set of genes showed life-lasting expression differences among adults reared at the alternative seasonal conditions.

Sex-specific transcriptional response to ageing

Overall, expression of *ca.* 10% of all genes on the array was affected by age. This is on the lower side of proportions of age-regulated genes found in previous studies in *D. melanogaster*, where it ranged from 4-19% of genes in males (Girardot *et al.* 2006; Landis *et al.* 2004; Zhan *et al.* 2007) to 23-38% in females (Doroszuk *et al.* 2012; Pletcher *et al.* 2002). This is consistent with the overall high level of variation observed in the present study that was not related to age. In the PCA, the second axis separated young from old and very old individuals but accounted for only 4% of total variation. Unlike our study, most similar studies used virgin individuals (e.g. Doroszuk *et al.* 2012). It is likely that mating introduces additional gene expression variation, for example as a result of spermatophore size (*cf.* Karlsson 1998). Not only the number of age-related genes, but also the number of Gene Ontology (GO) terms significantly enriched among the ageing-related genes was relatively low (Table 2, table in Fig. 2c), in particular when compared to similar studies in *D. melanogaster* (Doroszuk *et al.* 2012). This is likely a consequence of the fact that for *B. anynana* we were only able to assign a GO term to 27% of all transcripts on the array, with relatively few GO terms per annotated transcript. Some GO terms that we found in the present study to be significantly enriched among ageing-related genes have also been described in similar studies in *D. melanogaster*. For example, several studies reported

up-regulation with age of genes associated with stress response, and down-regulation of genes involved in processes related to reproduction, including in studies using virgins (Doroszuk *et al.* 2012; Girardot *et al.* 2006; Pletcher *et al.* 2005). DNA metabolism and transcription factor activity were also repressed with age in our study, similar to previous findings in *D. melanogaster* (Doroszuk *et al.* 2012) and in *Mus musculus* (Park *et al.* 2009). Mitochondrial metabolism-associated genes have often been found to be down-regulated with age (Girardot *et al.* 2006; Pletcher *et al.* 2005), but in this study no such down-regulation was observed, as in (Doroszuk *et al.* 2012). There were only two GO terms slightly enriched among the down-regulated genes that were related to mitochondrial metabolism: electron carrier activity (Table 2) and carbohydrate metabolic process (table in Fig 2c). Most studies on ageing-related transcriptional changes focused on an earlier part of the ageing trajectory, when mortality is still relatively low. In contrast, in our study the youngest individuals were sampled at 10% cohort mortality and may actually almost be considered middle-aged (see Fig. 1). This confirms that down-regulation of mitochondrial metabolism occurs earlier in life (Pletcher *et al.* 2005), and is in line with one other study in *D. melanogaster* that compared middle-aged with old individuals and found no age-related repression of mitochondrial gene expression (Doroszuk *et al.* 2012).

There were marked differences between males and females in their transcriptional response to ageing. Approximately half of all ageing-related genes were affected in both sexes, and the other half showed age-related expression changes in a single sex only. Of this latter group, *ca.* two thirds was affected in males while only one third was affected in females, indicating a stronger transcriptional response to ageing in males compared to females (Fig. 2a). This difference could partly be explained by seasonal differences in the ageing profile. Wet season males down-regulated many more genes with age than dry season males (Fig. 5a), and this seasonal difference in the transcriptional response to ageing was somewhat less pronounced in females (Fig. 4a). However, even if only considering genes differentially expressed with age in both seasonal morphs, the numbers of genes affected was substantially higher in males than in females, in particular among the down-regulated genes. Relaxing significance thresholds and comparing fold change in expression with age between the sexes suggested more concordance in the transcriptional response (see Results). For the majority (*ca.* 60%) of genes, expression was affected by age in the same direction in females and males. This suggests that to some extent, expression variation in both sexes is not independent. Nevertheless, the magnitude of this correlated expression variation was generally limited, resulting in the observed sex-specificity of genes that showed a statistically significant age effect. In our experimental setup, the adult condition is permissive of reproduction. As females are mated, their gene expression is likely to be geared towards high rates of egg production, which might mask any subtler age-related expression changes. For males this is not the case, which could explain why more genes are affected by age in that sex. Interestingly, the difference among the sexes in numbers of age-related genes is largest in wet-season reared animals. This fits with the idea that males and females are likely to be more similar in the dry season, when reproduction is repressed and both sexes express a survival strategy.

Sexual dimorphism in ageing-related expression changes was also apparent at the level of enriched GO terms, where we found evidence for both overlapping and sex-specific enrichment (table in Fig. 2c). Although many studies have reported pervasive sexual dimorphism in a variety of life history characteristics including ageing (e.g. Maklakov *et al.* 2008; Zajitschek *et al.* 2009), few have examined associated gene expression patterns. In one notable exception, Berchtold and colleagues (2008) found substantial sexual dimorphism in gene expression associated with ageing in a variety of human brain regions, with most age-related changes occurring in males (Berchtold *et al.* 2008).

Developmental imprint on adult gene expression: a role for the Insulin signalling pathway

We found 21 genes showing a significant signature of developmental conditions across the adult lifespan, of which six showed significant similarity to an annotated *D. melanogaster* gene (Table 3). Three of these six genes are connected to the Insulin signalling pathway. The transcript coding for Protein kinase C 53E (PkC53E), an intracellular signalling protein, is up-regulated in dry season females. Both in *D. melanogaster* and humans, it has been found to directly activate the transcription factor FoxO, affecting nuclear localization, mRNA expression and transcriptional activity (Mattila *et al.* 2008). An earlier study in humans suggested a role for this protein as a constitutive inhibitor of Insulin signalling, as it binds to and phosphorylates the Insulin Receptor Substrate (IRS) in absence of Insulin (Sampson & Cooper 2006). Together this suggests that FoxO is more active and hence Insulin signalling is lower in *B. anynana* females reared in dry season conditions, which in the field correspond to a more thrifty nutritional environment (Brakefield & Zwaan 2011). In addition to its role in Insulin signalling, PkC53E has also been linked to Ecdysteroid signalling (Wang *et al.* 2012). Ultraspiracle (USP), together with its heterodimeric partner Ecdysone Receptor (EcR), acts as a nuclear receptor and transcription factor that plays a central role in the cellular transcriptional response to Ecdysteroids (Klowden 2007). Recently, it was shown that USP phosphorylation by PkC53E is necessary for Ecdysteroid signalling (Wang *et al.* 2012). In *B. anynana*, Ecdysteroids link larval seasonal temperature with the developmental induction of alternative adult phenotypes (see Chapters 2 and 3). The seasonal bias in PkC53E expression observed in the present study might therefore indicate that Ecdysteroid signalling is also involved in maintaining the developmental imprint throughout adult life, long after the transient exposure to the juvenile environment. This is consistent with the role of Ecdysteroids in other insects, where they are involved in regulation of larval and pupal diapause (Denlinger 2002), but also play an important role in regulating several aspects of adult female reproduction (Schwedes & Carney 2012). Recently, USP has been found to be involved in behavioural plasticity in response to nutrition in honey bees (Ament *et al.* 2012). The two other Insulin signalling-related genes among the six annotated developmentally imprinted genes are downstream transcriptional targets of Insulin signalling. The first one is *target of brain insulin* (*tobi*), up-regulated in dry season females. It codes for an alpha-glucosidase expressed in the fat body, near the ovaries and in and around the gut. Insulin-producing cells in the brain, where Insulin-like peptides

(ILP) 2, 3 and 5 are expressed (Gronke *et al.* 2010; Toivonen & Partridge 2009), regulate expression of *tobi* in response to diet. Expression is highest under a high protein and low sugar diet, generally associated with increased reproduction and decreased lifespan, and lowest under a low protein and high sugar diet (Buch *et al.* 2008; Lee *et al.* 2008). Thus, both in *B. anynana* and in *D. melanogaster*, *tobi* shows expression plasticity in response to environmental conditions. Strikingly, both the inducing environment (temperature *vs*. diet) and the developmental stage in which the response is induced (larval *vs*. adult) differ between the plastic responses in these insects. The other target of Insulin signalling whose adult expression was affected by developmental seasonal conditions was *pudgy*, showing up-regulation in wet season females. It codes for a long-chain fatty acid-CoA ligase that in *D. melanogaster* has been found to be a direct transcriptional target of FoxO. It shows reduced expression under high Insulin signalling conditions, but strong up-regulation following fasting. The Pudgy protein activates free fatty acids both for catabolism and for anabolism, thus acting as a regulator of lipid homeostasis, linking nutrient sensing with fat metabolism (Xu *et al.* 2012). *B. anynana* adults of the two seasonal forms differ in abdominal lipid content (see Brakefield & Reitsma 1991; Chapter 2). Our microarray results may thus indicate that plasticity in *pudgy* expression links environmental input during development with season-specific adult lipid physiology.

In a wide range of animal taxa, the Insulin signalling pathway plays a central role in the regulation of growth, metabolism, reproduction and ageing in response to variation in nutrition. This neuroendocrine pathway links information on the nutritional state of the organism from the central nervous system via circulating Insulin-like peptide hormones and an intracellular phosphorylation cascade to activity of FoxO. This transcription factor regulates expression of a multitude of downstream effector genes that presumably govern the observed phenotypic effects (Broughton & Partridge 2009; Edgar 2006; McElwee *et al.* 2007; Tatar *et al.* 2003). The regulatory cascades by which Insulin signalling exerts its phenotypic effects are likely to be more complex and involve additional regulators other than FoxO (e.g. Slack *et al.* 2011). In the context of life history theory, the Insulin signalling pathway has been interpreted as a nutrient-sensitive endocrine switch between a reproductive and non-reproductive mode with "pro and slow" ageing consequences, respectively (Fielenbach & Antebi 2008; Tatar *et al.* 2003).

A number of classic examples of developmental plasticity in invertebrates has been linked to Insulin signalling. Perhaps the most well studied example of life history plasticity is dauer-formation in the nematode *C. elegans*, where worms enter a long-lived and stress-resistant diapause state when food conditions are adverse. Insulin signalling plays a crucial role in this transition, although other pathways such as steroid hormone signalling are also involved (Fielenbach & Antebi 2008). In insects, Insulin signalling has also been implicated in diapause regulation. Early experiments in *Pieris brassicae* showed that bovine insulin can terminate diapause (Arpagaus 1987). More recently, it was shown using RNAi that FoxO and Insulin Receptor (InR) are critical regulators of diapause in the mosquito *Culex pipiens* (Sim & Denlinger 2008). Another dramatic example of life history plasticity is reproductive division of labour in eusocial insects. In the honey bee *Apis mellifera*, caste determination

during development as well as adult maintenance of division of labour have both been linked to Insulin signalling (Ament *et al.* 2008; Cardoen *et al.* 2011; Smith *et al.* 2008). In eusocial ants this has been studied less intensively, but again expression of genes in the Insulin signalling pathway has been found to associate with reproductive caste (M. Corona, unpubl. data; Lu & Pietrantonio 2011; Okada *et al.* 2010). Finally, male beetle horn dimorphism is a beautiful example of developmental plasticity linking juvenile nutrition with adult reproductive potential. In a recent study, Emlen and colleagues (2012) showed that in the rhinoceros beetle *Trypoxylus dichotomus*, horn-specific sensitivity to Insulin plays a crucial role in the conditional development of this sexually selected trait (Emlen *et al.* 2012). In *B. anynana*, three of the six annotated genes differentially expressed across the adult lifespan as a result of developmental history were related to Insulin signalling. Our findings thus fit an emerging body of work pointing to a general role for Insulin signalling in regulating phenotypic plasticity, linking an environmental signal to alternative phenotypes for life history or morphology.

In addition to the three Insulin-related genes, three other annotated genes also showed a developmental imprint on adult expression (Table 3). C1424, most similar to *D. melanogaster* CG12398, showed season-biased expression in females. This transcript codes for a putative glucose dehydrogenase, which in *D. melanogaster* is expressed in follicle cells, potentially playing a role in vitelline membrane formation (Fakhouri *et al.* 2006). Consistent with this, CG12398 was observed in a different study to be up-regulated in mated females (McGraw *et al.* 2004). The observed up-regulation of this gene in *B. anynana* females reared in the wet season is likely related to their higher reproductive investment in this season. Interestingly, CG12398 transcription has been found to be directly regulated by Ecdysteroids in *D. melanogaster*: the EcR/USP complex physically binding to a region close to the CG12398 locus (Gauhar *et al.* 2009). Such binding, if conserved in *B. anynana*, would provide a direct mechanistic link between Ecdysteroid signalling, known to be involved in developmental induction of alternative phenotypes (see Chapters 2 and 3), and adult expression variation of life history-related genes.

The next gene that showed season-biased expression was *farnesyl pyrophosphate synthase* (*Fpps*), being more highly expressed in wet season males. This enzyme forms part of the mevalonate pathway and catalyses the synthesis of Farnesyl Diphosphate, which in insects is a precursor of JH (Bellés *et al.* 2005). JH plays important and well established roles in female insect reproduction (Klowden 2007), but its role in male reproduction is poorly understood. JH is probably involved in inducing protein synthesis in male accessory glands and potentially in mating behaviour (Wilson *et al.* 2003). Our results suggest that JH signalling is higher in wet season males, supporting a role for this pathway in adult male reproduction. An additional product of the mevalonate pathway are pheromones, potentially linking Fpps expression with pheromone synthesis (Bellés *et al.* 2005), and also supporting a role for Fpps in male reproductive investment.

The final season-biased gene, only affected in males, was most similar to blue-sensitive visual pigment (in the butterfly *Dryas iulia*) and to Rhodopsin 5 in *D. melanogaster*. This sequence had already been annotated for *B. anynana* as blue-sensitive visual pigment

(Genbank AAY16527.1) in a study on the evolution of butterfly eye pigments (Sison-Mangus *et al.* 2006). Insect visual pigments are G-protein coupled photoreceptors with peak absorbance at a particular range of wavelengths, and are expressed in photoreceptor cells in the eye (Briscoe & Chittka 2001). It is therefore puzzling to observe expression at all in our abdomen samples. Intuitively, the simplest explanation for this could be contamination of one or more abdomen samples with fragments of head tissue. However, this is unlikely to be the case. Previous gene expression work in *B. anynana* including head, thorax and abdomen samples (Chapter 4) showed that tissue-specificity strongly dominates overall expression variation, accounting for > 50% of variance. This was confirmed in a transcriptome-wide study in *B. anynana* using RNA seq on abdomen and thorax samples, where tissue-specificity contributed to > 21% of all expression variation (V. Oostra, C. Wheat, M. Saastamoinen and B. Zwaan, unpubl. data). In *D. melanogaster*, different body parts also show distinct expression profiles (e.g. Girardot *et al.* 2006). Any contamination with RNA from a different tissue would thus have left a profound mark on the expression profile, but we found no evidence in the PCA for any outlier sample with a markedly different expression profile. In addition, the expression difference between dry and wet season males was not driven by one or a few outliers, but by an average increase across the majority of replicates, lending further support that our findings are not a sampling artefact. In *D. melanogaster*, Rhodopsin 5 is highly expressed in head, as expected, but also shows some expression, albeit low, in adult hindgut, fat body, heart and spermatheca (Robinson *et al.* 2013) as well as in tested and larval imaginal discs (Contrino *et al.* 2012). In *B. anynana*, most clones from which the blue-sensitive visual pigment transcript was assembled originated from head RNA (e.g. Genbank GE680994), but some clones originated from developing wings in larvae and pupae (e.g. Genbank GE725280). Thus, both in *D. melanogaster* and *B. anynana*, Rhodopsin 5 is also expressed in tissues other than head or eye, although its biological function in those tissues is unknown. A recent study in *B. anynana* showed that in adult heads, expression of blue-sensitive visual pigment is season-biased (Everett *et al.* 2012). As we observed in the present study, adults reared in wet season conditions express more blue-sensitive visual pigment mRNA than those reared in dry season conditions. However, this effect was restricted to females, whereas in our study only males were affected by seasonal conditions.

Seasonal differences in transcriptional response to ageing

The majority of the ageing-related expression changes was not limited to one of the seasonal morphs. In both sexes, the percentage of ageing-related genes that were morph-specific summed to *ca.* 35% (Fig 4a, Fig 5a). However, the morph-specific gene sets were markedly different. Both in females and males, the fraction of genes differentially expressed with age was substantially higher in adults reared in warm, wet season conditions (*ca.* 29%) compared to those reared in cool, dry season conditions (*ca.* 7%). Wet season females specifically up-regulated immune response genes, and down-regulated genes coding for chorion proteins. In males, the percentage of wet-season specific genes among all down-regulated genes was 42% (compared to only 3% for dry season-specific genes). Several

of these genes are related to reproduction (e.g. genes related to Ecdysteroid and Juvenile Hormone signalling) as well as to DNA metabolism and carbohydrate metabolic processes. The imbalance between the seasonal morphs in age-related expression changes was smaller among the up-regulated genes, with only 18% of all up-regulated genes being specific to the wet season-reared males, and no particular category of genes dominating. These results indicate that for both sexes, part of the normal transcriptional response to ageing is abrogated in adults reared in dry season conditions. In males, where this reduction is most pronounced, it is accompanied by increased lifespan (Fig 1b). The genes underlying this response can thus be interpreted as candidate markers of longevity, or healthy ageing (*cf.* Doroszuk *et al.* 2012; Pletcher *et al.* 2005). We expect these genes to be effector genes responsible for the differences in adult life history phenotype, and to be downstream of the regulatory environment-sensitive pathways active during development. A natural long-lived *D. melanogaster* strain showed a much weaker transcriptional response to ageing compared to the control line. In particular, expression of reproduction genes did not show the normal decline with age observed in control flies, while expression of stress-related genes did not show the normal increase with age (Doroszuk *et al.* 2012). Similar results were obtained for flies under dietary restriction, which lacked the normal ageing-related down-regulation of reproduction genes observed in individuals given a richer diet (Pletcher *et al.* 2005). Long-lived mutants with reduced Insulin signalling (*chico* heterozygotes) showed a myriad of associated transcriptional differences, the most striking of which was up-regulation of genes related to P450 xenobiotic metabolism (McElwee *et al.* 2007). However, in that study, adults were only analysed at a single time point, making it unclear how reduced Insulin signalling affects transcription at later age.

Transcriptomics in a non-model organism

Studying expression profiles in a non-model species without a sequenced genome presents inherent limitations. First, the GO framework, a hierarchical structure of gene annotations (Ashburner *et al.* 2000), is most suitable for genetic model organisms such as *D. melanogaster*. For these models it is a powerful tool that can leverage findings across many genes to gain a more meaningful biological interpretation. However, this model organism-centred approach makes it less suitable for organisms that are just beginning to be genomically characterised, as the GO framework depends on the ability to assign GO terms to genes. This ability in turn depends on sequence homology between genes of interest and the annotated model organism genes, and thus by definition suffers from a bias towards more conserved genes (Pavey *et al.* 2012). Our attempts at electronic annotation of the *B. anynana* predicted gene set via sequence similarity illustrate these challenges. We were only able to assign a GO term to 27% of all transcripts on the array, of which each annotated transcript was associated on average with 4.0 GO terms. This likely contributed to the relatively small set of enriched GO terms associated with ageing observed in *B. anynana* (see above).

Second, the oligo arrays used in this study were based on an EST database that was derived primarily from developing wing tissue in larvae and pupae, and only to a limited

extent from adult abdominal tissue, in addition to embryonic tissue. It thus seems likely that the abdominal transcriptome, the target of the present study, is incompletely represented on these arrays. Direct sequencing of RNA derived from tissues of interest, enabled by the decreasing cost of nucleotide sequencing, can provide better coverage of the transcriptome. RNA seq, integrating gene discovery, expression profiling and analysis of sequence variation, is emerging as the new tool of choice for evolutionary biologists and ecologists interested in genomics of non-model species (see Ekblom & Galindo 2011; Hornett & Wheat 2012; Stapley *et al.* 2010). In recent years, the transcriptomes of several ecologically relevant butterflies have been characterised using next generation sequencing (e.g. Carter *et al.* 2013; Vera *et al.* 2008). In addition, for two species the genome sequence has now been published (The Heliconius Genome Consortium 2012; Zhan *et al.* 2011). In *B. anynana*, a large RNA seq project has recently been completed, aimed at understanding the response to larval food stress in both seasonal morphs, targeting both adult thoraces and abdomens (V. Oostra, C. Wheat, M. Saastamoinen and B. Zwaan, unpubl. data). Together with the current efforts of the *Bicyclus* community to sequence the *B. anynana* genome (see http://www.bicyclus.org/), this dataset holds great promise for an unprecedented genomic dissection of life history adaptations to seasonal environments.

Conclusions

Probing the transcriptional profile of young and old *Bicyclus anynana* butterflies revealed substantial ageing-related expression changes. Approximately half of all expression changes were sex-specific, with females up-regulating stress response genes and down-regulating reproduction-related genes with age. In adults reared in dry season conditions, which live longer and delay reproduction, age-related expression changes were abrogated compared to the shorter-lived wet season morph. In particular, dry season butterflies lacked the up-regulation of immune genes with age and the down-regulation of reproduction and Ecdysteroid signalling genes that we observed in wet season butterflies. These gene sets are expected to act downstream of environment-sensitive pathways directly involved in the developmental switch. Their observed seasonal differences in ageing-related expression likely contribute to the phenotypic differences in seasonal life history strategies. We also identified a small number of genes whose expression across the adult lifespan was constitutively affected by the seasonal conditions experienced during development, and thus are likely more directly linked to the developmental switch. Several of these seasonally imprinted genes were related to Insulin signalling, a nutrient-sensing pathway involved in life history plasticity in a variety of other animals. We speculate that the evolution of seasonal plasticity in *B. anynana*, an adaptation to specific ecological circumstances, has been accompanied by the co-option of this highly conserved endocrine pathway. This illustrates the versatility of hormonal systems that can be redeployed to play additional roles in different life stages or environments.

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Supplementary tables

Both supplementary tables are in the separate MS Excel file "Chapter 5 – Supplementary Tables xls", which can be found at

https://www.dropbox.com/s/tpjmdsrjxn765dv/Chapter%205%20-%20Supplementary%20Tables.xls

Supplementary Table 1. Annotations for genes differentially expressed between young and old females reared either in dry season conditions (dry season-specific ageing-related genes) or in wet season conditions (wet season-specific ageing-related genes). See Figure 4 in main text.

Supplementary Table 2. Annotations for genes differentially expressed between young and old males reared either in dry season conditions (dry season-specific ageing-related genes) or in wet season conditions (wet season-specific ageing-related genes). See Figure 5 in main text.

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