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Author: Oostra, Vicencio
Title: Hormonal and transcriptional mechanisms underlying developmental plasticity of life histories in a seasonal butterfly
Issue Date: 2013-06-26

Seasonally induced expression variation in life history genes in the butterfly *Bicyclus anynana*

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Abstract

Developmental plasticity – the potential of organisms to time their reproductive schedules and accompanying lifespan to the predicted adult environment – is a key adaptation to predictable environmental heterogeneity. The butterfly *Bicyclus anynana* has evolved developmental plasticity of adult life history strategy as adaptation to its habitat's contrasting seasonal environments. In response to variation in juvenile temperature, larvae develop into either fast-reproducing and short-lived wet season adults, or longer-lived dry season adults that delay reproduction. We analyse transcriptional variation in young adults developed under alternative seasonal conditions by measuring expression of 27 candidate life history-related genes as putative molecular effectors of the two seasonal strategies. Seasonal expression differences were most marked and easily interpretable for genes involved in innate immunity and metabolism, effector genes likely to be tightly linked to observed phenotypes. Immune genes showed a bias towards increased expression in the wet season, reflecting a likely higher immune risk in ▶ ▶

►► the warm wet season, when adults are reproductively active. It may also suggest dry season adults can afford to down-regulate innate immunity, avoiding negative consequences of an overactive immune system. Lipid and carbohydrate metabolic genes were more highly expressed in the dry season, indicating not only increased acquisition and storage, but also increased reliance on previously stored reserves for energy demands compared to the wet season. The developmental environment left a less clear-cut signature on expression of endocrine pathways, although Insulin signalling seems to be higher in the dry season. This study illustrates how molecular phenotypes underlying adaptations to fluctuating environments can be characterised and contributes to a mechanistic understanding of natural variation in life histories.

Introduction

Animals have evolved many ways to deal with the fluctuations in ecological opportunities, such as variation in food availability, that they encounter throughout their life (Piersma & van Gils 2010). One major mechanism underpinning this is phenotypic plasticity, the ability to express a different phenotype in response to environmental variation (Schlichting & Pigliucci 1998). Of key importance is the potential to adjust the life history strategy, including the timing of reproductive schedules and accompanying lifespan, to prevailing or future conditions (Visser *et al.* 2010). Examples include seasonal timing of breeding in birds (Dawson 2008), crickets expressing a long-winged morph specialised for dispersal (Zera 2009), alternative male reproductive strategies in salmon (Aubin Horth & Dodson 2004; Dodson *et al.* 2013) and female reproductive diapause in fruit flies (Schmidt 2011). Also in humans it has been hypothesised that plasticity plays a role in linking the nutritional environment during gestation with adult health and lifespan (Rickard & Lummaa 2007). Understanding genetic mechanisms of adaptation, i.e. how genetic variation maps to fitness in relevant environments, is a major aim for evolutionary biology (Dalziel *et al.* 2009). Considerable progress has been made in using rapidly developing sequencing technology to probe how the environment and the genome interact to produce ecologically relevant phenotypes (Aubin-Horth & Renn 2009). However, the combined life history traits often result in complex phenotypes, which makes it hard to uncover the genetic regulatory mechanisms underlying natural life history variation (Flatt & Heyland 2011).

Detailed genetic studies in the traditional laboratory model organisms have greatly enhanced our understanding of the genetic regulation of life histories, in particular ageing. Perhaps the most important insight that has emerged over the last two decades is that the Insulin signalling pathway is a major regulator of ageing. This nutrient sensing pathway plays a crucial regulatory role in growth and metabolism, and genetic alterations in this pathway in several model organisms produce a range of metabolic phenotypes that affect lifespan and health (Baker & Thummel 2007; Fontana *et al.* 2010; Tatar *et al.* 2003). The hundreds of downstream genes whose expression is affected by these mutations are being characterised (e.g. McElwee *et al.* 2007). Sugar homeostasis and lipid metabolism are key pathways regulated by Insulin signalling, and are strongly associated with health and lifespan in humans (e.g. Heijmans *et al.* 2006). Indeed, studies in invertebrates, in particular in *D. melanogaster*, have uncovered the mechanistic links between nutrient intake, Insulin signalling, carbohydrate and lipid metabolism and lifespan (Baker & Thummel 2007). Innate immunity is another important biological process that has been linked to ageing and variation in lifespan in invertebrate model organisms (Doroszuk *et al.* 2012; Pletcher *et al.* 2005) as well as in humans (Kuningas *et al.* 2009). It has been suggested that the innate immune system may underlie a trade-off between early and late life survival (DeVeale *et al.* 2004), and lifespan variation in response to adult nutrition is correlated with immunity related gene expression (Doroszuk *et al.* 2012; Pletcher *et al.* 2005). Despite this impressive progress, studies in laboratory models have a main limitation: the natural ecology of these models is relatively poorly understood, making the evolutionary relevance of candidate

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life history pathways unclear (Flatt & Heyland 2011). It is therefore crucial to broaden mechanistic studies on the genetic regulation of ageing to include organisms that have a well-studied ecology and thus place discovered mechanisms in an evolutionary context (Partridge & Gems 2006). Testing whether life history pathways and genes discovered in model organisms are also relevant in an ecologically realistic setting as well as in natural populations is an important step towards this goal.

The East African savannah butterfly *Bicyclus anynana* displays extensive plasticity in life history, and has become an important model for bridging the gap between ecological and mechanistic studies of life history (Brakefield & Zwaan 2011). In the field, it expresses a short-lived, fast reproducing morph in the wet season, when resources are abundant. In the food-restricted dry season, adults postpone reproduction and use stored reserves to survive until the start of the wet season (Brakefield & Reitsma 1991). Important aspects of this seasonal life history variation can be induced in the laboratory by exposing animals to the alternative temperatures that in the field are associated with the different seasons. Developmental plasticity is an important though not sole determinant of the adult phenotype (Brakefield *et al.* 2007). Larvae that develop at low temperatures, corresponding to dry season conditions in the field, develop into adults that start their life with more fat reserves. Young, recently eclosed adults that have developed as larvae in warm, wet season conditions allocate relatively more mass to the abdomen and start with a higher rate of egg laying (Brakefield & Zwaan 2011). Differential gene regulation presumably underlies the phenotypic differences between the morphs, given that they both develop from the same genetic background. The genomic tools available for *B. anynana* (e.g. Beldade *et al.* 2006; Beldade *et al.* 2009) have only recently been deployed in the context of life history evolution (de Jong *et al.* 2013; Pijpe *et al.* 2011). Hormonal mechanisms are known to be involved in the developmental induction of the adult phenotypes (see Chapters 2 and 3 of this thesis), but relatively little is known about regulation of adult gene expression, or how that interacts with developmental hormonal pathways.

Here, we use quantitative real-time PCR (qPCR) in the two seasonal forms to analyse adult expression of 27 candidate life history-related genes, as putative molecular effectors of the two seasonal strategies. Expression was measured in adults at very young age, when expression differences as a result of developmental experience are expected to be largest. The targeted genes in candidate life history pathways have been studied extensively in model organisms and have been shown to be important for life history variation. The main focus is on genes involved in innate immunity, reproduction, lipid and carbohydrate metabolism, and hormone signalling. Some of the genes studied have functionally been linked to relevant life history variation while others are known players in relevant pathways. In addition, some of these pathways have obvious connections to phenotypic or ecological differences between the seasonal morphs in *B. anynana*, e.g. genes involved in lipid metabolism. Ecdysteroid signalling plays an important regulatory role during metamorphosis as mediator of seasonal developmental plasticity (see Chapters 2 and 3), but this pathway has not been studied in the adult stage. However, in other insects it has been found that adult Ecdysteroids are involved in the regulation of male and female reproduction (reviewed in Schwedes & Carney 2012)

and have major effects on lifespan (King-Jones & Thummel 2005; Tricoire *et al.* 2009). A more trivial criterion whether to include genes in our study is the availability of genomic data from cDNA sequencing projects (Beldade *et al.* 2006; Beldade *et al.* 2009). A first goal of this study is to characterise the adult seasonal morphs at the molecular level. A second goal is to understand whether genes known to be responsible for life history adaptation in other organisms are also involved in the seasonal adaptation in *B. anynana*. We analyse whether the selected genes are up or down-regulated in young adults differing in their developmental history. The third goal is to assess tissue-specific expression patterns. We sampled and measured expression in head, thorax and abdomen separately. These body parts represent different physiological functions and thus likely differ substantially in their expression patterns.

Materials and methods

1. Experimental design, animal rearing, and sampling

We employed a full factorial design to assess the effects of developmental temperature and sex on gene expression in the three principal body parts (head, thorax and abdomen). This experiment was part of a much larger experiment aimed at understanding responses to seasonal and reproductive conditions throughout adult life, which will be described elsewhere. We therefore combined sampling and rearing efforts for these experiments. Parallel cohorts of larvae were reared at either 19 or 27°C to induce the alternative adult seasonal morphs. Adults were allowed to eclose at their developmental temperature and in this experiment were sampled one day later (following Pijpe *et al.* 2011), collecting head, thorax and abdomen separately for each individual. The experiment was started by collecting eggs from the wild type laboratory stock population (Brakefield *et al.* 2009) on young maize plants (*Zea mays*). Eggs were kept at 23°C until larvae hatched, which were then randomly divided over two high precision environmental climate chambers (Sanyo Versatile Environmental Test Chamber model MLR-351H) representing the dry (19°C) and wet (27°C) season conditions. Photoperiod (12:12 L:D) and relative humidity (70%) were identical for both treatments. All larvae were reared on young maize plants, with 40 larvae per plant and ten plants per experimental temperature (N = 400 per temperature). A total of *ca.* 600 adults eclosed successfully (N = 150 per sex per temperature), of which 48 (N = 12 per sex per developmental temperature) were used in this experiment and the remainder was kept for the larger experiment. After eclosion, adults were kept for 24h at the temperature at which they had developed, and then sampled by flash-freezing in liquid N_2. Using microscissors and small forceps, the head, thorax and abdomen were separated from one another and put into separate tubes (kept in liquid N_2). After sampling, samples were transferred to -80°C were they were kept until RNA isolation. Sampling were always taken at the same time of the day (+/- two hours), in the dark phase of the diurnal cycle.

2. RNA isolation and cDNA synthesis

Total RNA was extracted from 864 samples (288 individuals) divided over nine 96-well plates, using the Nucleospin 96 RNA kit (Machery-Nagel, Germany). These samples belonged to

288 individuals, of which 24 were sampled for the experiment described in this paper (N = 6 per sex per developmental temperature per body part) and the other 264 individuals for a separate experiment (see above). Samples were homogenized in 350 μl RA1 lysis buffer (with 1% v:v β-mercaptoethanol), using glass beads in a 96-well plate TissueLyser II (Qiagen) at 25 Hz for 2 x 2.5 minutes. A filtering step was included prior to binding of homogenate to the silica membrane, and RNA was incubated on column with DNase for 15 min to digest genomic DNA. Each RNA sample was eluted with 100 μl H_2O and concentration and purity were measured spectrophotometrically using a ND1000 NanoDrop. RNA quality was assessed by visually inspecting fragment size distribution of each sample run on a 1.1% agarose gel, and stored until further processing at -80°C. Abdomens yielded on average more RNA (320 ng / μl) than thorax (141 ng / μl) or head (80 ng / μl) samples. Degraded samples, samples with a concentration < 40 ng / μl and samples with an absorbance at 260 nm: absorbance at 280 nm < 1.8 were excluded from subsequent analysis. Prior to cDNA synthesis, one standard sample was prepared by combining aliquots from 22 samples from all body parts, both sexes and developmental temperatures, and diluting to 200 ng / μl. From this sample, a dilution series was prepared of 0.32, 1.6, 8, 40 and 200 ng/μl. All biological RNA samples were diluted to 40 ng / μl. cDNA synthesis was performed at ServiceXS (Leiden, The Netherlands) using the DyNAmo cDNA synthesis kit (Finnzymes / Thermo Scientific Molecular Biology) according to manufacturer's recommendations. Random hexamers were used as primers, and reactions were performed in a final volume of 20 μl with 5 μl RNA sample. For one reaction per plate, H_2O was used instead of RNA, serving as a negative RT control. Thermal conditions consisted of 10 min primer extension at 25°C, followed by cDNA synthesis for 30 min at 37°C and reaction termination for 5 min at 85°C. The resulting cDNA samples were diluted 2.5 fold.

3. Candidate gene selection, primer design and qPCRs

We compiled a list of genes of interest from the candidate life history pathways (see Introduction), including reference genes. Choice of putative reference genes was based on comparable studies in insects on gene expression in relation to life history (e.g. Corona *et al.* 2005; Lourenco *et al.* 2008; Ponton *et al.* 2011) and on reference genes used previously in this species (Pijpe *et al.* 2011; V. Oostra, unpubl. data). Coding sequences for all genes were obtained by screening a database of *ca.* 100,000 *B. anynana* expressed sequence tags (ESTs; Beldade *et al.* 2009). For each gene, we compiled a list of protein sequences from other insects for which annotated sequence information was available for that gene (e.g. *Bombyx mori*, *Drosophila melanogaster*, *Apis mellifera* or *Tribolium castaneum*). This list was used as query in an NCBI's tblastn search with standard parameters against the *B. anynana* ESTs database, performed locally using blastall (Altschul *et al.* 1990). Each putative *B. anynana* gene obtained in this fashion was subsequently used as query in a blastx search against NCBI's non-redundant (nr) protein database (http://blast.ncbi.nlm.nih.gov/), using standard parameters, in order to confirm correct annotation. From this curated list of candidate genes, 40 life history related genes and eight reference genes were chosen. We used the Biomark 96.96 Dynamic Array (Fluidigm, USA) in combination

with custom Taqman Expression Assays (Applied Biosystems, USA) to measure gene expression (protocol detailed below). This platform allows measuring on one array (plate) up to 96 cDNA samples, each for 96 different genes simultaneously (or 48 in duplicate, or 32 in triplicate). Prior to running the nine experimental Biomark Arrays (see below), we performed a pilot with one array to test primers and probes for these genes as well as to assess expression stability of the putative reference genes (to pick the most appropriate reference genes). On a single 96.96 Biomark Dynamic Array (Fluidigm, USA) expression of 48 genes was measured in duplicate for a subset of 96 biological samples. Based on this pilot, we chose 28 genes of interest with the best amplification curves, as judged by the shape of the curve and the signal level reached. Of the eight tested putative reference genes, we chose the four that showed the most stable expression across all, very disparate, experimental conditions. Details of these 32 genes are presented in Table 1 and Table S1.

We used nine experimental Biomark 96.96 Arrays, each with 96 samples, to measure gene expression of 32 genes (in triplicate) for the full experiment. To prevent amplification of any genomic DNA left in the samples, primers and probe were designed to amplify coding sequence that spanned an exon-exon boundary. Intron positions were inferred from genomic sequence of corresponding *B. mori* genes, at the time of the study the only other Lepidopteran with a sequenced genome (Xia *et al.* 2004). See Table S1 for primer and probe sequences of all 48 genes. Prior to PCR, a specific target amplification consisting of 14 cycles was performed on each cDNA sample, using the Taqman PreAmp Master Mix (Applied Biosystems, USA) and a cocktail of primer/probe mixes for all 32 genes. Subsequently, samples were diluted 5-fold and amplified on nine different 96.96 BioMark Arrays (Fluidigm, USA). Of the 864 original RNA samples, 792 cDNA samples belonging to 264 individuals were used in the qPCRs. Body parts were distributed over separate arrays, with three arrays for the abdomen samples, three for the thoraces, and three for the heads. This resulted in 88 biological samples on each array. The remaining eight positions were allocated to the dilution series, the negative RT control and a no template control using H_2O as a template. On all nine arrays, the same 32 genes were measured, each in triplicate, making pair-wise combinations with each cDNA sample. PCRs were performed according to standard Taqman PCR protocol, with 35 cycles and an annealing temperature of 60°C, and data was collected at the end of each cycle. Both cDNA synthesis and qPCRs (except primer design) were performed at ServiceXS (Leiden, The Netherlands).

4. Data pre-processing and normalisation

Data were acquired using the BioMark Real-Time PCR Analysis software (v2.1.1). The quality threshold for the amplification curves was set at the default value. In qPCR data analysis, the C_t value is the metric of expression. This value indicates at which amplification cycle the signal threshold, as a measure for amplicon abundance, reaches a pre-defined threshold. Thus, a low C_t value indicates an early crossing of this threshold, caused by high initial abundance of the cDNA template as a result of high expression. C_t values were obtained setting the signal threshold at automatic, allowing for manual threshold adjustment per gene. For each gene the threshold was kept constant.

Table 1. Candidate *B. anynana* life history and reference genes. Primer and probe sequences as well as additional 16 genes evaluated in the pilot are given in Table S1.

Gene abbreviation	Full gene name	Biological process	Gene type	EST contig ID
AGBE	1,4-Alpha-Glucan Branching Enzyme	carbohydrate metabolism	Life history gene	C5600
GlyP	Glycogen phosphorylase	carbohydrate metabolism	Life history gene	S6487
Pepck	Phosphoenolpyruvate carboxykinase	carbohydrate metabolism	Life history gene	C7079
EcR	Ecdysone Receptor	ecdysteroid signalling	Life history gene	P1
Hr46	Hormone receptor-like in 46	ecdysteroid signalling	Life history gene	C5241
Att	Attacin	innate immunity	Life history gene	C7762
BGRP	beta 1,3-glucan recognition protein	innate immunity	Life history gene	C1792
Cec	Cecropin	innate immunity	Life history gene	C6939
Glov	Gloverin	innate immunity	Life history gene	C7882
Pgrp-1	peptidoglycan recognition protein 1	innate immunity	Life history gene	C2529
Spz	spatzle	innate immunity	Life history gene	C2954
TLR-2	Toll-like receptor 2	innate immunity	Life history gene	S8409
Ilp-1	Insulin-like peptide 1	insulin signalling	Life history gene	C7575
Ilp-3	Insulin-like peptide 3	insulin signalling	Life history gene	C8175
Pi3k21B	Pi3 kinase 21B	insulin signalling	Life history gene	S2613
Pk61c	Protein kinase 61C	insulin signalling	Life history gene	S796
ApoD 1	Apolipoprotein D 1	lipid metabolism	Life history gene	C2737
ApoD 2	Apolipoprotein D 2	lipid metabolism	Life history gene	C850
ApoLp III	insect Apolipophorin III	lipid metabolism	Life history gene	C7929
ApoLp I-II	insect Apolipophorin I and II	lipid metabolism	Life history gene	C7601
Desat	Desaturase	lipid metabolism	Life history gene	C7463
Fatp	Fatty acid (long chain) transport protein	lipid metabolism	Life history gene	S4364
Lcfacl	Long-chain-fatty-acid--CoA ligase	lipid metabolism	Life history gene	C3392
Lip	Lipase	lipid metabolism	Life history gene	C2218
Lpin	Lipin	lipid metabolism	Life history gene	S1885
Vg	Vitellogenin	reproduction	Life history gene	C7110
VgR	Vitellogenin receptor	reproduction	Life history gene	S7915
Eif4e *	Eukaryotic initiation factor 4E	translation	Life history gene*	C3876
Ef1a48D *	Elongation factor 1 alpha 48D	translation	Reference gene*	C3199
RpL32	Ribosomal protein L32	translation	Reference gene	C2683
RpS18	Ribosomal protein S18	translation	Reference gene	C2277
VhaSFD	Vacuolar H+-ATPase SFD subunit	ATP hydrolosis coupled proton transport	Reference gene	C4173

* Excluded from analysis (see Methods).
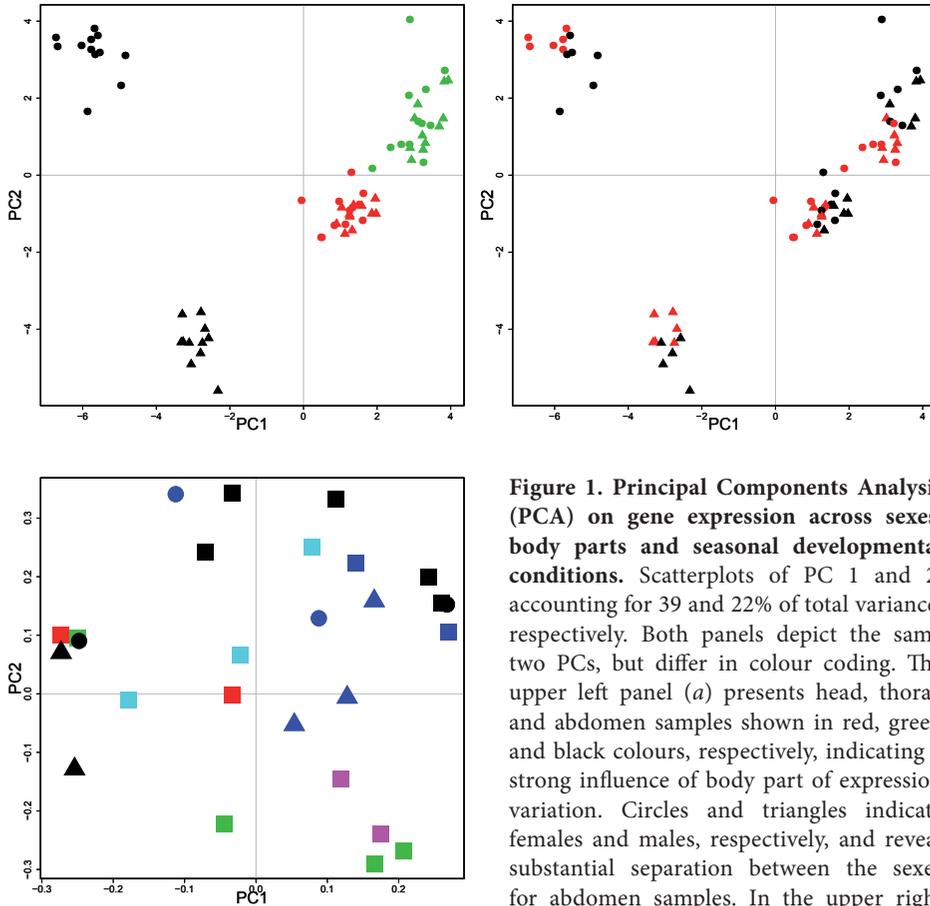
Figure 1. Principal Components Analysis (PCA) on gene expression across sexes, body parts and seasonal developmental conditions. Scatterplots of PC 1 and 2, accounting for 39 and 22% of total variance, respectively. Both panels depict the same two PCs, but differ in colour coding. The upper left panel (*a*) presents head, thorax and abdomen samples shown in red, green and black colours, respectively, indicating a strong influence of body part of expression variation. Circles and triangles indicate females and males, respectively, and reveal substantial separation between the sexes for abdomen samples. In the upper right panel (*b*) again the sexes are again coded by circles and triangles, and black and red colours represent individuals reared at dry or wet season conditions, respectively, showing the effect of season within each body part. In the lower left panel (*c*) loadings of all 27 genes on the first two PCs are plotted, with different colours indicating different biological processes, and different symbols representing an additional subdivision within each biological process. In blue are immune genes, with pathogen recognition proteins, Toll signalling proteins and antimicrobial peptides indicated by squares, circles and triangles, respectively. Reproduction-related genes, carbohydrate metabolic genes, Insulin signalling genes, and Ecdysteroid signalling genes are depicted in magenta, cyan, green, and red, respectively. Lipid metabolic genes are indicated in black, with lipid transport, synthesis and breakdown proteins indicated by squares, circles and triangles, respectively. Exact loadings for each gene along the first three PCs are presented in Table S2.

Including the exact same dilution series of five samples on all nine arrays allowed us to correct for technical variation in expression across arrays. The regression of expression (C_t) on the (base 2) logarithm of the dilution factor for the five samples in the dilution series varied both in intercept and slope across the nine arrays. Assuming that this linear relationship should be identical across arrays, as the samples are identical, we used the array-specific deviation from the across-arrays average slope and intercept to correct expression of all biological samples. First, we regressed, for each array separately, C_t on dilution factor for the five samples of the dilution series and calculated array-specific slope and intercept for this regression. Second, we computed averages across the nine arrays for the intercept and slope of the regressions. Third, we subtracted from each individual C_t value of the biological samples, the array-specific intercept and divided by array-specific slope. Finally, we multiplied this by the average slope and added the average intercept to obtain the corrected C_t values. Regressions for the dilution series were now identical, and the biological samples were much more similar across the nine arrays. All these computations were performed for each gene separately.

The four most stable reference genes tested in the single array pilot were used in the nine experimental arrays. To examine whether these genes indeed showed stable expression across all experimental treatments, stability of all 32 genes was evaluated and ranked using the internal control gene stability measure as defined by (Vandesompele *et al.* 2002), implemented in the R / Bioconductor package SLqPCR (Kohl 2007). The three most stably expressed genes included three of the four *a priori* defined reference genes (*Ef1a48D*, *RpL32* and *RpS18*), and these genes were used to normalise expression of all other genes. First, for each sample separately the geometric mean of C_t values for these three genes was computed. Then, for the same sample this normalisation factor was subtracted from each C_t value of the other genes (Vandesompele *et al.* 2002). Normalisation was done for each sample separately. These normalised C_t values were used as expression values without additional normalisation to a reference sample. Prior to normalisation, the fourth and least stable of the reference genes (*VhaSFD*) was removed from the analysis. We also removed *Eif4e*, as this gene showed a very stable expression, similar to that of the four *a priori* defined reference genes. Thus, of the original 32 genes measured, three were used as reference gene and two were discarded, leaving 27 genes of interest.

Figure 2 (next three pages). Expression of 27 candidate life history genes as measured by qPCR. Each row depicts expression for a single gene as a function of seasonal developmental condition (DSF: dry season form; WSF: wet season form) for females (solid lines) and males (dotted lines) in head (left), thorax (centre) or abdomen (right). Gene expression on the y axes is presented as inverse C_t values (measured on a ^{2}log scale), with high values indicating high expression and low values low expression. Note the difference in scale for the different graphs. Single asterisks above the lines in each graphs indicate a significant effect of season on gene expression (FDR = 0.10) for both sexes pooled, unless there was a significant sex by season interaction (see Methods). In that case, asterisks are indicated for females and males separately and marked with an apostrophe. For all two-way Anovas (including uncorrected and FDR corrected p values) see Table S3. ▶▶
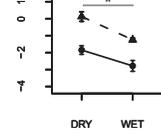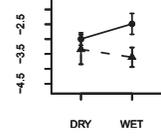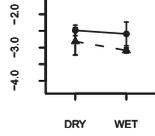
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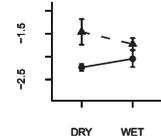| Biological process	Gene	HEAD	THORAX	ABDOMEN
carbohydrate metabolism glycogen synthesis	*AGBE*			
carbohydrate metabolism glycogen breakdown	*GlyP*			
carbohydrate metabolism	*Pepck*			
insulin signalling	*Ilp-1*			
insulin signalling	*Ilp-3*			
insulin signalling	*Pi3k21B*			
insulin signalling	*Pk61c*			
ecdysteroid signalling	*EcR*			
ecdysteroid signalling	*Hr46*			
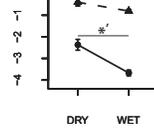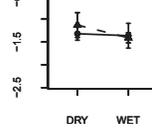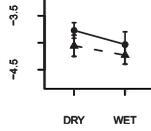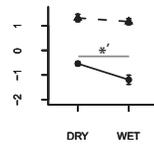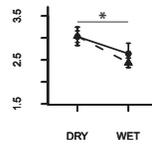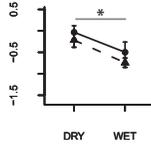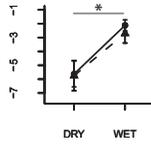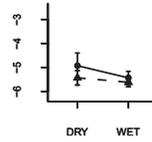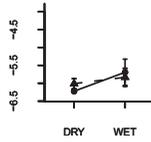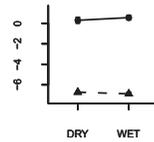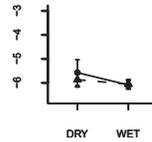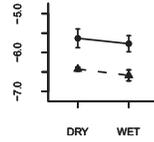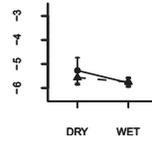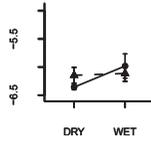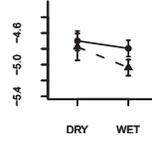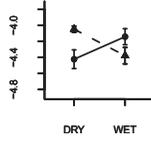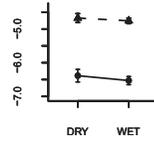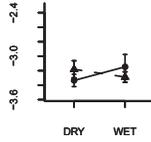
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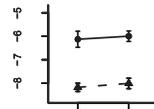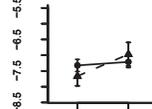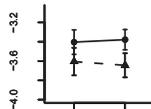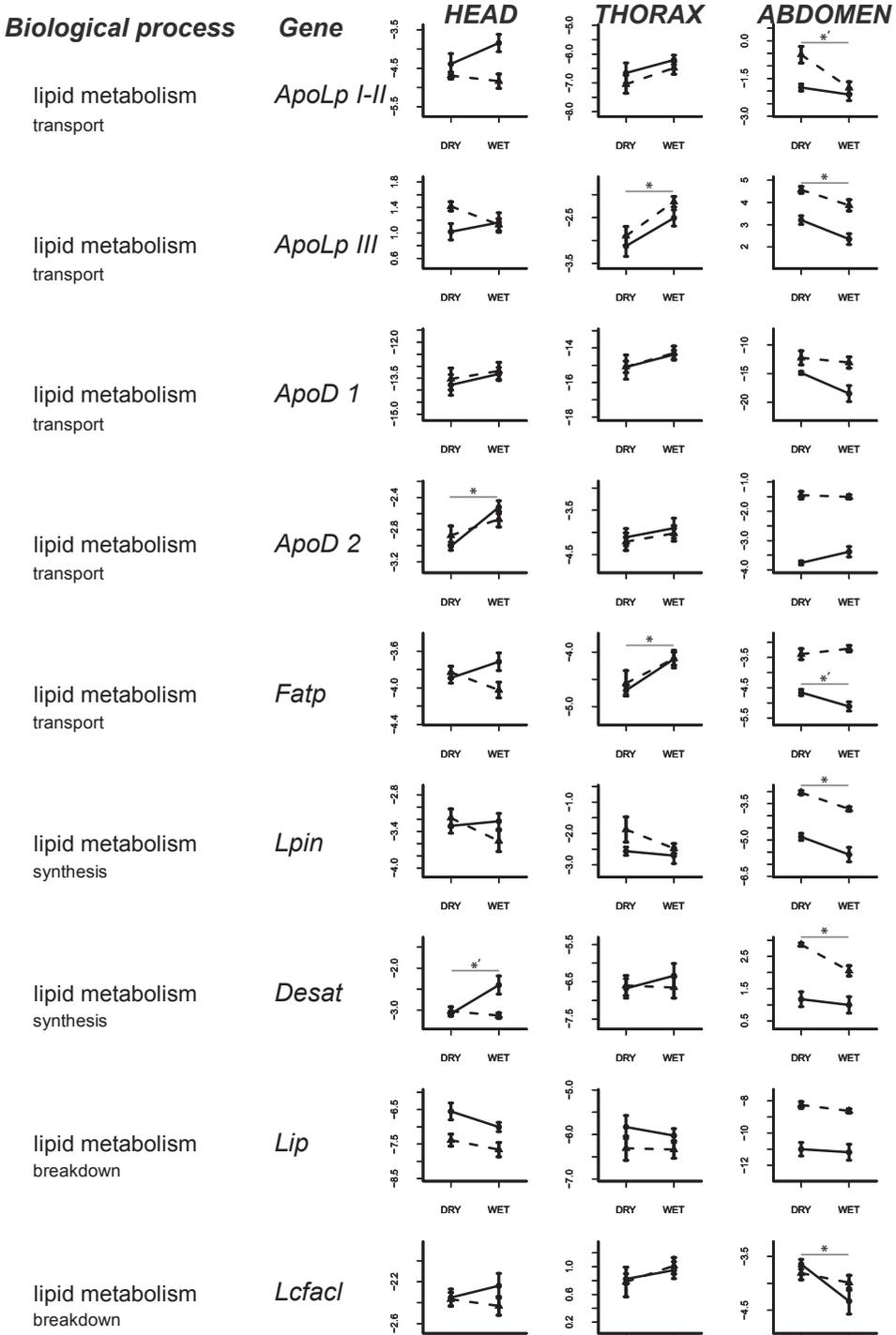
5. *Data analysis*

To gain preliminary insight into the variance structure of the expression data, an unsupervised Principal Components Analysis (PCA) was performed on expression of the 27 life history-related genes. Given the extensive variation associated with body part as observed in the PCA (see Results), gene expression was subsequently analysed separately for the three body parts. For each gene and each body part, a two-way Anova was constructed with sex and seasonal morph as fixed effects (see Table S3 for an overview of all models). For combinations of genes and body parts that showed a significant (p < 0.1) interaction of sex by morph, *t*-tests betwe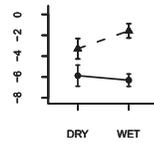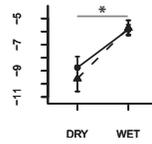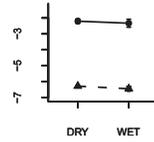en dry and wet season-reared individuals were performed for females and males separately. P values presented in Figure 2 refer to these *t*-tests, after correcting for multiple testing using false discovery rates (FDR; Benjamini & Hochberg 1995). For those cases where the sex by morph interaction was not significant, p values for the seasonal morph term of the two-way Anovas is presented in the figures, also after correction for multiple testing. For the purpose of presentation (Fig. 2), C_t values were multiplied by -1, so that high expression is on the upper part of the y axes and low expression on the lower part. Otherwise, expression metrics were not transformed, and are thus expressed on a ^{2}log scale relative to average expression of the three reference genes. All analyses were performed in R (R Development Core Team 2010).

Results

PCA revealed a clear separation of body parts and sexes, and, to a more limited extent, of seasonal morphs (Fig. 1*a, b*). The first axis, accounting for 39% of total variance, separated the body parts from one another, in particular the abdomens from the two other body parts. Within the abdomen, there was a clear grouping of females and males along this axis. *Ecr*, *Pk61C* and three lipid metabolic genes showed high negative loadings for PC 1, while *PGRP* and *ILP-3* and three other lipid genes had high positive loadings (Fig 1b, Table S2). These genes showed marked sexual dimorphism in expression, but only in the abdomen (Fig 2). The second axis, explaining 22% of total variance, separated male from female abdomens even more strongly than PC1, but again did not separate the sexes in the two other body parts. The genes most strongly associated with this axis were *Vg* and three Insulin signalling genes with negativ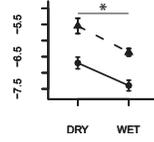e loadings and *Spz* and two lipid transport genes with positive loadings (Fig 1b, Table S2). The former genes were highly expressed in females compared to males, while for the latter males showed markedly higher expression than females (Fig. 2). In all cases except *Vg*, this sexual dimorphism was restricted to the abdomen. Within each body part, there was some separation of the seasonal morphs along both the first and the second axis. Interestingly, the seasonal morphs separate along PC1 for the female abdomen. In contrast, in male abdomen the seasonal morphs show a perpendicular response, grouping along PC2. This indicates that the main transcriptional differences induced by the seasonal environment differ between the sexes, at least in the abdomen. In heads of both sexes, the main separation between the seasonal morphs occurs along PC 1, while in thoraces both PC 1 and 2 separate the morphs. Finally, the third axis, accounting for 15% of total variance,

mainly separated the heads from the two other body parts, although it also separated female from male abdomens to some extent (data not shown). The genes driving this response were *Tlr-2* and two carbohydrate metabolic genes, with high, negative loadings, and *Hr46*, with a high positive loading (Table S2).

Innate immunity

Of the genes involved in innate immunity, two code for non-self recognition proteins (*BGRP* and *PGRP-1*) that are involved in detection of bacterial or fungal pathogens outside the cell. Two code for proteins in the Toll signalling pathway (*Spz* and *TLR-2*), and transduce the signal into the cell via membrane-bound Toll-like receptors. The three final immune genes studied code for antimicrobial peptides (*Att*, *Cec* and *Glov*) that are secreted outside the cell and directly affect bacterial cells (Broderick *et al.* 2009). Several immune markers showed significantly increased expression in adults of both sexes reared in wet season conditions. *Pgrp-1* was upregulated in head and thorax, *TLR-2*, in thorax, and *Glov* in all body parts. *Att* and *Cec* also seemed to show some induction in the wet season in head and thorax, but this was not significant (p = 0.12-32). On the other hand, in the abdomen two genes showed the reverse pattern. Expression was decreased in the wet season for *BGRP* in both sexes and for *Spz* in males, but not females (Fig. 2). Thus, in head and / or thorax, expression of five immune genes was higher in the wet season. In the abdomen, this was only the case for Glov. Two other genes showed highest abdominal expression in the dry season. Interestingly, for both these genes male expression was much higher.

Female reproduction

The two female reproduction-related genes studied here, *Vg* and *VgR*, are both involved in vitellogenesis, the uptake of nutrients into the oocytes. *Vg* codes for the yolk protein Vitellogenin that provides the major source of nutrients for the oocyte. During vitellogenesis, Vg proteins are transported into the oocyte via endocytosis mediated by the Vitellogenin Receptor, encoded by *VgR* and expressed in oocytes (Klowden 2007; Tufail & Takeda 2009). In *B. anynana*, whole-body expression of Vg proteins has previously been found not to differ among females kept at the two seasonal temperatures as adult, even though egg size and laying rate are different (Geister *et al.* 2008). In the honey bee *Apis mellifera*, *Vg* is associated with plasticity of lifespan between workers and queens (Corona *et al.* 2007; Munch & Amdam 2010). Here, *Vg* expression in males was either at very low levels (in abdomen) or absent (in head and thorax), and in all cases expression was much lower than in females (Fig. 2). Wet season-reared females showed higher *Vg* expression than dry season-reared females in head, while the abdomen showed no such difference. Thorax *Vg* expression was also higher in the wet season, but this effect was not significant (p = 0.09). Irrespective of developmental conditions, females showed much higher expression in abdomen compared to head or thorax. *VgR* was not expressed above background levels in males. In females, expression was highest in the abdomen but there was no evidence for season-biased expression in that body part. In contrast, in female heads, *VgR* expression was significantly higher in the wet season.

Carbohydrate metabolism

In addition to lipids, insects store energy reserves as glycogen. Although glycogen has a lower energy content than lipids per unit mass, it is more readily broken down when needed (Arrese & Soulages 2011). *GlyP* codes for an important enzyme that catalyses this process, freeing stored energy and making it available for processes such as flight. The reverse process, the conversion of free circulating trehalose to stored glycogen, or glycogenesis, is catalysed by the enzyme encoded by *AGBE* (Arrese & Soulages 2011; Gäde & Auerswald 2003). In *B. anynana*, *GlyP* expression was highest in thorax compared to the other body parts (Fig. 2). In all body parts, individuals developed in dry season conditions expressed *GlyP* at significantly higher levels than in the wet season, although in abdomens this was only the case for females. In contrast, *AGBE* did not show a significant imprint of developmental conditions, except in female abdomens where expression was higher in the dry season. Interestingly, this dry season-biased induction was much stronger than for *GlyP*, suggesting that in the female abdomens, the balance between glycogen storage (*AGBE* expression) and breakdown (*GlyP* expression) shifts more towards storage in the dry season. The third carbohydrate metabolic gene measured was *Pepck*. In mammals, this gene codes for a key enzyme involved in gluconeogenesis, the production of glucose from non-carbohydrate carbon substrates such as lactate, glycerol, and glucogenic amino acids. This enzyme controls the rate of gluconeogenesis, and its expression, induced by fasting and repressed by dietary carbohydrates, is an important regulator of blood glucose levels (Croniger *et al.* 2002a; Croniger *et al.* 2002b). We observed a strong induction of *Pepck* expression in females and males developed under wet season conditions compared to those of the dry season (Fig. 2). In abdomen, no such evidence for season-biased expression was found. Furthermore, females showed much lower *Pepck* expression in their abdomen than males, while no such difference was found for other body parts.

Hormone signalling

We measured the expression of four genes in the Insulin signalling pathway for which sequence data in *B. anynana* was available: transcripts coding for two Insulin-like peptides (Ilps), and for two kinases (Pi3k21B and Pk61c, also known as Pdk1) involved in the intracellular phosphorylation cascade that starts with Ilp binding to the Insulin receptor (InR) and ends with phosphorylation, cytoplasmatic localisation and inactivation of FoxO (Broughton & Partridge 2009; Edgar 2006). Of these four genes, only one was differentially expressed in response to seasonal developmental conditions. In both male and female abdomens, *Pk61C* showed increased expression in the dry season, indicating high Insulin signalling and low FoxO activity. The other hormone pathway probed in this study was Ecdysteroid signalling. Neither *EcR*, coding for the nuclear hormone receptor and transcription factor Ecdysone Receptor, nor *Hr46*, coding for a different nuclear hormone receptor and transcription factor downstream of EcR (Riddiford & Truman 1993; Swevers & Iatrou 2003), showed any seasonal expression bias (Fig 2). In abdomens, *EcR* was expressed at substantially higher levels in males compared to females, while for *Hr46* the reverse was the case.

Lipid metabolism

We measured season-related expression of nine genes whose products are involved in lipid metabolism, covering three main aspects: a) Lipid transport genes included genes involved in lipid transport from the gut to the fat body for storage and from the fat body to the rest of the soma for catabolism (*ApoLp I-II*, *ApoLp III*, *ApoD 1*, *ApoD 2*), and a gene involved in dietary uptake of fatty acids in the gut as well as release into target tissues such as flight muscle or brain (*Fatp*). b) Lipid synthesis genes included *Lpin* (triglyceride synthesis) and *Desat* (fatty acid synthesis), both involved in storage of dietary lipids. c) Lipid breakdown genes included *Lipase*, coding for an enzyme breaking down triglyceride, and *Lcfacl*, coding for an enzyme involved in the activation of fatty acids prior to beta oxidation (Canavoso *et al.* 2001).

For six of these genes (*ApoLp I-II*, *ApoLp III*, *Fatp*, *Lpin*, *Desat*, and *Lcfacl*), abdominal expression was highest in adults developed in dry season conditions, although for *Fatp* this was only the case in females and for *ApoLp I-II* only in males (Fig. 2). In contrast, for four genes this pattern was reversed, either in thorax (*ApoLp III*, *Fatp*) or head (*ApoD 2*, *Desat*), with highest expression in wet season adults. While overall expression in abdomen was an order of magnitude higher than in thorax, there is an interesting shift in the balance between *ApoLp III* expression in thorax and abdomen towards lower abdominal and higher thoracic expression in the wet season. For *Fatp*, this shift is also observed, although here it is restricted to females. Overall, expression was highest in abdomen compared to the other body parts. This was particularly pronounced for *Desat* and several lipid transport genes. In contrast, the two genes involved in lipid breakdown showed the highest expression in thorax.

Discussion

The expression patterns observed in this study provide an important molecular characterisation of seasonal developmental plasticity in *B. anynana*. We used a very similar design to previous studies in this species that were aimed at analysing reaction norms at the phenotypic level (e.g. de Jong *et al.* 2010; Pijpe *et al.* 2007; Chapters 2 and 3). As gene expression can be considered an intermediary phenotype, the obtained 'genomic reaction norms' (*sensu* Aubin-Horth & Renn 2009) measured in this study can be viewed as a much more detailed characterisation of life history plasticity than possible at phenotypic level. Several methods have proven very powerful in characterising phenotypic variation at the molecular level, such as metabolomics or enzyme activity of pathways of interest. One particularly insightful approach has been the direct measurement of flux through lipid metabolic pathways, in the context of life history trade-offs among dispersal morphs of the cricket *Gryllus firmus* (Zera & Harshman 2011). Here we measured gene expression at the mRNA level as the molecular phenotype of choice. It is a relatively easy way to characterise phenotypes in fine detail, and to test specific hypotheses regarding molecular genetic mechanisms putatively involved in plasticity. Provided that sequence data is available, the same methods can be applied to any gene of interest across a variety of pathways.

Reproduction and immunity

The wet season adult female morph, induced in the laboratory by rearing larvae at high temperatures, is characterised by an increased rate of egg laying and a decreased egg size compared to dry season females (Fischer *et al.* 2003). Although the core of reproductive function in insects is located in the abdomen (Klowden 2007), and *Vg* expression was indeed much higher in the abdomen than in the head or thorax, there was no increased abdominal *Vg* or *VgR* expression in wet season relative to dry season females (Fig 2). The effect of having a higher egg production might be counteracted by the smaller size of those eggs, making the overall Vg and VgR requirements more or less equal. An additional explanation for the lack of seasonal bias in abdominal *Vg* expression might stem from the fact that females were sampled at young age (day 2 of adult life). At this age, females are just starting to mate, egg production rate is still very low (see e.g. Fig. 5 in Chapter 3) and virgin females have likely not started any egg production at all. Consistent with this, Vg protein levels are below detection level in freshly eclosed *B. anynana* females (Geister *et al.* 2008). In contrast, we did observe wet season-biased *Vg* expression in head and thorax, in line with the wet season being the core reproductive season. It is possible that the lower overall expression levels in these body parts might allow detection of more modest differences between the developmental temperature treatments compared to the abdomen, where the overall very high expression may swamp more subtle expression variation. Although finding any *Vg* expression at all outside the abdomen might seem surprising, there is fat body tissue in all three body parts (Klowden 2007) and *Vg* expression has been observed previously in head and thorax of the honey *Apis mellifera* (Corona *et al.* 2007). So overall, the expression of these reproduction related genes support the link between development into the wet season form and reproduction at the molecular level.

In *B. anynana*, the general effect of developmental seasonal environment on expression of immunity genes was one of increased immune gene expression in the wet season morph (Fig 2). In thorax (*TLR-2*), head and thorax (*PGRP-1*) or all three body parts (*Glov*) expression was highest in the wet season. Although not statistically significant, the expression in genes coding for the two other Antimicrobial peptides *Att* and *Cec* was also biased towards the wet season. This is consistent with the association that has been observed in other insects between reproductive activity and increased infection risk for the female. Up-regulating immune defences during periods of reproduction may be beneficial to reduce the mating-related immune risk. Remarkably, such up-regulation has been observed in males as well (Lawniczak *et al.* 2007; Siva-Jothy 2009). Furthermore, the warmer temperatures of the wet season might represent an additional immune risk, as microorganisms grow more readily at higher temperatures. Ageing studies in a variety of animals including insects and vertebrates, have shown increased expression of genes involved in innate immunity at old age (Doroszuk *et al.* 2012; Pletcher *et al.* 2005; Sarup *et al.* 2011). In long-lived animals this up-regulation is often abrogated, suggesting that an (over)active immune system can be detrimental for lifespan, a hypothesis known as inflammageing (Franceschi *et al.* 2007). If the dry season represents a situation of reduced immune risk, both due to reduced mating

frequency and a lower temperature, down-regulating innate immunity might allow adults to reach an older age to survive the six month long dry season.

Not all immune genes showed increased expression in the wet season. *BGRP* and *Spz* were down-regulated in the wet season compared to the dry season, although this was only the case in the abdomen. This would support a negative trade-off between immune activity and reproductive investment, which has often been observed, and is proposed to be driven by a competitive shift in resource allocation or by pleiotropic effects of reproductive hormones on the immune system (Harshman & Zera 2007).

The individuals measured in this study were young, virgin, and kept under non-infectious conditions. Expression differences between the seasonal morphs were thus solely due to temperature variation experienced during development. As reproductive activity decreases with age, it would be interesting to see if the effect of seasonal conditions on expression of immune genes alters with age. In addition, allowing adults to reproduce and comparing them with virgins of the same age might reveal which immune genes associate with reproductive status (*cf*. McGraw *et al.* 2004).

Hormonal regulation

The seasonal strategies in *B. anynana* involve a suite of ecologically relevant traits closely related to reproduction and lifespan. Hormones acting during pupal development, in particular Ecdysteroids, have been shown to be a crucial regulatory link between the inducing larval environment and the development of these alternative phenotypes (see Chapters 2 and 3). Nevertheless, it is unknown whether they are also associated with seasonal plasticity in the adult stage, although this would seem likely given their role in regulating adult reproduction in insects (Schwedes & Carney 2012). In terms of mRNA expression, we found no indication that Ecdysteroid signalling genes in young adults are associated with the seasonal morphs (Fig 2). This would suggest that this hormonal pathway plays a role in the environmental induction of the adult morphs during development, but not in their maintenance in the adult stage. One important caveat is that we only measured expression for two genes in this pathway, leaving open the possibility that regulation happens at other points in the pathway. EcR and only a few other proteins are the key transducers of the Ecdysteroid signal, and *ca.* 26 other proteins are directly involved in initiating the large transcriptional cascade that characterises the response to Ecdysteroids (Gauhar *et al.* 2009). Furthermore, there are a number of key enzymes involved in Ecdysteroid synthesis that may also be subject to environmental regulation (Huang *et al.* 2008). Finally, no direct measures of Ecdysteroid titres were taken in these adults.

The other major hormonal pathway probed here was the Insulin signalling pathway. Mutations in genes of this pathway have large and pleiotropic effects on lifespan and reproduction across a range of organisms (Fontana *et al.* 2010; Tatar *et al.* 2003). In addition, this pathway has been shown to regulate phenotypic plasticity in a range of other insects (e.g. in honey bees; Corona *et al.* 2007). In *B. anynana* adults, one gene seemed to strongly associate with seasonal morph. In both males and females, abdominal *Pk61C* expression was higher in the dry season. This is indicative of low FoxO and high Insulin activity, which

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is contrary to expectations, as high Insulin signalling is generally associated with increased reproduction and short lifespan (Tatar *et al.* 2003). Our findings for *Pepck*, which showed substantial up-regulation in wet season adults, also point to higher Insulin signalling the dry season. *Pepck*, which we measured in the context of carbohydrate metabolism, is not a player in the Insulin signalling pathway, but it is a direct transcriptional FoxO target. Its expression is tightly linked to Insulin signalling and has in fact been used as a read-out for activity of this pathway, with high *Pepck* levels indicating low Insulin signalling and high FoxO localisation in the nucleus (Mattila *et al.* 2008). In other body parts and for other Insulin genes no significant effect of seasonal morph was found, but slightly lowering the False Discovery Rate (FDR = 0.10 – 0.17) reveals a slightly more complex picture. In the head, males showed a dry season-biased *Pk61C* expression similar to the abdomen but for females the response was reversed. In addition, for *Ilp-1*, *Ilp-2* and *Pi3k21B* expression was also wet season-biased. Despite these more subtle effects, the main finding seems to be that in young adults, Insulin signalling is higher when they have been reared in the dry season. This unexpected result may be a (non-adaptive) imprint from the larval stage, when individuals need to sustain a higher metabolic rate in order to complete growth in the cooler dry season (see Chapter 2). If this is true, it would be predicted that these effects of developmental conditions on Insulin signalling will be reversed by the direct action of adult temperature conditions, as is the case for metabolic rate (Pijpe *et al.* 2007). The same caveat as for Ecdysteroid signalling applies here: we could only measure four genes of a large and complex pathway. Furthermore, these measurements were taken at a single time point at the start of adult life. Once reproduction starts, the patterns may become clearer.

Lipid and carbohydrate metabolism

For *B. anynana*, the dry season in the field is characterised by limited nutrient availability. Larvae that develop at the end of the progressively deteriorating wet season accumulate more lipids and eclose as adults with more stored lipid reserves (Brakefield & Reitsma 1991). Previous studies in the laboratory found abdominal lipid content to differ between seasonal morphs (e.g. Chapter 2), but total fat mass is a relatively crude measure of several aspects of lipid metabolism that may be differently regulated. Measuring expression of nine genes involved in lipid synthesis, transport and degradation reveals a more detailed picture. In the abdomen, where fat content is highest (see Chapter 2), expression of lipid metabolic genes was usually higher than in the other body parts. The exception were two genes involved in lipid breakdown, which were more highly expressed in the thorax (Fig. 2). In the abdomen, six of the nine genes measured showed increased expression in dry season adults. Some of these genes are involved in lipid transport (*ApoLp I-II, ApoLp III, Fatp*), some in lipid synthesis from non-lipid nutrients (*Lpin*, *Desat*), and one in lipid breakdown (*Lcfacl*). This indicates an increased overall lipid turnover in the dry season, in both the income and the expenditure side, as well as in various aspects of transport. This pattern is also observed for two carbohydrate metabolic genes, which were both up-regulated in dry season adults (Fig. 2). *AGBE* is involved in converting circulating sugars to stored glycogen, contributing to survival under starvation. *GlyP* catalyses the reverse process of freeing sugars from

reserves (Arrese & Soulages 2011; Gäde & Auerswald 2003). It has been suggested that glycogen storage and metabolism might be particularly important in the dry season (de Jong *et al.* 2013). It is known from other insects that flight performance decreases at lower temperatures (Lehmann 1999; Niitepold 2010). To still be able to sustain flight in the cooler dry season, animals that need to remain active may place increased demands on glycogen, a critical source of flight fuel (de Jong *et al.* 2013). Our results for *AGBE* and *GlyP*, both up-regulated in the dry season, support this hypothesis.

Together, our results for lipid and carbohydrate metabolism suggests that in the dry season adults not only store more of their incoming adult food as reserves, but also rely more on previously stored reserves for their energy demands than in the wet season. Expression differences observed in these young adults are a reflection of larval allocation decisions, which likely differ from those during the adult stage (Boggs 2009). It has been shown previously in *B. anynana* that relative lipid content increases during adult life, mainly due to use of non-lipid resources for survival (Zwaan *et al.* 2001), but this has only been studied for the wet season morph. Examining expression of lipid metabolic genes at older age could reveal whether the initial differences in lipid metabolism between the seasonal morphs continue to differ during adult life. If so, this might be due to differential acquisition from adult food, or due to differential expenditure of acquired resources.

One lipid metabolic gene measured in *B. anynana* has interesting associations with life history phenotypes in other species. In humans, *ApoD* expression is elevated in a number of neurological pathologies, including Alzheimer's disease and stroke, presumably as a consequence of oxidative damage associated with those diseases (Muffat *et al.* 2008). Over-expression of this human gene in *D. melanogaster* increases resistance against oxidative stress, and the fly ortholog of this gene, *Glial Lazarillo* (*Glaz*), also protects against oxidative stress. In *B. anynana*, *ApoD 2* showed increased expression in heads of adults reared in wet season conditions. This suggests that in the wet season, when adults are more active and have higher metabolic rates due to the higher temperatures (Pijpe *et al.* 2007), they increase protection against oxidative damage in the brain.

Conclusions

Measuring expression reaction norms for 27 life history genes in the seasonally plastic butterfly *B. anynana* revealed sexual dimorphism in abdominal gene expression as a major driver of overall expression variation, mainly due to sex-specificity of genes involved in lipid metabolism and hormone signalling. In addition, adult expression for several interesting pathways showed evidence for a signature of seasonal conditions experienced during development. Immune genes showed a general bias towards increased expression in the wet season. This likely reflects the relatively higher immune risk in the warm wet season, when adults are reproductively active. It may also suggest that in the dry season, adults can afford to down-regulate innate immunity, avoiding some of the negative consequences on lifespan of an overactive immune system, and contributing to their longer lifespan in the field. Lipid and carbohydrate metabolic genes were generally more highly expressed in

the dry season, indicating not only increased acquisition and storage, but also increased reliance on previously stored reserves for energy demands compared to the wet season. We hypothesise that this developmental signature on expression of metabolic genes in young adults reflects an adaptive response to ensure an adequate balance between income and expenditure in each season. In general, seasonal expression differences were most marked and easily interpretable for genes involved in innate immunity and metabolism. These effector genes are more likely to be tightly linked to observed phenotypes at higher levels of organization. Studying these pathways in older adults will reveal whether the observed developmental plasticity can be reversed when adult conditions change. For the targeted regulatory pathways, the seasonal signature was less clear-cut. This might be due to the importance of heterochrony in hormonal pathways (*cf*. Chapter 2). Conclusively implicating a regulatory pathway in environmental responses likely requires sampling a larger number of the involved genes. This study illustrates how molecular phenotypes underlying adaptations to fluctuating environments can be characterised and contributes to a mechanistic understanding of natural variation in life histories.

Acknowledgments

Supplementary tables

All three supplementary tables are in the separate MS Excel file "Chapter 4 – Supplementary Tables xls", which can be found at

https://www.dropbox.com/s/l5s11ma5bm7eyy3/Chapter%204%20-%20Supplementary%20Tables.xls

Table S1. Primer and probe sequences for candidate *B. anynana* life history and reference genes. In addition to the 32 genes presented in Table 1, this table S1 also includes the 16 genes evaluated in the pilot (see Methods).

Table S2. Loadings of 27 life history genes on first three Principal Components. See also Figure 1.

Table S3. Statistical models (two-way Anova) for gene expression as a function of seasonal morph (temperature) and sex. A separate model was fitted for each combination of gene and body part. Each row represents results for one of three terms in the model (sex, seasonal morph, and sex by seasonal morph interaction). FDR correction was applied only for the 'seasonal morph' term across all genes and body parts, and only for cases where the interaction between sex and season was not significant (see Methods).

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