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Hormonal and transcriptional mechanisms underlying developmental plasticity of life histories in a seasonal butterfly

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voor papa en mama

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Hormonal and transcriptional mechanisms underlying developmental plasticity of life histories in a seasonal butterfly

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Hormonal and transcriptional mechanisms underlying developmental plasticity of life histories in a seasonal butterfly

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1 General introduction

Dealing with the unknown

Temporal variation in abiotic and biotic variables such as temperature, rainfall, food availability or predation pressure profoundly affects the abilities of organisms to survive and reproduce successfully. Most organisms are remarkably flexible in the face of such heterogeneity in the quality of their habitat, and have evolved a range of behavioural, physiological or morphological responses (Beldade *et al.* 2011; Piersma & van Gils 2010; West-Eberhard 2003; Whitman & Ananthakrishnan 2009). Such flexibility is can be regarded as adaptive phenotypic plasticity, which can formally be defined as 'the property of a given genotype to produce different phenotypes in response to distinct environmental conditions' (Schlichting & Pigliucci 1998). Some notable examples of phenotypic plasticity are briefly described in Box 1.

When environments are seasonal—and thus predictable—there is scope for anticipating and preparing for changes in the environment before they occur. Seasonal plasticity is a widespread feature of animal life, and includes diverse adaptations including migration, diapause and plumage moult (Denlinger 2002; Piersma & van Gils 2010). Studying how organisms have adapted to these annual cycles of ecological opportunity and threat is a key topic for evolutionary ecology (Visser *et al.* 2010). In addition, understanding how animals cope with environmental challenges is an important requirement for predicting biotic responses to climate change (Hofmann & Todgham 2010; Meylan *et al.* 2012).

A special case of phenotypic plasticity is developmental plasticity, where the phenotypic changes induced by the environment originate during the course of development (Beldade *et al.* 2011). A striking example is environmental sex determination in many reptile species, in which the sex of the developing embryo is determined by the temperature at which the egg is incubated (Sarre *et al.* 2004). Studying developmental plasticity provides a unique window into the developmental processes that translate genotypes into phenotypes, and reveals how environmental modulation of these processes can be a source of phenotypic variation (Gilbert 2012; West-Eberhard 2003).

A powerful concept in studies of plastic responses is the reaction norm (Schlichting & Pigliucci 1998). Here, the phenotypic value for a trait is plotted as a function of environmental variation. Genotypes responding readily to environmental variation have steep reaction norms, whereas those less sensitive to the environment (i.e. more canalised) have flatter reaction norms (Fig. 1*a*). Measuring phenotypic variation for several genotypes across environments can reveal the extent of genetic variation for environmental responses (gene-by-environment interaction), a prerequisite for evolutionary change towards increased or decreased plasticity. If genotypes differ only in the intercepts of their reaction norms and not in the slopes, evolutionary change in plasticity is constrained within that population, at least in the short term (Debat & David 2001; Schlichting & Pigliucci 1998; Stearns 1989). In addition, reaction norms can also be compared among different traits to gain some insight into the extent of integration between these traits (Fig 1*b*). If traits have similarly shaped reaction norms they might also share the underlying developmental mechanisms generating the environment-sensitive responses. Such phenotypic integration can be driven

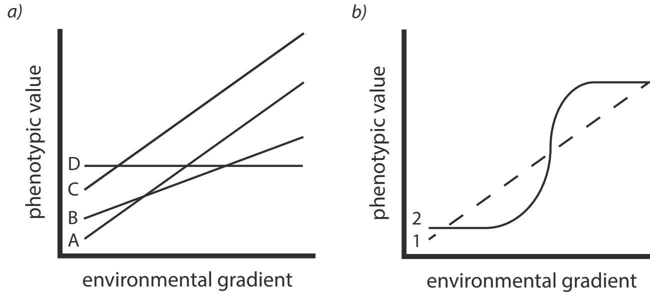

Figure 1. Reaction norms and phenotypic plasticity. *a)* Four genotypes illustrating different responses to the environmental gradient. Genotype A and B show different degree of phenotypic plasticity, as indicated by a different slope for the reaction norms, and express a different phenotype in some but not all environments. Genotype A and C show the same degree of plasticity, as the reaction norms have the same slope. The different intercepts for the reaction norms indicate that in each environment the genotypes express a different phenotype. Genotype D is a canalised genotype, showing no plasticity and expressing the same genotype across the environmental gradient. *b)* Two phenotypically plastic traits in the same organism. Trait 1 (dashed line) responds in a linear manner to the environment, expressing intermediate phenotypes across along the whole environmental gradient. Trait 2 (solid line) responds in a threshold-like manner to the environment, expressing a single phenotypic value over the lower part of the environmental gradient and switching to a different single phenotypic value over the higher part of the gradient. Note that trait 2 is canalised within each of the two parts of the environmental range, but the plasticity lies in the ability to switch to the other phenotypic state. If the environment is discrete, and individuals only experience one of two extremes, both traits display a polyphenism but produced from differing underlying mechanisms (Nijhout 2003).

by correlated selective pressures, for example due to variation between seasons affecting many organismal traits simultaneously (Ketterson *et al.* 2009; Nijhout 2003; Pigliucci 2003). Correlated selection pressures do not necessarily lead to shared mechanisms regulating the phenotypic reaction norms. Nevertheless, comparing reaction norms among traits can be a useful first step in revealing potentially shared underlying regulatory mechanisms of plasticity, and eventually understanding how such mechanistic integration has evolved.

Mechanisms of plasticity in animals

Phenotypic plasticity entails the production of different, alternative phenotypes from a single genotype, dependent on the experienced environment. Strikingly, the genetic information needed to produce those phenotypes is thus encoded in a single genome. This places the study of environment-dependent regulation of gene expression at the heart of a mechanistic understanding of phenotypic plasticity (Beldade *et al.* 2011; Bossdorf *et al.* 2008; Evans & Wheeler 2001; Gilbert 2005). In the simplest model, environmental conditions induce the expression of particular genes, leading to expression of a particular phenotype in that environment. Alternative environmental conditions induce expression

Box 1. Examples of phenotypic plasticity in the animal kingdom

In several groups of **horned beetles** (e.g. the dung beetle *Onthophagus taurus*), male larvae reared under high nutritional conditions develop into dominant males, with large horns that are used in male-male competition. Males reared on poor food not only fail to develop these anatomical structures but also employ a sneaker male reproductive tactic (Emlen & Nijhout 2001). Female larvae in **social insects** (e.g. the honey bee *Apis mellifera*, and the fire ant *Solenopsis invicta*) also respond to nutrition, and develop into fertile and long-lived queens when fed rich nutrients during development (Ross & Keller 1995; Smith *et al.* 2008). Larvae fed normal food develop into short-lived, less reproductive or sterile adult workers. **Wing dimorphic insects** such as crickets (e.g. *Gryllus spp.*) and aphids (e.g. *Acyrthosiphon pisum*) develop into a winged dispersive or into a flightless reproductive morph, dependent on a variation in density, photoperiod, temperature or food quality during development (Brisson 2010; Zera 2009). A special case of this is **phase polyphenism in locusts**, where solitary locust nymphs (e.g. *Schistocerca gregaria*) can switch to a gregarious phase upon crowding, forming devastating swarms that can last several generations (Simpson *et al.* 2011). **Diapause** is a ubiquitous feature of animal life in seasonal environments. When the growing season ends, animals switch to a metabolically dormant 'waiting mode' characterised by arrested development or delayed initiation of reproduction (Hahn & Denlinger 2011). In insects, there is a huge diversity in the life cycle stage in which diapause occurs, including in embryos (e.g. the Asian tiger mosquito *Aedes albopictus*; Lounibos *et al.* 2003), larvae (e.g. the Indian meal moth *Plodia interpunctella*; Bell *et al.* 1979), pupae (e.g. the green-veined white butterfly *Pieris napi*; Friberg *et al.* 2012), or adults (e.g. the fruit fly *Drosophila melanogaster*; Schmidt 2011). In temperate regions, diapause is typically triggered by changes in photoperiod, although temperature and other factors also often play a role (Bradshaw & Holzapfel 2010). The **nematode dauer stage** is also a form of diapause, when instead of continuing development to adulthood, larvae in poor conditions arrest development as stress-resistant, long-lived 'dauer larvae' (e.g. in *Caenorhabditis elegans*; Fielenbach & Antebi 2008). Many animals respond to **dietary restriction** with an extension in lifespan and a reduction in reproductive investment, as a presumably adaptive response to temporal reductions in environmental quality, reminiscent of a mild diapause syndrome (Nakagawa *et al.* 2012; Shanley & Kirkwood 2000). This form of plasticity has been described in nematodes (Sutphin & Kaeberlein 2008), fruit flies (Tatar 2011), and rodents (Swindell 2012) and likely provides health benefits in primates as well (Colman *et al.* 2009; Mattison *et al.* 2012).

Some notable and well-studied examples of phenotypic plasticity in insects. *a)* Horned and hornless male morphs of the dung beetle *Onthophagus nigriventris*. Photo courtesy of Doug Emlen. *b)* Queen, workers and larvae of the black garden ant *Lasius niger*. Photo courtesy of Romain Libbrecht. *c)* Solitary (left) and gregarious (right) *Locusta migratoria* locusts. Photo courtesy of Gabriel Miller, reprinted from Simpson *et al.* (2011) with permission from Elsevier.

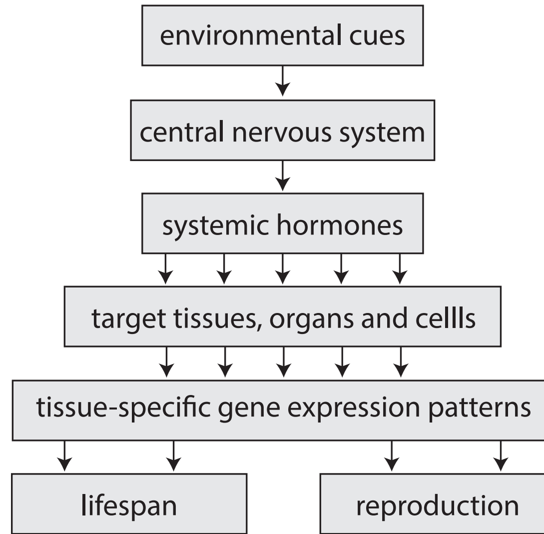

Figure 2. Potential mechanisms regulating phenotypic plasticity in life histories. Environmental variation, for example a reduction in nutritional levels, is processed by the central nervous system. Allocation decisions are translated via systemic hormone signalling to target tissues, which can respond in a tissue- or trait-specific manner, dependent on the local sensitivity to the hormone. This results in altered gene expression patterns in these tissues in response to the hormone signal, which affects the phenotype. Together, these phenotypic changes contribute to an adjusted life history strategy, for example a thriftier phenotype with reduced investment in reproduction and increased investment in somatic maintenance. This framework allows a centrally regulated, coordinated response to the environment as well as local trait-specific fine tuning of the response. Adapted from Tatar *et al.* (2003).

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of other genes, leading to a different phenotype in the other environment. A pivotal mechanism linking centrally processed environmental cues with regulation of gene expression in the various tissues and cells responsible for the phenotypic changes, are hormones (Evans & Wheeler 2001; Finch & Rose 1995; Gilbert 2005; Zera *et al.* 2007). On the one hand, systemic hormone titres are centrally regulated from the central nervous system in response to signals sensed from the environment. This allows the organism to mount a systemic, integrated and coordinated response to environmental variation. On the other hand, how the tissues and cells that ultimately bring about the phenotypic changes respond to the hormone is a local property of those tissues (Fig 2). Hormone sensitivity can be regulated via variation in expression of hormone receptors, intracellular activity and localisation of those receptors, and chromatin state of the genomic transcriptional targets of the hormone. Whichever way it is achieved, local hormone sensitivity allows for a cell-, tissue- or trait-dependent differentiated response to the circulating hormone (Klowden 2007).

In almost all cases of adaptive plasticity studied in animals, hormones play a crucial role in translating an environmental stimulus into expression of alternative phenotypes (Beldade *et al.* 2011; Finch & Rose 1995; Nijhout 2003). One important hormonal pathway that has been studied in this context is Insulin signalling in *Drosophila melanogaster*. The circulating hormones are a class of Insulin-like peptides (ILPs) that are produced mainly in the brain and whose expression is determined by the organism's nutritional state. Under high nutritional conditions, ILPs bind to Insulin Receptors in the target tissues, where they activate an intracellular phosphorylation cascade that leads to cytoplasmic localisation and deactivation of the growth-inhibiting transcription factor FoxO. Disruption of this nutrient-sensing pathway by genetic mutation in some of its components (e.g. the Insulin Receptor Substrate) results in lifespan extension and often a reduction in fecundity (Partridge 2010; Tatar *et al.* 2003). FoxO has many transcriptional targets, which are presumably responsible for the observed phenotypic effects, either directly or via additional layers of hormonal and transcriptional regulation (see Alic *et al.* 2011).

Ecdysteroids are another important class of insect hormones. Their canonical function is that of a developmental timer in the larval stage, with short pulses of high concentrations signalling the transition to the next larval moult or to pupation. Unlike ILPs they are not peptide but steroid hormones. In the cell, Ecdysteroids bind to nuclear hormone receptor complexes that also act as transcription factors, containing both ligand binding and DNA binding domains (Klowden 2007). The genomic transcriptional targets of Ecdysteroids are numerous, and include many other transcription factors (Gauhar *et al.* 2009). In addition to their role as developmental timers in the larval stage, Ecdysteroids have also been implicated in diapause regulation (Denlinger 2002) and in adult female reproduction (Schwedes & Carney 2012).

Plasticity, development, and ageing: a life history perspective

Life history theory aims to explain the diversity in life history traits such as growth rate, size at maturity, reproductive investment and lifespan in relation to spatial and temporal variation in ecological opportunities. A key component of life history theory is understanding observed positive and negative correlations between life history traits (e.g. trade-offs) as a result of nutrient acquisition and their allocation to competing physiological functions. Life history strategies can thus be interpreted as optimal allocation decisions, maximising fitness given a particular environment (Boggs 2009; de Jong & Noordwijk van 1992; Roff 1992; Stearns 1992). One typical prediction of such environment-dependent variation in life history strategies is a trade-off between reproduction and lifespan. When resources are abundant, rapid reproduction is favoured over investment in a durable body, resulting in a short lifespan. Facing (temporarily) harsh conditions, a strategy of delayed reproduction and increased investment in somatic repair is instead favoured, resulting in increased lifespan.

Life history theory thus sets the stage for an evolutionary understanding of ageing, including in humans. Termed the disposable soma theory, ageing is understood to evolve in this context as a by-product of selection for investment in early reproduction at the cost of investment in a durable soma and late survival (Kirkwood 1977; Kirkwood & Rose 1991). This theory links evolutionary theories of ageing, focusing on life history trade-offs, with a mechanistic perspective, focusing on molecular pathways affecting lifespan. It can make predictions of what classes of genes might be involved in variation in ageing, such as those involved in somatic repair. The disposable soma theory therefore provides a powerful integrative framework for a mechanistic and evolutionary understanding of ageing (Zwaan 1999).

Two hypotheses have been proposed to try to understand human ageing and age-related morbidity from an evolutionary perspective. The 'thrifty genotype hypothesis' aims to explain the dramatic increase in prevalence of obesity and metabolic syndrome among affluent populations (Neel 1962). Briefly, it assumes that our life history strategy has evolved in a feast-famine environment. Such an environment would favour genotypes that maximise glucose intake and rapid storage of any excess resources into fat reserves, providing energy in recurring times of food scarcity. In the modern food-rich environment these 'thrifty genotypes' are hypothesised to have become detrimental because resource accumulation when energy-rich foods are constantly available can lead to obesity, insulin resistance and other related health problems (Neel 1962). However, this hypothesis makes many assumptions regarding ancestral diets and selective forces which are very hard to test (Bouchard 2007; Zwaan 2003). In addition, the few genes that have been found to have pleiotropic effects on fitness between pre-industrial and modern environments are not related to metabolism, but to immunity (Kuningas *et al.* 2009).

The second hypothesis aimed at understanding human ageing patterns from a life history perspective is the 'thrifty phenotype' or Barker hypothesis, which is strongly focused on development. It stems from the observation that fetal malnutrition, especially during early gestation, has a wide range of adverse effects on health at middle and old age. It is proposed that altered Insulin signalling mediates this developmental response (Hales & Barker 1992, 2001). Moreover, it has been hypothesised that it is an adaptive response to variation in nutrition (Gluckman *et al.* 2005), a hypothesis known as the 'predictive adaptive response'. This hypothesis states that the developmental nutritional response evolved as an adaptation to fluctuations in food resources. In this scenario, the physiological imprint of malnutrition during embryonic development would be predictive for future conditions of scarcity and prepare offspring for said conditions (Gluckman & Hanson 2004; Gluckman *et al.* 2005). The observed adverse health outcomes would then be a result of a mismatch between developmental and adult food conditions. However, this adaptive explanation has been heavily criticised by evolutionary biologists (Rickard & Lummaa 2007; Wells 2007), most notably because it would require conditions during gestation to be predictive for those during middle age.

Although the observations are real, a more likely scenario would be that the developmental response is not adaptive for adult life, but rather for early childhood survival. As this period is closer in time to embryonic development, nutrition levels experienced during gestation

might have some predictive value here. In this scenario, the benefits of a thrifty phenotype during early life would have detrimental consequences later in life, and the developmental response would thus represent a trade-off between early and late life survival. Alternatively, fetal malnutrition in humans may always adversely affect the offspring's juvenile and adult health, irrespective of adult nutritional conditions (i.e. 'scarring', see Brakefield *et al.* 2005; Rickard & Lummaa 2007). These theories may sound plausible, but lack clear or convincing hypotheses for genetic and physiological mechanisms involved. Using model organisms to study these hypotheses unleashes the power of manipulative experimentation to uncover mechanistic and evolutionary links between development and adult health span. For example, distinguishing between adaptive (mismatch) and non-adaptive (scarring) explanations for the detrimental effects of fetal malnutrition requires evaluating fitness in response to variation in juvenile and adult environments separately. Extending such an approach with an ecological component is particularly important for understanding human ageing and ageing-related morbidity.

Mechanisms of life history plasticity: towards an ecological approach

It is hard to overstate the importance of model organisms in contributing to our mechanistic understanding of ageing. The powerful genetic methods available for *C. elegans* and *D. melanogaster* have allowed an unprecedented dissection of the physiological and genetic regulation of ageing and of plastic food responses (Fontana *et al.* 2010; Toivonen & Partridge 2009). Of particular relevance here is the nutrient-sensitive plastic reallocation known as the dietary restriction (DR) response. In many, although not all, animals studied so far, moderate DR results in an increased lifespan, usually accompanied by a decreased reproductive output (Nakagawa *et al.* 2012). This can be interpreted as an adaptive response that evolved to cope with temporary reductions in nutrient availability. Animals respond by activating physiological processes that promote lifespan extension, such as somatic repair, and repressing reproductive processes while retaining the ability to reproduce later when conditions improve (Kirkwood & Shanley 2005; Shanley & Kirkwood 2000). However, adaptive explanations are often hard to test in traditional model organisms. For most of these, little is known about the natural ecological situation, where the mechanisms underlying lifespan extension in response to DR presumably evolved. This is especially problematic for species where the average lifespan in the field is much shorter than in the laboratory, as is the case for *D. melanogaster*. Genetic manipulation studies may reveal molecular pathways with major effects on lifespan and reproduction under laboratory conditions, but this does not mean that such effects are relevant under ecologically realistic conditions. These challenges highlight an important limitation of the traditional model organisms. To understand how and where mechanisms contributing to life history variation evolved, it is necessary to look beyond the laboratory models and supplement them with organisms more amenable to ecological studies (Partridge & Gems 2006).

The study of phenotypic plasticity has a rich and productive history in the evolutionary and ecological literature, and plasticity of life history traits has been widely recognised as highly relevant for fitness in variable environments (Nylin & Gotthard 1998; Stearns 1989). This provides an excellent resource for studying, in an evolutionary context, the mechanisms underlying environmental responses that contribute to variation in ageing (Flatt & Schmidt 2009). Impressive progress has been made in connecting the traditions of evolutionary biology with molecular genetics and physiology towards an integrative understanding of mechanisms of life history evolution, including phenotypic plasticity (Flatt & Heyland 2011b; Zera & Harshman 2001; Zera *et al.* 2007). In particular, the emerging field of ecological and evolutionary genomics holds great promise for using high-throughput DNA sequence data to probe how the environment and the genome interact to produce ecologically relevant phenotypes. Despite the complex nature of life history phenotypes, comprising the interactive effects of many physiological, morphological and behavioural traits (Flatt & Heyland 2011a), considerable advances are being made (Aubin-Horth & Renn 2009).

Ecological and evolutionary genomics aims to understand how genes function in the real world outside the laboratory. It proposes to develop an Ecological Association Ontology, conceptually similar to the existing Gene Ontology framework but instead aimed at an ecological annotation of genes (Pavey *et al.* 2012). An exciting development in this light is the more than exponential decrease in DNA sequencing costs over the last decade, and the corresponding explosion of available analysis tools. This has allowed ecologists and evolutionary biologists to develop and deploy a variety of genomic tools to organisms studied for their ecological and evolutionary relevance (Ekblom & Galindo 2011; Orsini *et al.* 2013).

The obvious drawback of using non-model organisms is the lack of available resources, in particular genetic tools. Although genomics has now become accessible for ecologists, other tools such as manipulation of gene function are still largely lacking for many non-model species (Sommer 2009). Developing such tools for these organisms requires substantial investment in time and resources, and this is especially difficult for organisms that are only being studied by a handful of research groups. For example, RNA interference (RNAi) has revolutionised the study of gene function and expanded it into systems where this was not previously possible (Boutros & Ahringer 2008; Tomoyasu *et al.* 2008). This has allowed not only to test involvement of candidate genes in phenotypes of interest (e.g. Emlen *et al.* 2012) but also the application of genome-wide functional screens (e.g. the iBeetle project in *Tribolium castaneum*, see http://ibeetle.uni-goettingen.de/). However, in Lepidoptera RNAi has not lived up to its promise. Results in this group of insects have been mixed and it is unclear whether this is solely due to technical issues or whether Lepidopteran biology might make this group less responsive to RNAi (Terenius *et al.* 2011).

Despite the technical challenges involved, adding an ecological component to the study of mechanisms underlying life history variation will also contribute to a better understanding of human ageing and ageing-related morbidity. As the knowledge of genetic

factors underlying human ageing increases, it is becoming clearer that the interaction between genes and the environment is critical in determining a healthy lifespan. Our genetic makeup was shaped by very different selective forces than those in place today. Using laboratory models for which knowledge on natural life histories is at best anecdotal will only get us so far in understanding how our old genes produce healthy and unhealthy phenotypes in the new modern environment. Combining extensive ecological knowledge with the ability to perform manipulative experiments in the laboratory, the butterfly *Bicyclus anynana* provides an excellent model system to study mechanistic and evolutionary links between development and ageing.

Study system: the seasonal butterfly *Bicyclus anynana*

In this thesis, I use a captive laboratory population of an ecologically well characterised butterfly to try to understand mechanisms of developmental plasticity in life histories. The Nymphalid butterflies belonging to the genus *Bicyclus* comprise *ca.* 90 species distributed throughout Africa, including Madagascar. *Bicyclus anynana* inhabits the seasonal grassland savannahs and open woodland ecosystems of tropical and subtropical East Africa, roughly from South Africa to Ethiopia. *B. anynana* adults feed on fermenting fruit fallen from the trees, and the larvae are grass feeders. The savannah habitat is characterised by strong seasonality. In Malawi, where the laboratory population originates, the dry season is relatively cool and the wet season is warm. During the dry season, grasses dry out completely, precluding survival for any caterpillars. Adult butterflies do get by, feeding on the limited fruit that is still occasionally available. After bad times come good times, and the rains of the wet season bring larval food in the form of fresh grasses.

To cope with these seasonal fluctuations in ecological opportunities, *B. anynana* is able to express a distinct life history strategy in each season. Adult butterflies bridge the harsh dry season by being relatively inactive and temporarily relinquishing reproduction. In contrast, wet season adults are active and reproduce readily. Another striking difference between the seasons is in the ventral wing pattern. Dry season adults have uniformly brown, cryptic wings, allowing them to rest inconspicuously among the dead leaf litter. In contrast, butterflies of the wet season display prominent eyespots along the margin of the wing, hypothesised to deflect predator attacks away from the vulnerable adult body when attached at rest (Fig. 3). Phenotypic plasticity is widespread in the *Bicyclus* genus and related genera, but this has only systematically been studied for wing pattern, not life history (Brakefield & Frankino 2009; Brakefield & Reitsma 1991; Brakefield & Zwaan 2011). Bringing this species into the laboratory and establishing a captive population has allowed a detailed examination of how *B. anynana* is able to express these two distinct phenotypes in the two seasonal conditions.

Developmental plasticity has turned out to be crucial. Larvae developing at cooler temperatures, which in the field occur over the transition from the wet to the dry season before host plants completely dry out, eclose with a dry season phenotype. When larvae experience high temperatures during development, which in the field indicate the

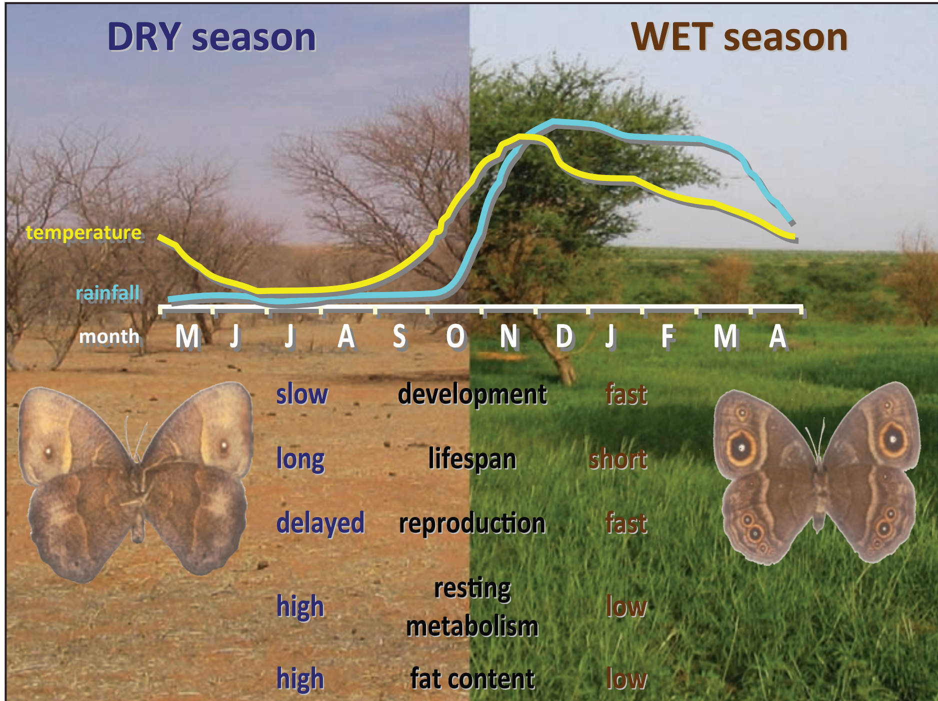

Figure 3. Seasonal phenotypic plasticity in *Bicyclus anynana*. Seasonal climate and alternative phenotypic morphs in *B. anynana* for the dry (left) and wet (right) season. The graphs at the top depict rainfall (yellow) and temperature (turquoise) throughout the year (horizontal axis), redrawn from Brakefield & Reitsma (1991). The table and photos of adult butterflies indicate phenotypic differences between the seasonal morphs. The photos in the background were taken at the same location on the savannah in Sudan during the dry and wet season, respectively, and serve to illustrate the seasonally fluctuating habitat quality (photos courtesy of Jonas Ardö, adapted from Ardö *et al.* (2008), used with permission).

wet season, they develop into wet season adults. Thus temperatures in the field act as a predictive cue for the forthcoming environment the adults will experience. Developing individuals are particularly sensitive to temperature during the last (fifth) larval instar and the early pupal period, although this has only been established for wing pattern plasticity. Young adults developed at alternative temperatures differ in a variety of traits related to life history, including egg laying rate, resting metabolic rate (RMR), size and fat content (Fig 3). However, temperature does not induce the full extent of life history plasticity as observed in the field. In particular, lifespans of up to six months as observed in the dry season in the field have never been observed in the laboratory. Wing pattern pigmentation is an irreversible process completed during the pupal stage, and young adults eclose with a dry or wet season wing pattern fixed for life. Life history on the other hand is more malleable. Adult butterflies are able to acclimate after a certain adjustment period when conditions change in the adult

stage from those experienced during the larval stage. Most notably, female egg laying rates in dry season-reared adults can increase from their initially low values if the females are placed in warm wet season condition as adult (Brakefield *et al.* 2007; Fischer *et al.* 2003). However, this acclimatisation does take some time and thus developmental plasticity is an important mechanism that allows adults to emerge with the best matched phenotype from the very start of adult life. This gives them a time advantage compared to if they had to rely solely on adult acclimation (Brakefield & Frankino 2009; Brakefield & Zwaan 2011).

Hormonal systems have emerged as crucial mediators of developmental plasticity in *B. anynana*. Individuals reared at alternative temperature conditions differ in dynamics of Ecdysteroid signalling during pupal development, with an early hormone peak in the wet season morph and a late peak in the dry season morph. Lines artificially selected to diverge in plasticity for ventral wing pattern recapitulate this difference in hormone dynamics when reared at a single temperature. Furthermore, exogenous Ecdysteroids applied to young pupae reared at dry season conditions are able to induce a wet season-like wing pattern (Brakefield & Frankino 2009; Brakefield & Zwaan 2011; Koch *et al.* 1996; see also Chapters 2 and 3 of this thesis).

An important and successful genetic tool that has allowed *B. anynana* to rise to prominence in evolutionary studies of wing pattern, seasonal adaptation and life history is artificial selection. Using an outbred laboratory population derived from the wild, it has been possible to obtain phenotypically divergent selection lines for a wide variety of traits, including body size, development time, wing pattern, starvation resistance and lifespan (Brakefield 2003; Brakefield *et al.* 2003; Pijpe *et al.* 2007; Pijpe *et al.* 2006; J. Pijpe unpubl. data). Antagonistic selection experiments on different traits or sexes have yielded important insights into the role of developmental and hormonal constraints in short term evolutionary change (Allen *et al.* 2008; Beldade *et al.* 2002; Zijlstra *et al.* 2002; Zijlstra *et al.* 2004).

Box 2. LifeSpan: integrating research into development and ageing

The work described in this thesis was carried out in the context of the **Network of Excellence LifeSpan**, a collaborative and integrative research programme aimed at studying mechanistic links between development and ageing. Central to LifeSpan was the realisation that ageing cannot be understood without considering the whole life history, including development. LifeSpan (January 2007 – December 2011) comprised of seventeen research groups from ten European countries, working on different aspects of ageing research. This provided the opportunity to interact between observational studies in humans and manipulative studies in model organisms. For example, genetic, epigenetic, transcriptomic and immunological aspects of human longevity were studied in several cohorts of long-lived people and their families, as well as in monozygotic twins. At the same time, model organisms (including. rodents, fruit flies, worms, butterflies, ants, worms) were used to identify novel ageing and longevity genes, as well as to functionally test involvement of candidate genes. Model organisms were also used to examine mechanisms underlying environmental responses, in particular how variation in juvenile conditions affects adult health and lifespan. In addition to generating and disseminating scientific knowledge, LifeSpan also provided a valuable platform for initiating and maintaining interdisciplinary collaborations (see for details http://www.lifespannetwork.nl/).

Another field where *B. anynana* has become an important model is evo-devo (Brakefield & French 1999). The marginal eyespots on the four wings have proved a fruitful system to study the genetics of morphological diversity, the role of modularity in evolutionary change, and the origin of evolutionary novelties (Beldade & Brakefield 2002; Brakefield *et al.* 2009; Saenko *et al.* 2008). The evo-devo studies in particular have pushed forward the development of genomic tools for this species. Genomic tools available today include several expressed sequence tag (EST) data bases, microsatellite and AFLP markers, a linkage map, BAC libraries and a custom designed microarray (Beldade *et al.* 2006; Beldade *et al.* 2009a; Beldade *et al.* 2009b; Conceição *et al.* 2011; Van 't Hof *et al.* 2008). Although originally developed for studies of wing pattern development, these tools have also started to be used for genomic analyses of life history variation (e.g. de Jong *et al.* 2013; Pijpe *et al.* 2011; see also Chapters 4 and 5 of this thesis). With several on-going sequencing projects, the genomic toolbox for *B. anynana* is only increasing.

Aims and outline of this thesis

The general aim of this thesis is to gain insight into the hormonal and transcriptional patterns that underlie developmental plasticity of life history in the seasonal butterfly *B. anynana*. An additional goal is to use these findings to enhance mechanistic and evolutionary understanding of variation in human health and ageing.

Using complementary approaches, the thesis deals with two main aspects of the seasonal adaptation in *B. anynana*. The first question is how the environment experienced during larval and pupal development induces the two adult seasonal forms via conserved hormonal pathways. In particular, I investigate the extent to which Ecdysteroids, known to be involved in wing pattern plasticity in this species, are also instrumental in mediating the response of adult life history to developmental conditions (**CHAPTERS 2** and **3**). The second major question covered in this thesis is what transcriptional changes in the adult are associated with the seasonal forms. Using a candidate gene approach (**CHAPTER 4**) in parallel to an unbiased screen (**CHAPTER 5**), I analyse gene expression variation in adults that have developed as larvae under alternative seasonal conditions. Finally, **CHAPTER 6** provides a broader evolutionary perspective and asks to what extent developmental plasticity, as measured in the laboratory, is retained in the rainforest species *Bicyclus martius* under relaxed selection.

In **CHAPTER 2**, I use a fine gradient of developmental temperatures to study the precise thermal response of adult life history traits involved in the seasonal adaptation. The extent to which the adult traits show similarly shaped reaction norms—from linear to threshold-like—may indicate whether the regulatory mechanisms underlying the thermal responses are shared among traits. Crucially, within the same experiment I also study thermal reaction norms for pupal Ecdysteroids and Juvenile Hormones, the mechanisms hypothesised to regulate the environmental response.

CHAPTER 3 provides a functional test of the involvement of Ecdysteroids in mediating developmental plasticity of the adult life history syndrome. First, it is established

whether application of exogenous Ecdysteroids during pupal development can induce the phenotypic changes normally induced by developmental temperature. Hormones are injected during the pupal stage, at four time points that represent different parts of natural Ecdysteroid titre dynamics. This is done for three different seasonal temperatures, allowing to test environment-specific windows of sensitivity. Within the same experiment, but not published in this thesis, adult wings of the injected individuals have been analysed to assess the effect of Ecdysteroids on wing pattern (R. Mateus *et al.*, unpubl. data). Second, in a follow-up experiment it is assessed to what extent the adult phenotypic changes induced by exogenous Ecdysteroids during development affect reproductive output, lifespan and starvation resistance. Together, these experiments establish whether Ecdysteroids play a functional role in translating predictive information on environmental quality during development into adaptive alterations in a suite of adult traits.

Next, **CHAPTER 4** examines how the seasonal forms differ in the expression of selected life history-related genes. As both adult seasonal forms develop from the same genetic background, environmentally induced phenotypic differences ultimately depend on transcriptional regulation. Young adults start their life expressing distinct life history strategies as end points of divergent developmental pathways. These pathways are in turn induced by the alternative seasonal conditions experienced as larvae and pupae, mediated by hormone signalling. I use qPCR to examine expression in young adults of 27 candidate life history-related genes, as putative molecular effectors of the two strategies. The genes are related to reproduction, immune defence, carbohydrate metabolism and lipid metabolism. Thus, a first goal 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*. I analyse whether the selected genes are up- or downregulated in young adults differing in their developmental history.

In **CHAPTER 5**, microarrays are used to analyse how ageing affects the global transcriptional profile, and how this is modulated by the adults' seasonal developmental history. The first aim is to characterise the whole-genome transcriptional signature of ageing for this organism which has not traditionally used as a model in ageing studies. Special emphasis is placed on sex-specificity in ageing-related expression changes. The second goal is to analyse to what extent seasonal conditions experienced during juvenile development leave a life-long transcriptional imprint in middle-aged and old adults, when those conditions are no longer experienced. Finally, I compare the transcriptional response to ageing among cohorts reared under the alternative conditions. This allows assessing which transcriptional changes contribute to the alternative seasonal life histories, including lifespan.

Taking a broader evolutionary perspective, **CHAPTER 6** examines the fate of seasonally plastic traits in a butterfly under relaxed natural selection. *Bicyclus martius* inhabits the West-African rainforest, a generally wet season-like habitat with limited fluctuation in temperature, larval food availability or reproductive opportunities. The lack of seasonal exposure to harsh dry season conditions likely reflects a situation of relaxed selective

pressures, both on dry season-specific adaptations and on plasticity itself. This chapter aims to establish the extent to which *B. martius* has retained the ability to express alternative phenotypes when exposed in the laboratory to a range of temperatures not normally encountered in the field. In the savannah butterfly *B. anynana*, these temperatures induce plasticity for a variety of traits, some of which share a hormonal regulator (e.g. Chapters 2 and 3 of this thesis). Analysing variation between traits in environmental responses in *B. martius* allows determining the extent to which hormonal regulation precludes independent evolution of plastic responses among traits.

Finally, in **CHAPTER 7**, I summarise the findings presented in the previous chapters and provide a perspective on how these data contribute to a better mechanistic understanding of plastic responses as adaptation to environmental variation. In addition, I discuss how these findings can increase our knowledge on mechanisms linking development and ageing in humans, and how events during early development can affect health and lifespan.

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Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly

2

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Abstract

Polyphenisms—the expression of discrete phenotypic morphs in response to environmental variation—are examples of phenotypic plasticity that may potentially be adaptive in the face of predictable environmental heterogeneity. In the butterfly *Bicyclus anynana*, we examine the hormonal regulation of phenotypic plasticity that involves divergent developmental trajectories into distinct adult morphs for a suite of traits as an adaptation to contrasting seasonal environments. This polyphenism is induced by temperature during development and mediated by ecdysteroid hormones. We reared larvae at separate temperatures spanning the natural range of seasonal environments and measured reaction norms for ecdysteroids, juvenile hormones (JHs) and adult fitness traits. Timing of peak ecdysteroid, but not JH titres, showed a binary response to the linear temperature gradient. Several adult traits (e.g. relative abdomen mass) responded in a similar, dimorphic manner, while others (e.g. wing pattern) showed a linear response. This study demonstrates that hormone dynamics can translate a linear environmental gradient into a discrete ►►

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►► signal and, thus, that polyphenic differences between adult morphs can already be programmed at the stage of hormone signalling during development. The range of phenotypic responses observed within the suite of traits indicates both shared regulation and independent, trait-specific sensitivity to the hormone signal.

Key words: ecdysone, hormonal regulation, life history, phenotypic plasticity, reaction norm, seasonal polyphenism

Introduction

Phenotypic plasticity is the ability of individual genotypes to produce different phenotypes when exposed to environmental variation (Stearns 1989, Schlichting & Pigliucci 1998). Potentially, it allows organisms to persist in variable environments, and it is therefore of major evolutionary significance. Furthermore, phenotypic plasticity reveals how the developmental mechanisms that translate genotypes into phenotypes can be modulated by the environment and how sensitivity to the environment can be a source of phenotypic variation (Brakefield *et al.* 2003, West-Eberhard 2003, Gilbert & Epel 2008). The reaction norm concept describes phenotypic variation as a function of the environment and provides an experimental framework for studying developmental sensitivity to the environment (Debat & David 2001, Sultan 2007). A flat reaction norm represents a canalized phenotype, whereas a steep reaction norm represents a plastic phenotype. Polyphenisms can be seen as an extreme case of phenotypic plasticity, where alternative discrete phenotypes develop in response to environmental variation (Shapiro 1976, Stearns 1989, West-Eberhard 2003).

Hormones play crucial regulatory roles in coordinating the expression of physiological, behavioural and morphological traits into an integrated life history (Nijhout 1994, Zera *et al.* 2007, Ketterson 2009). The two major classes of insect hormones, ecdysteroids and juvenile hormones (JHs), have been implicated in many cases of insect polyphenisms, such as horned beetles, butterflies, social insects and sand crickets (Nijhout 2003, Zera 2007, Smith *et al.* 2008, Brakefield & Frankino 2009). While various studies have measured reaction norms across an environmental gradient for phenotypic traits (e.g. Trotta *et al.* 2006, Liefting *et al.* 2009), and others have measured differences in hormone dynamics between morphs at the extreme ends of a reaction norm (e.g. Brakefield *et al.* 1998), these approaches have rarely been combined (but see Anstey *et al.* 2009). It is therefore unknown whether discrete differences between adult morphs are already present at the endocrine level during development.

A polyphenism typically involves a suite of morphological, physiological and life-history traits that may respond to the same environmental signal (e.g. Pijpe *et al.* 2007, Brisson 2010). The central regulation of systemic hormone titres enables integration of traits at the organismal level, but can thereby potentially constrain their independent evolution (Ketterson & Nolan 1999, Zijlstra *et al.* 2004, McGlothlin & Ketterson 2008). On the other hand, sensitivity of the local tissue determines the response to the hormone, indicating scope for differentiated regulation of the traits comprising the polyphenism (Nijhout 1994). In contrast with theoretical advances (e.g. McGlothlin & Ketterson 2008), there is little empirical knowledge on the extent to which suites of traits regulated by the same hormone constitute integrated phenotypes across environmental gradients, or can respond independently.

With the present study, we aim to understand how hormonal mechanisms regulate a suite of fitness traits involved in the phenotypic plasticity in *Bicyclus anynana*. This afrotropical butterfly has evolved developmental plasticity as an adaptation to its seasonal environment (Brakefield & Reitsma 1991, Brakefield *et al.* 1996). In the warm wet season,

butterflies have large, prominent eyespots on the ventral surface of their wings, which are probably involved in the deflection of predatory attacks (Lyytinen *et al.* 2004). Butterflies of the cool dry season express a cryptic wing pattern with small to virtually absent eyespots. In the dry season in the field there is strong natural selection against conspicuous eyespots (Brakefield & Frankino 2009). Furthermore, these butterflies express an alternative physiology and life-history strategy that allows them to bridge the period of (nutritional) stress that the dry season represents (Brakefield *et al.* 2007). During the dry season, adults have altered metabolic rate, and accumulate more mass and higher fat content during the larval stage, important fitness traits associated with adult starvation resistance (Zwaan *et al.* 1991, Chippindale *et al.* 1996, Pijpe *et al.* 2007, De Jong *et al.* 2010). Finally, reproduction is delayed until the end of the dry season (Brakefield & Reitsma 1991, Fischer *et al.* 2003, Brakefield *et al.* 2007). The seasonal adult morphs are induced in response to temperature during a critical period of pre-adult development (Brakefield & Reitsma 1991, Brakefield *et al.* 1996). Analyses of the reaction norm for wing pattern have revealed a linear response to developmental temperature (Brakefield & Reitsma 1991, Wijngaarden *et al.* 2002), but it is unknown how the life-history traits respond to a gradient in environmental temperature.

Ecdysteroids have been found to be involved in regulating wing-pattern plasticity in *B. anynana* (Koch *et al.* 1996, Brakefield *et al.* 1998, Zijlstra *et al.* 2004), but it is unknown whether these hormones have a role in regulating the full suite of traits involved in the seasonal adaptation. Furthermore, it is unknown how ecdysteroid titres change along a continuous gradient in environmental temperature and how this response relates to those of the phenotypic traits. In this study, we apply the reaction norm concept to the hormone dynamics underlying the phenotypic response. The extension of the use of the reaction norm perspective to developmental and molecular processes regulating the phenotype promises to be a useful tool in the integrative study of phenotypic plasticity (Aubon-Horth & Renn 2009).

We manipulated the developmental environment by rearing cohorts of larvae under five different temperatures spanning the natural range of seasonal environments, with the lowest temperature corresponding to the dry-season environment and the highest to the wet-season environment. We measured the reaction norms for ecdysteroids and JHs during the critical pupal stage, as well as for size at maturity, relative abdomen to total body mass (as a measure of allocation of resources to early life reproduction versus flight ability), metabolic rate, fat reserves and ventral wing pattern—key fitness traits involved in the seasonal polyphenism.

Materials and methods

Experimental design

Cohorts of *B. anynana* used in this experiment were derived from an outbred wild-type population established in the laboratory in 1988. The experiment was carried out in two phases, one for the measurement of phenotypic traits and the other for measurement of hormone titres. In each phase, 2000 larvae were reared from egg to adult (n = 400 per

temperature treatment). Eggs were collected from the wild-type population on a single day and kept at 23.5°C until hatching. Larvae were reared on maize (*Zea mays*) in climate-controlled chambers at 70 per cent relative humidity (RH) with a

12 L : 12 D light/dark cycle. After hatching, larvae were randomly divided over each of five climate-controlled chambers (19°C, 21°C, 23°C, 25°C and 27°C, ± 0.5°C) representing five temperature treatments, with a different allocation of temperature treatments to chambers in the two phases of the experiment. The lowest temperature corresponds to dry-season conditions in the field and the highest temperature to wet-season conditions Brakefield & Reitsma 1991. Temperature and RH were logged throughout the rearing process using data loggers (± 0.2°C) to ensure stability of environmental conditions.

Life-history traits

For each individual, we recorded development time as the number of days between hatching of the egg and eclosion of the butterfly. Pupae were weighed within 36 h after pupation to the nearest 0.1 mg. One day after eclosion, 100 males and 100 females per temperature treatment were haphazardly selected for resting metabolic rate (RMR) measurements. Fifty butterflies per rearing temperature per sex were measured at 19°C, and 50 at 27°C, in a climate-controlled chamber during the dark phase of the diurnal cycle. RMR was measured as the individual rate of CO_2 respiration (millilitre per hour) over a period of 20 min, following (Pijpe *et al.* 2007). Following RMR measurements, wings were cut off after which the butterflies were dried for 48 h at 55°C and weighed to the nearest 0.01 mg. Total fat (triglyceride and free fatty acids) was extracted by incubating the dried butterflies at room temperature in 2 : 1 (v/v) dichloro-methane : methanol for 96 h, followed by drying and weighing, yielding fat-free dry weight. Fat content was calculated by subtracting the fat-free dry weight from the initial dry mass. In order to estimate allocation of resources to different parts of the body, thorax and abdomen were dried and weighed separately.

Wing pattern

The ventral surface of one hindwing of each individual was photographed using a digital still camera connected to a binocular microscope (Leica). The images were analysed with ImagePro 6.0 software to measure the following wing pattern elements: (i) distance between the first and the fifth eyespot; (ii) radius of the inner black disc of the fifth eyespot; (iii) radius of the white centre of the fifth eyespot; and (iv) width of the median band (after Wijngaarden & Brakefield 2001).

Hormone titres

For female pupae of each temperature treatment, we measured ecdysone (Ecd), 20-hydroxyecdysone (20E), and JH-I, JH-II and JH-III titres at 11 time points throughout the earlier 55 per cent of the pupal stage, with five replicate pupae per time point. To correct for the direct effect of temperature on pupal development time, we scaled the time points for each temperature treatment separately to the total average duration of the pupal stage. For each temperature treatment, we chose 11 time points after pupation, corresponding to

approximately 5 to 55 per cent of total pupal developmental time, spanning the relevant time window for ecdysteroid dynamics (Zijlstra *et al.* 2004). We took 20 µl haemolymph samples from individual pupae, sampling each pupa only once. Hormone titres were measured from haemolymph by liquid chromatography–mass spectrometry (LC-MS), using the method developed by Westerlund & Hoffmann (2004) and Westerlund (2004), with minor modifications to the protocol (for details see Supplementary Material). This method allows for simultaneous quantification of all hormones from the same sample.

Statistical analyses

Life-history traits and wing pattern

Data were analysed using two-way analysis of variance (ANOVA) for each trait separately, with temperature treatment and sex as fixed effects. RMR and fat content were first analysed with dry weight as the only independent variable, of which the residuals were used as the dependent variables in the ANOVAs. Likewise, for relative abdomen mass we used the residuals of the model with abdomen dry weight as dependent, and total dry weight as independent variable. Finally, the four wing-pattern measurements were reduced using a principal component analysis (PCA; cf. Wijngaarden & Brakefield 2001), pooling data across the sexes. The first principal component (PC1) explained 50.5 per cent of the total variation and was associated with the traits that are indicative of seasonality (radius of the inner black disc of the fifth eyespot, radius of the white centre of the fifth eyespot and width of the median band). PC2 explained 30.3 per cent of the total variation and was associated with the distance between the first and the fifth eyespot, an index of size rather than seasonality. Thus, only PC1 was further analysed. *Post hoc* comparisons between specific levels of a factor were performed using Tukey's honest significant differences (HSD) tests.

Hormone titres

Previous work on *B. anynana* has shown that ecdysteroid titres peak at around 20 to 40 per cent of pupal development (hours after pupation as percentage of total pupal development time), with lower titres before and after. Titres of the two seasonal morphs have similarly shaped curves and similar absolute values, but show a difference in timing of peak titres (Koch *et al.* 1996, Brakefield *et al.* 1998, Zijlstra *et al.* 2004). To compare hormone dynamics across temperature treatments, we estimated the timing of the peaks for Ecd and 20E by fitting, for each hormone separately, the function

$$Y = e^{bt-at^2}$$

to the time series of each temperature, where Y is the hormone concentration (picograms per microlitre) at time t (relative time after pupation as fraction of total pupal time), and a and b are parameters determining the height and timing of the peak.

For each treatment, and for Ecd and 20E separately, we randomly drew one data point for each time point, using the five replicate pupae per time point, yielding five replicate time series per treatment per hormone. Through each time series, we fitted the function with parameter values minimizing residual sum of squares. We thus obtained, per temperature treatment per hormone, five independent estimates of the two parameter values based on the

five replicate pupae per time point. For this function, the timing of the peak t_{peak} is given by $b/2a$ (calculated by setting the first derivative of the function to 0), yielding five independent estimates of t_{peak}. This t_{peak} was subsequently used as dependent variable in a one-way ANOVA with temperature treatment as fixed factor. *Post hoc* comparisons between specific treatment levels were performed using Tukey's HSD tests. As we had no *a priori* expectations regarding JH-III concentration dynamics during the pupal stage, we used JH-III concentration as the dependent variable in a linear model with temperature treatment as fixed factor and relative time after pupation (as fraction of total pupal time) as covariate.

To estimate the potential effect of diurnal cycle on hormone concentrations (cf. Zhao & Zera 2004), we used, for each hormone separately, one-way ANOVA with hour of day at which a pupa was sampled as fixed factor and hormone concentration as dependent variable, followed by Tukey's HSD tests.

Results

Reaction norms of phenotypic traits

All phenotypic traits involved in the seasonal variation showed a significant response to the gradient of developmental temperature. However, the precise shape of each reaction norm differed across traits. Some traits changed gradually and linearly along the temperature gradient, while other traits showed a discontinuous change at intermediate temperatures. Furthermore, for some traits there were marked differences between males and females in their response, while for other traits no such sex specificity was found.

In both sexes, development time decreased continuously with increasing developmental temperature; larvae developed faster under wet-season conditions. Though males developed faster than females ($p < 0.001$), the shape of the reaction norm was virtually identical between the sexes (Fig. 1a).

Across the temperature gradient, pupal mass was lower for males than for females ($p < 0.0001$), and in both sexes pupae were larger when reared at lower temperatures, corresponding to dry-season conditions. However, the shape of the reaction norm differed between the sexes. In males, pupal mass decreased in a continuous, linear manner with increasing developmental temperature, with intermediately sized pupae at intermediate temperatures. By contrast, in females, pupal mass did not change within the lower or higher ends of the reaction norm but showed a significant decrease between 21°C and 23°C (Fig. 1b).

Relative abdomen mass, as a measure of relative allocation to reproduction versus flight, was higher in adult females than in males, but only when they had developed at the three higher temperatures (i.e. wet-season conditions; $p < 0.01$). At lower temperatures, males did not differ from females. In females, the response to developmental temperature was discontinuous between the two lower and three higher temperatures, with a significant increase between 21°C and 23°C. For males, the pattern was qualitatively similar (i.e. a relatively larger abdomen under wet-season compared with dry-season conditions), but the overall difference between the highest and the lowest temperature was smaller than for females (Fig. 1c).

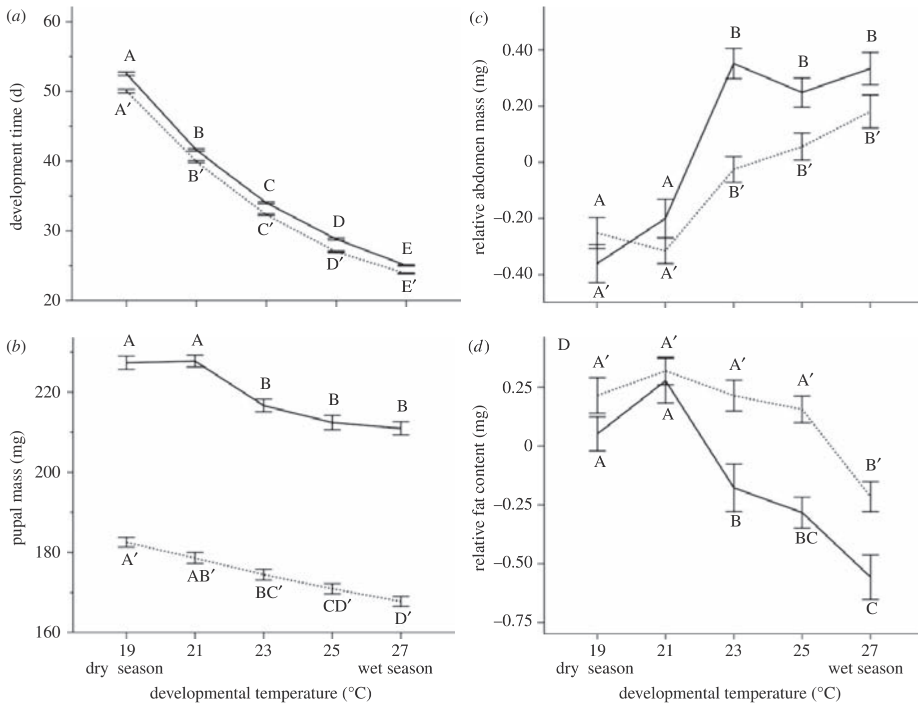

Figure 1. Effects of developmental temperature on (A) development time, (B) pupal mass, (C) relative abdomen mass (residuals from regression of abdomen dry mass on total dry mass) and (D) relative fat content (residuals from regression of fat content on dry mass). Females and males are represented by the solid and dotted lines, respectively. Error bars represent ± 1 s.e. with $50 < n < 150$. Significant differences across the temperature treatments (Tukey's HSD, $p < 0.05$) are indicated by different letters, coding for females and males separately.

At the three higher temperatures, females had lower adult relative fat content than males ($p < 0.01$), while at 19°C and 21°C males did not differ from females. Male relative fat content did not change along the temperature gradient, with the exception of 27°C, where it was lowest when compared with the other temperatures. In females, relative fat content decreased discontinuously with increasing developmental temperature (i.e. females developed highest fat content under dry-season conditions; Fig. 1d).

For both sexes and all developmental temperatures, adult RMR (the rate of CO_2 respiration at rest) was lower when measured at 19°C than when measured at 27°C ($p < 0.0001$; compare Fig. 2a with 2b). RMR at 19°C was higher for males when compared with females across the developmental temperature gradient ($p < 0.0001$). In both sexes, RMR measured at 19°C showed a discontinuous shift along the developmental temperature gradient; adults developed at lower temperature (dry-season conditions) had higher RMR at

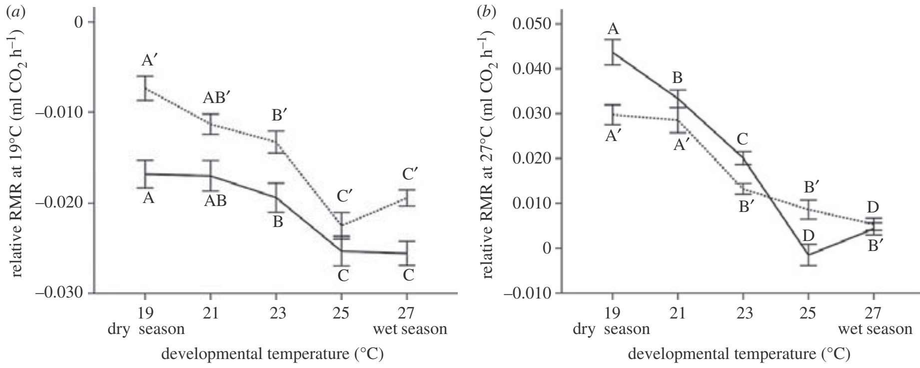

Figure 2. Effects of developmental temperature on adult relative RMR (residuals from regression of RMR on mass) measured at (A) 19°C and (B) 27°C. Note the difference in scale. Females and males are represented by the solid and dotted lines, respectively. Error bars represent ± 1 s.e. with n = 50. Significant differences across the temperature treatments (Tukey's HSD, $p < 0.05$) are indicated by different letters, coding for males and females separately.

19°C than those developed at higher temperature (Fig. 2a). In both sexes, RMR measured at 27°C showed a similar decrease with increasing developmental temperature (i.e. butterflies developed highest RMR when reared under dry-season conditions). In males, the response was discontinuous while in females it was almost linear, with the exception of the highest developmental temperature. Furthermore, at the lowest developmental temperature, RMR measured at 27°C was lower in males than in females, while this was not the case at the higher temperatures (Fig. 2b).

Finally, PC1 of wing pattern changed linearly along the temperature axis in both males and females. Butterflies developed larger eyespots when reared under wet-season conditions, and the reaction norms differed in elevation between the sexes ($p < 0.001$; Fig. 3).

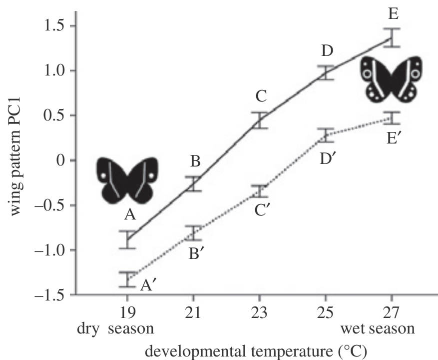

Figure 3. Effects of developmental temperature on the first principal component (PC1) of wing pattern, explaining 50.5% of variation in eyespot and band size. Females and males are represented by the solid and dotted lines, respectively. Error bars represent ± 1 s.e. with n = 50. Significant differences across the temperature treatments (Tukey's HSD, $p < 0.05$) are indicated by different letters, coding for females and males separately.

Dynamics and reaction norms of female hormone concentrations during pupal stage

Lack of diurnal cycle in hormone concentrations

Careful visual inspection of Ecd, 20E and JH-III concentrations plotted against time of day at which a sample was taken revealed no indication for a diurnal cycle for any of these hormones (cf. Zhao & Zera 2004). For Ecd and JH-III, no effect of time of day on concentration was found ($p > 0.1$). For 20E, there was a small but significant effect of time of day on concentration ($p = 0.05$), but *post hoc* comparisons between specific levels (i.e. hours) were not significant ($p > 0.1$).

Ecdysone and 20-hydroxyecdysone

Both ecdysteroids showed qualitatively similar dynamics during the pupal stage, with low early concentrations, peak concentrations between 20 to 40 per cent of pupal development (hours after pupation as percentage of total pupal development time), and low late concentrations (Fig. 4). For both hormones, concentrations were in a similar range across all temperature treatments (Ecd: approx. 30-1800 pg μl^{-1}; 20E: approx. 100-2600 pg μl^{-1}), but varied with time after pupation. Ecd concentrations were below detection levels very early and very late in the pupal stage.

While the absolute concentrations were similar across temperature treatments, the timing of increase, peak and decrease of hormone concentration showed a marked shift between the temperature treatments. We formally compared hormone dynamics throughout the pupal stage across temperature treatments by constructing, for each ecdysteroid separately, nonlinear regression models with hormone concentration as dependent variable and relative time after pupation (as fraction of total pupal time) as independent variable (see Materials and Methods). All models were significant (95% confidence interval (CI) for p: 0.0002-0.0040) and captured most of the variation (95% CI for R^2: 0.79-0.86). Using the estimated parameters for each model, we calculated peak concentrations and their timings.

Peak Ecd concentrations did not differ across temperature treatments ($p > 0.7$). However, there was a significant shift in the timing of peak concentrations with increasing developmental temperature. Concentrations peaked late at lower temperatures (dry-season conditions) and early at higher temperatures (wet-season conditions; $p < 0.0001$), with a discontinuous shift between these two types of dynamics occurring between 21°C and 23°C, and no changes between the two lower or among the three higher temperatures (Fig. 4a, c).

Similarly, peak 20E concentrations did not differ across temperature treatments ($p > 0.1$), but there was a significant shift in the timing of peak concentrations with increasing developmental temperature. At lower temperatures (dry-season conditions), 20E concentrations peaked late when compared with the higher temperatures (wet-season conditions; $p < 0.0001$), with a discontinuous shift occurring between 21°C and 23°C. Again, this shift was the only change in timing along the temperature gradient and no intermediate types of dynamics were observed at intermediate temperatures (Fig. 4b, c).

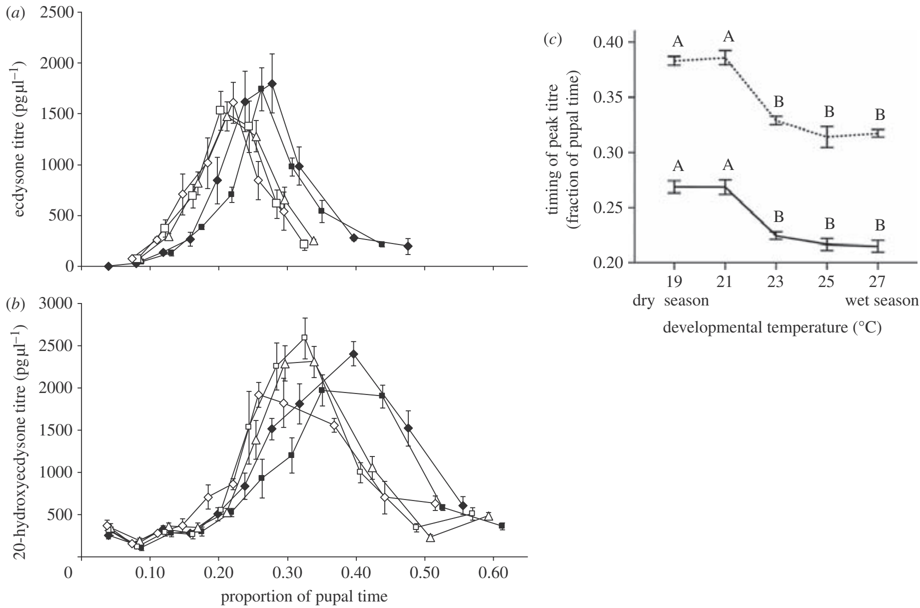

Figure 4. Effects of developmental temperature on ecdysone (Ecd) and 20-hydroxyecdysone (20E) dynamics during the pupal stage. Titres (± s.e.) throughout the pupal stage (fraction of total pupal development time) across the five temperature treatments (filled diamonds, 19°C; filled squares, 21°C; open triangles, 23°C; open squares, 25°C; open diamonds, 27°C) of (A) Ecd and (B) 20E. (C) Reaction norms of estimated time of peak (± s.e.) Ecd (solid line) and 20E (dashed line) titre for developmental temperature.

Ecd concentrations peaked earlier than those of 20E, with a time lag of approximately 10 per cent of pupal time (hours after pupation as percentage of total pupal development time), which was constant along the temperature gradient (Fig. 4c).

Juvenile hormones

JH-I was only detected in haemolymph of approximately 14 per cent of all pupae, with concentrations ranging from 1 to 35 pg μl^{-1} and no effect of developmental temperature. JH-II was below detection level in all analysed pupae. JH-III was detected in haemolymph of pupae of all developmental stages across all developmental temperatures, in a comparable range of concentrations (30-100 pg μl^{-1}; Fig. 5). There was no effect of relative time after pupation (as fraction of total pupal time) on JH-III titres ($p > 0.1$), but there was a small but significant effect of developmental temperature ($p < 0.0001$), with pupae developed at 19°C having highest, and pupae developed at 21°C lowest, JH-III concentrations.

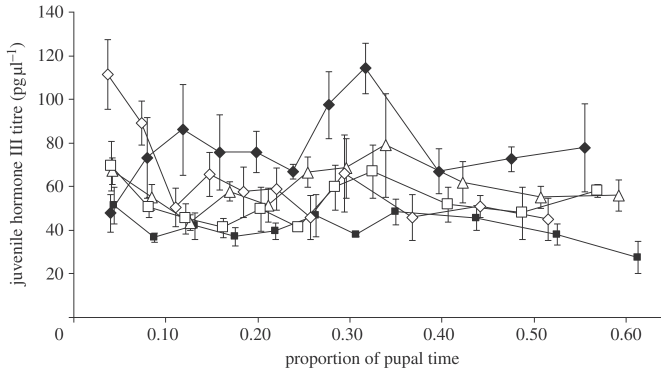

Figure 5. Dynamics during pupal stage of juvenile hormone (JH)-III titres (± s.e.) across the five temperature treatments. JH-I was only detected in approximately 14 per cent of all pupae, and JH-II was detected in none of the pupae. Filled diamonds, 19°C; filled squares, 21°C; open triangles, 23°C; open squares, 25°C; open diamonds, 27°C.

Discussion

Hormone dynamics

The discontinuous expression of a polyphenic trait across an environmental gradient requires some form of a switch mechanism between alternative developmental trajectories. This developmental switch could arise by a change in: (i) the hormone titre; (ii) the sensitivity to the hormone; (iii) the hormone timing; and (iv) the window of sensitivity (Nijhout 2003). Experimental studies have linked each of these scenarios to polyphenisms, either by direct measurement of titres, titre regulators or sensitivity, or indirectly by hormone manipulation. Examples include Ecd titre changes linked to horn length in beetles (Emlen & Nijhout 1999); morph-associated differences in JH dynamics in a wing-polymorphic crickets (Zhao & Zera 2004); and differences in the timing of Ecd release between the two wing-pattern morphs of butterflies (Brakefield *et al.* 1998). However, these studies typically concern the hormonal dynamics under only two environmental conditions, which makes it impossible to discern whether these hormonal changes are continuous or discrete. Analysing the precise shape of the reaction norm at the hormone level reveals how a continuous environmental trajectory is translated into discrete alternative developmental trajectories (e.g. Anstey *et al.* 2009).

For the first time, we show that the dichotomy between adult phenotypic morphs can already be programmed at the stage of hormone signalling during development. Our results reveal a discontinuous shift in the timing of peak Ecd and 20E titres in response to a linear gradient of developmental temperatures (Fig. 4c). Both hormones show this shift in timing between 21°C and 23°C, while there are no significant differences in timing between the two lower temperatures (dry-season conditions), nor among the three higher temperatures (wet-season conditions). None of the measured JHs show a clear response to the temperature gradient (Fig. 5) and all are, therefore, unlikely to be involved in regulating the

polyphenism. Our results indicate that a discontinuous response of ecdysteroid dynamics to the continuous environmental gradient underlies the polyphenism in *B. anynana*.

Phenotypic responses

In females, but not in males, the response of pupal mass to developmental temperature was discontinuous. Thus, despite large, continuous changes in development time in response to the temperature gradient (Fig. 1a), female larvae ultimately develop a discontinuous pattern in pupal mass (Fig. 1b). The correspondence between the responses of female pupal mass and ecdysteroid dynamics (Fig. 4) to the temperature gradient suggests that these traits share upstream regulators.

In adults, the relative contribution of the abdomen to total body mass showed a clear discontinuous response to developmental temperature (Fig. 1c), which was particularly pronounced in females. These results indicate a higher relative allocation to flight during the harsh dry season and to reproduction during the favourable wet season, especially in females. Adult fat content in males remained fairly constant across most developmental temperatures, with the exception of the highest, whereas the response was more discontinuous in females (Fig. 1d). The response of RMR ranged from clearly discontinuous to more linear (Fig. 2). Overall, the responses of a number of adult traits to the temperature gradient are strikingly similar to the discontinuous response of pupal ecdysteroid dynamics (Fig. 4), indicating a regulatory role for ecdysteroid signalling during the pupal stage in shaping adult physiological and allocation traits.

In accordance with earlier results (Brakefield & Reitsma 1991, Wijngaarden *et al.* 2002), we found a linear response of ventral wing pattern to the temperature gradient (Fig. 3), contrasting with the discontinuous reaction norm for pupal ecdysteroid dynamics (Fig. 4). Previous studies, using hormone manipulation and artificial selection experiments, have demonstrated a functional role of pupal ecdysteroids in the regulation of wing pattern polyphenism, as well as genetic correlations between dynamics of ecdysteroid titres and wing pattern (Koch *et al.* 1996, Brakefield *et al.* 1998, Zijlstra *et al.* 2004). Combined, these findings strongly imply an additional level of regulation between the hormone signal and the response of the developmental pathways producing the wing pattern. This regulation is likely to involve changes in the window of hormone sensitivity (for example by altered timing of Ecd receptor expression (Nijhout 2003, Brakefield *et al.* 1998).

Conclusions

By applying a continuous temperature gradient to developing larvae and pupae, we showed that a discontinuous ecdysteroid signal during the pupal stage underlies the seasonal polyphenism in *B. anynana*. Furthermore, several fitness traits (such as relative abdomen mass, RMR, female pupal mass and fat content) displayed a similar, dimorphic response, indicating shared regulation of these traits. In contrast, ventral wing pattern, known to be regulated by ecdysteroids (Zijlstra *et al.* 2004), responded in a linear manner. Taken together, our findings suggest that the diversity in shapes of reaction norms of the traits

involved in the phenotypic plasticity stems from variation in how each trait responds to the ecdysteroid dynamics.

In view of our results and of earlier findings that revealed a short window of sensitivity of the ventral wing pattern to 20E injections (Koch *et al.* 1996) and a complete lack of sensitivity of the dorsal wing pattern (Brakefield *et al.* 1998), we propose that variation across traits in windows of sensitivity to the systemic hormone signal can be a general mechanism underlying both linear and discrete responses to environmental gradients within suites of traits that share a hormonal regulator. This diversity in responses allows for both flexibility and integration of traits underlying adaptations to divergent environments.

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Supplementary material

Hormone titre measurements by liquid chromatography – mass spectrometry (LC-MS)

Hormone quantification from haemolymph followed, with minor modifications, the LC-MS method developed by Westerlund & Hoffmann (2004) and Westerlund (2004), which allows for simultaneous quantification of all hormones from the same sample. Pupal haemolymph (20 μl) was extracted with a glass capillary and deposited into 300 μl 1 : 1 methanol : isooctane (v/v) solution. The mixture was vortexed for 20 s, allowed to stand at room temperature for 20 min and then centrifuged at 10,000 × g at 6°C for 20 min. With a glass Pasteur pipette both phases of the supernatant were transferred to a new glass vial and stored at -80°C until further analysis. Prior to hormone titre quantification by LC-MS, the isooctane phase of the sample was evaporated and sample volume was reduced to 20 μl using a vacuum centrifuge. The sample was then centrifuged at 10,000 × g for 10 min, after which the supernatant was transferred to a new glass vial and placed in the autosampler (Shimadzu SIL-10ADVP) connected to an Eldex Micro Pro HPLC system. Samples were separated on a 150 × 2 mm C18 reversed-phase column (ReproSil-Pur ODS-3, 5 μm, Dr. Maisch-GmbH, Ammerbuch, Germany) protected by a guard column (C18 cartridge, Phenomenex, Aschaffenburg, Germany) at a flow rate of 200 μl min^{-1} and column temperature of 37°C. The mobile phase consisted of a methanol/water gradient varying between 30% and 100% over 20 min. MS analysis was performed using electrospray ionisation in the positive mode on a Shimadzu LCMS-2010A operating under the following conditions: Probe high voltage was set at 4.50 kV, CDL voltage at -5.0 V and temperature at 250°C, the heat block at 200°C. The nitrogen flow rate was 4 l min^{-1}. Post-run analysis was performed using the Shimadzu LCMSsolution Ver.3 software. To achieve absolute quantification of hormones, calibration curves were compiled by measuring a dilution series which consisted of samples made by spiking a *B. anynana* haemolymph mixture with a range of known concentrations of synthetic hormones: JH-I, JH-II (both purchased from SciTech, Prague, Czech Republic), JH-III, 20-hydroxyecdysone and ecdysone (all three purchased from Sigma Aldrich, USA). All solvents were HPLC grade.

Ecdysteroids link juvenile environment to adult life history syndrome in a seasonal insect

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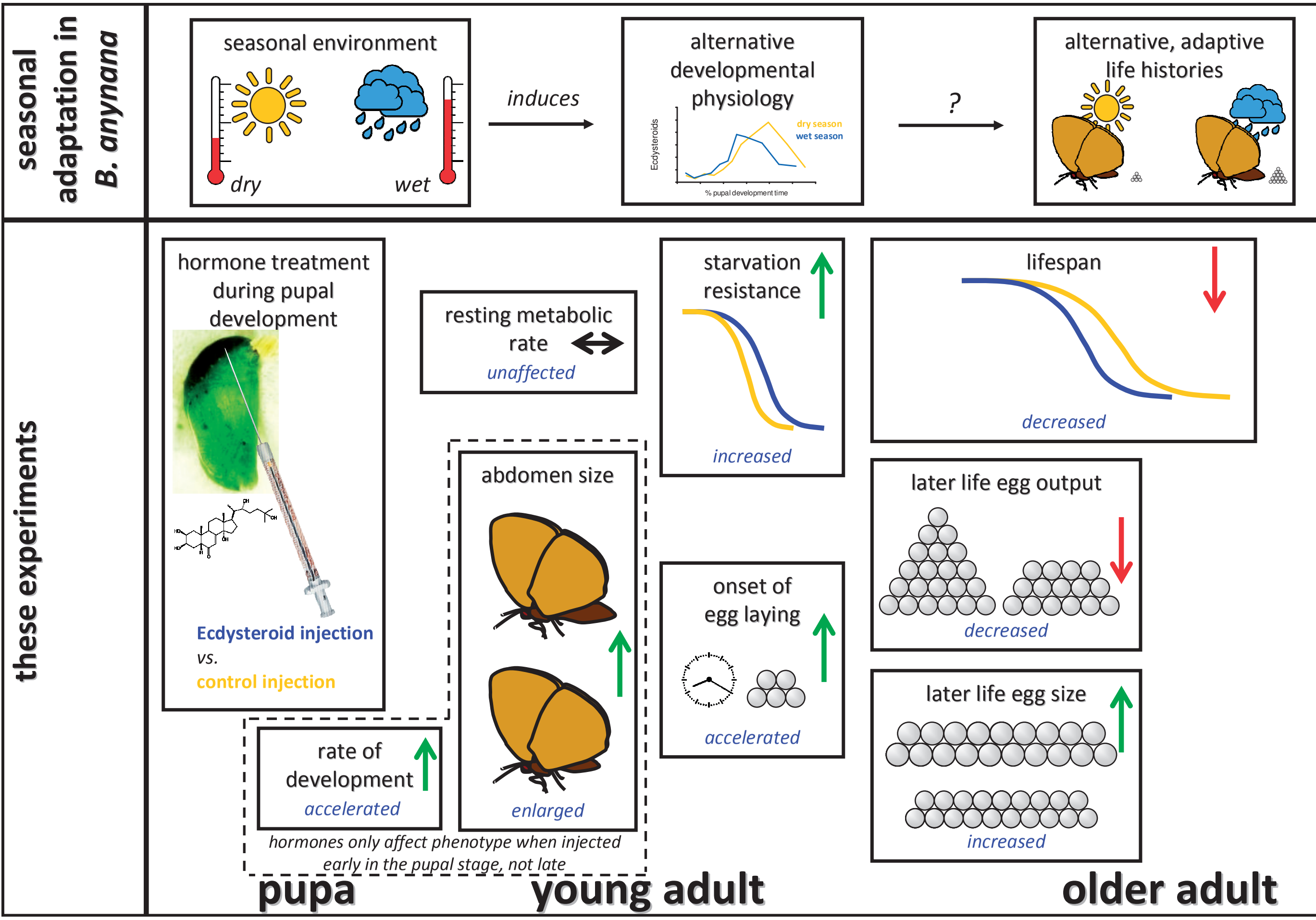
Abstract

The conditional expression of alternative life strategies is a widespread feature of animal life, and a pivotal adaptation to life in seasonal environments. The butterfly Bicyclus anynana expresses alternative adult life histories in its habitat's wet and dry seasons, as end points of divergent developmental pathways triggered by seasonal variation in pre-adult temperature. Pupal Ecdysteroid hormones also show distinct dynamics in each season, but whether they play a functional role in regulating the full seasonal syndrome is unknown. Here, we show that pupal Ecdysteroid levels can induce increased allocation of adult mass to the abdomen--a hallmark of the temperature-induced wet season morph. Crucially, this allocation shift is accompanied by changes in ecologically relevant traits, including timing of reproduction, lifespan and starvation resistance. Together, our results support a functional role for Ecdysteroids in translating predictive information on environmental quality during development into adaptive alterations in a suite of adult traits.

Manuscript submitted

Graphical abstract



Introduction

Understanding how animals cope with the seasonal fluctuations in environmental quality that characterise many temperate and tropical habitats is a key challenge in evolutionary ecology, and an important requirement if we want to predict ecological responses to climate change (Hofmann & Todgham 2010; Meylan *et al.* 2012; Visser *et al.* 2010). To optimally match suites of traits – i.e. the life history syndrome – to seasonally changing ecological opportunities, animals living in seasonal environments need mechanisms linking information on environmental quality to resource allocation decisions. In many animals, hormones provide such mechanisms (Beldade *et al.* 2011; Nijhout 2003; Simpson *et al.* 2011). They play crucial regulatory roles in transducing indicators of seasonal progression, such as temperature or photoperiod, into adaptive alterations of the phenotype, such as timing of reproduction or preparation for diapause (e.g. Brakefield & Zwaan 2011; Dawson 2008; Denlinger 2002). These same hormonal mechanisms are also involved in the regulation of some other instances of phenotypic plasticity when the environmental stimulus is not (directly) related to seasonality, such as crowding, e.g. in crickets and locusts (Simpson & Sword 2009; Zera 2009), nutrition, e.g. in nematodes, social insects and beetles (Emlen *et al.* 2012; Smith *et al.* 2008; Sommer & Ogawa 2011), or a combination of stimuli, e.g. in aphids (Brisson 2010). Understanding seasonal adaptations from an evolutionary perspective will require combining a detailed dissection of hormonal mechanisms of plasticity with ecological experiments seeking to examine the relationships between these mechanisms and fitness in the field (Beldade *et al.* 2011; Braendle *et al.* 2011; Gilbert 2012; Visser *et al.* 2010; Zera *et al.* 2007). However, in many cases of seasonal plasticity the opportunities to address the environmental sensitivity, the hormonal changes, the sensitivity of the target phenotype to the hormone, and the ecological relevance of the altered phenotype in the same system are limited. Here, we take an integrative approach and study seasonal adaptation in the butterfly *Bicyclus anynana* from the developmental and hormonal mechanism through to the alternative life history strategies relevant for natural populations.

The East African butterfly *B. anynana* expresses distinct life strategies in each season. During the warm wet season, larval and adult food is plentiful, larvae develop fast and adults live active lives with rapid reproduction and relatively short lifespans. In contrast, during the cool dry season characterised by no larval resources and adult food scarcity, adults display a higher investment in body reserves, have longer lifespans and postpone reproduction (Brakefield & Reitsma 1991; Brakefield & Zwaan 2011). These phenotypic differences are determined by the seasonal temperatures that the larvae and pupae experience during development, with a high temperature signalling the wet season and a declining temperature predicting the approaching dry season (Brakefield & Reitsma 1991). In the laboratory, several aspects of these alternate life histories can be induced by development at different temperatures (de Jong *et al.* 2010; Fischer *et al.* 2003; Pijpe *et al.* 2007; Steigenga & Fischer 2007). Recently, we showed that females reared at high temperatures (wet season conditions) develop a relatively larger abdomen compared to those reared at low temperatures (dry season conditions). This response is discontinuous,

with a threshold at an intermediate temperature (Oostra *et al.* 2011). Resting metabolic rate (RMR) in young adults is also affected by developmental temperature: butterflies developed at low temperatures have a higher RMR as adults, irrespective of adult temperatures (Oostra *et al.* 2011; Pijpe *et al.* 2007). The proximate mechanisms linking pre-adult temperatures to adult phenotype are unknown, but previous observations suggest an involvement of Ecdysteroid hormones during the pupal stage. Seasonal temperatures experienced during larval development drive dynamics of pupal Ecdysteroids, with an earlier peak in hormone concentration in pupae reared at high versus low temperatures (Brakefield *et al.* 1998; Koch *et al.* 1996). A detailed characterisation of hormonal reaction norms showed that the shift in hormone dynamics is discontinuous, with a similar shape and identical threshold temperature as the phenotypic reaction norm for female abdomen size (Oostra *et al.* 2011). Together, these correlative studies suggested that Ecdysteroid signalling is a regulator of the developmental plasticity in life history.

The first aim of the present study was to establish the extent to which pupal Ecdysteroids play a functional role in inducing the full seasonal syndrome in response to developmental temperature. We approached this question by manipulating Ecdysteroids in pupae reared at three different temperatures spanning the range of natural seasonal environments (Brakefield & Reitsma 1991), and then monitoring the phenotypic effects for a suite of seasonally plastic traits: 1) pupal development time, 2) adult RMR, 3) allocation of adult body mass to abdomen, and 4) adult fat content.

The second aim of this study was to assess windows of hormone sensitivity during the pupal stage. In our previous experiments, we observed differences in thermal responses among traits putatively regulated by the same hormone, and suggested that these could arise as a result of differences among traits in their windows of sensitivity to that hormone (Oostra *et al.* 2011). To assess hormone sensitivity across time, each pupa was injected at one of four separate time points, representing different stages of the natural dynamics in Ecdysteroid concentrations during the pupal stage (Brakefield *et al.* 1998; Oostra *et al.* 2011; Zijlstra *et al.* 2004).

Our third goal was to test in an independent follow-up experiment, the ecological consequences of any hormone-induced changes in morphology and physiology observed in the initial experiment. We again manipulated Ecdysteroids, focussing on a single temperature and injection time point, and monitored effects on multiple aspects of adult fitness: 1) onset of oviposition, 2) early life fecundity, 3) egg size, 4) lifespan and 5) starvation resistance.

In this study, we show that Ecdysteroids are responsible for the temperature-induced seasonal developmental plasticity of allocation of body resources to the abdomen in *B. anynana* females. In addition, we demonstrate that the Ecdysteroid-induced allocation changes have consequences for fitness: pupal hormone injections accelerate onset of oviposition and increase egg size, but reduce fecundity later in life as well as lifespan. These results support a functional role for Ecdysteroids in reproductive investment decisions during development in response to variation in environmental quality, and provide insight into mechanisms enabling organisms to persist in fluctuating environments.

Materials and methods

Experimental design

We first performed a full factorial experiment with three developmental temperatures and four injection time points. Immediately after hatching, larvae were divided over three temperature treatments: 19, 23 and 27°C. We recorded pupations to the nearest 15 minutes using time-lapse photography, and assigned female pupae to one of four injection time points: 3, 16, 29 or 34% of total pupal development time (DT). Pupae were injected with either 20-hydroxyecdysone (20E) or control solutions, after which they were allowed to continue development and eclose individually at their respective larval temperatures. After eclosion, we measured resting metabolic rate (RMR) and abdominal dry weight and fat content in N = 15-45 per temperature per injection time point per injection treatment. In a follow-up experiment, we reared larvae at 23°C, injected the pupae at 16% DT, and measured fecundity, lifespan and starvation resistance in the adult females (N = 50-80 per injection treatment). In both experiments, all larvae were derived from the same outbred *B. anynana* captive population and reared on young maize plants sprayed with an antifungal agent (Brakefield *et al.* 2009) for rearing protocols).

Hormone injections

Fresh injection solutions were prepared daily by combining 107 µl 1x Ringer's physiological solution with 3 µl Vital Red dye (Fluka) and either 10 µl 100% ethanol (control treatments) or 10 µl 1 mg / ml 20E (Sigma) in 100% EtOH (hormone treatments). Using a 10 µl Hamilton micro syringe with a 0.3 mm needle, we injected pupae laterally between the 4th and 5th abdominal segments, with 3 µl injection solution (0 or 0.25 µg 20E for the control and hormone treatments, respectively), injecting each female only once. Previous studies on pupal Ecdysteroids in *B. anynana* yielded detailed knowledge on natural 20E concentrations as well as dose-response curves for mortality (Brakefield *et al.* 1998; Koch *et al.* 1996; Zijlstra *et al.* 2004), enabling us to inject a hormone amount well within physiological ranges (Zera 2007).

Measurements of phenotypic responses

a. First experiment: pupal development time, RMR, abdominal dry weight and fat content

All pupae were weighed to the nearest 0.1 mg within 36 hours of pupation. In the first experiment, a subset of pupae (*ca.* 20%) was kept separately to measure pupal development time with 15 minutes precision. We monitored these pupae towards the end of the pupal period and recorded new eclosions every 15 minutes by time-lapse photography. One day after eclosion, we measured RMR for each female as the individual rate of CO_2 respiration (ml per hour) over a period of 20 min, following (Pijpe *et al.* 2007). All RMR measurements were done at 27°C during the dark phase of the diurnal cycle. Next, abdomens were cut off to measure their dry weight, extract total fat (triglyceride and free fatty acids) and measure fat-free dry weight following (Oostra *et al.* 2011). Fat content was calculated by subtracting the fat-free dry weight from the initial dry mass.

b. Second experiment: fecundity, lifespan and starvation resistance

One day after eclosion, we weighed each adult female to the nearest 0.1 mg and introduced her into a mating cage with 10-30 virgin males (3-10 days old), keeping the ratio of females to males in these cages below one. We inspected the cages every 15 minutes and separated mating pairs into cylindrical oviposition pots. After each mating had finished, we removed the male and provided the female with *ad libitum* food and a fresh cutting of *Oplismenus sp.* grass for oviposition. After 72 hours we moved the female to a new pot. This was repeated three times, yielding a total of four consecutive egg measurement periods with age classes of: 2-4, 5-7, 8-10, and 11-13 days. After each period, we counted the total number of eggs in the oviposition pot. To estimate egg size, we photographed the spherical eggs against a black background using a Leica DC200 digital still camera connected to a Leica MZ12 stereo microscope (3.2X magnification). On every image, we measured egg area as a measure of egg size (Fischer *et al.* 2003), using an automated macro in ImageJ software. After four egg measurement periods covering the 12 days after mating, we transferred females to larger cages, with a maximum of 10 females per cage, provided oviposition plants and *ad libitum* food, and monitored survival daily. We excluded from analysis females that laid only unfertilised eggs.

Each day, we separated a fraction of newly eclosed females and excluded them from the fecundity assay. Instead, we kept them virgin, introduced them into larger cages with a maximum of 15 females per cage, and provided them with *ad libitum* access to water (wet cotton) but not food to record starvation resistance (SR). We scored and removed dead females twice a day.

Statistical analyses

In the first experiment we analysed data for each time point separately, using a two-way analysis of variance (ANOVA) for each phenotypic trait, with rearing temperature and injection treatment as fixed variables. Pupal development time was natural log transformed. We analysed RMR, abdomen dry weight, abdomen fat content and abdomen fat-free dry weight first in separate linear regressions models with pupal mass as the only predictor variable (see Table S1 in Supporting Information), and subsequently used the residuals of these regressions as dependent variables in the two-way ANOVAs. Post hoc comparisons between 20E and control treated females at specific temperatures were performed with Tukey's honest significant differences (HSD) tests.

In the second experiment, fecundity was strongly non-normally distributed during the first egg measurement period (age 2-4 days), as a large fraction of females had not yet laid any eggs in this period. Therefore we chose to analyse this first period separately, treating fecundity as a categorical variables: females either had or had not started to lay eggs in this period. Numbers of females in each category were compared between injection treatments using a χ^2 test. For the three subsequent egg-laying periods (ages 5-13 days), we analysed fecundity using a repeated measures general linear model (GLM) with injection treatment and age as fixed variables, and individual as random variable. In order to obtain p-values for each main effect, we constructed a model without the main effect and compared it to the

full model with a likelihood-ratio test. For specific comparisons at each age class between 20E and control treated females, we obtained p-values using a Markov Chain Monte Carlo method (Baayen 2011). We also analysed egg size using a repeated measures GLM with injection treatment and age as fixed variables, and individual as random variable. We analysed lifespan and starvation resistance using a Cox proportional hazard model with adult mass as covariate and injection treatment as fixed variable; age at death was used as the dependent variable. All analyses were performed in R (R Development Core Team 2010) with packages *survival* (Therneau 2012), *lme4* (Bates *et al.* 2011) and *languageR* (Baayen 2011).

Results

Ecdysteroids accelerate pupal development and increase adult mass allocation to abdomen

20E treatment induced a substantial acceleration of pupal development when pupae were injected at 3 and 16, but not at 29% DT (Fig 1; Table S2 in Supporting Information). Pupae reared at 27°C showed the weakest response to early 20E treatment compared to pupae reared at the other temperatures, and at 34% DT 20E treatment had the reverse effect on these pupae: development was slowed rather than accelerated (Tukey's HSD $p < 0.0005$). The overall acceleration in development upon injections earlier in development was due to a higher proportion of butterflies eclosing a full day or more earlier, and was not accompanied by a change in time of day at which they eclosed (data not shown).

Relative abdomen mass (size-corrected abdomen dry mass) was substantially increased after pupal 20E injection at 3 or 16%, but not at 29 or 34% DT (Fig. 3; Table S2). In addition, at 3% DT, hormone treatment and rearing temperature interacted (Table S2) in such a way that pupae reared at 19°C or 23°C responded to 20E treatment (Tukey's HSD $p < 0.05$) while those reared at 27°C did not. This suggests a period of Ecdysteroid sensitivity during development of the abdomen, which appears to come earlier at the two lower temperatures relative to 27°C. The effect of 20E treatment on relative abdomen mass is similar in magnitude and direction to the effect of developmental temperature (Fig. 3; Table S2). Thus, exogenous Ecdysteroids phenocopy the temperature-induced seasonal differences in abdomen size.

We then asked whether this hormone-induced increase in abdomen mass was due to an increase in fat content, fat-free dry weight, or both. Abdominal fat content was higher in females injected as pupae with 20E compared to controls for manipulations at 3 and 16% DT, but not at 29 or 34% DT (Fig. 4; Table S2). Again, at 3% DT we observed a significant interaction with temperature (Table S2); pupae reared at 19 and 23°C showed a response to 20E (Tukey's HSD $p < 0.001$), whereas those at 27°C did not. Likewise, abdominal fat-free dry weight increased in response to pupal 20E injections, but again only when injected at 3 and 16% and not at 29 or 34% (Fig. S1 and Table S2 in Supporting Information). At 3% DT we observed an interaction between treatment and temperature (Table S2), with pupae reared at 23°C showing a significant response to 20E (Tukey's HSD $p < 0.05$), whereas those reared at 19°C (Tukey's HSD $p = 0.11$) or 27°C (Tukey's HSD $p = 0.40$) did not. Considered

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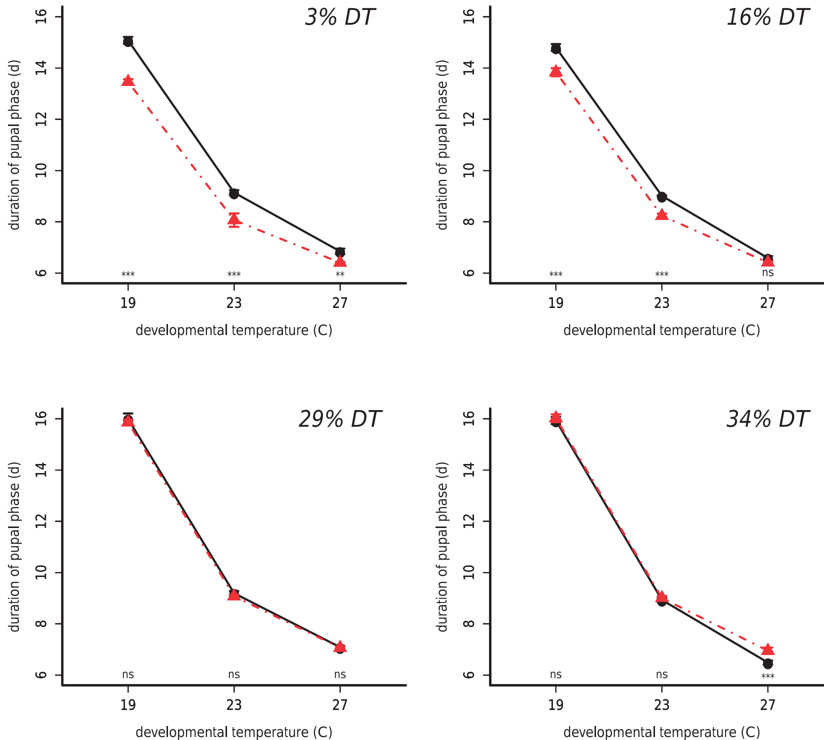

Figure 1. Early but not late 20-hydroxyecdysone (20E) treatment accelerates pupal development. Duration of pupal stage (days, ±SEM) is strongly affected by developmental temperature, as indicated by the shape of reaction norms and large differences between extreme temperatures (two-way ANOVA $p < 0.00001$). In addition, pupae injected with 20E (red triangles and line) at 3 or 16% of pupal development time (DT) show significant acceleration of development in comparison with controls (black circles and line; two-way ANOVA $p < 0.00001$), while those injected at 29 or 34% DT show no such effect. Late injections (34% DT) decelerate development, but only at 27°C (Tukey's HSD $p < 0.001$). See also Table S2. Asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant differences between control and 20E treated animals; in the case of significant temperature x treatment interaction in two-way ANOVAs, p values from post-hoc Tukey's HSD are reported; when this interaction was not significant, the overall treatment effect of the two-way ANOVA is given.

together, we conclude that the increase in abdomen mass in the females injected with 20E as pupae in the earlier time points was due to an increase in both fat and non-fat mass, with both traits showing an identical window of sensitivity to the 20E injections.

Developmental imprint on adult RMR is not affected by Ecdysteroids

We found no evidence for a role for Ecdysteroids in mediating the pre-adult temperature effect on adult RMR. As observed previously (Oostra *et al.* 2011; Pijpe *et al.* 2007), RMR corrected for body size (see Table S1) was higher in females developed at lower temperatures.

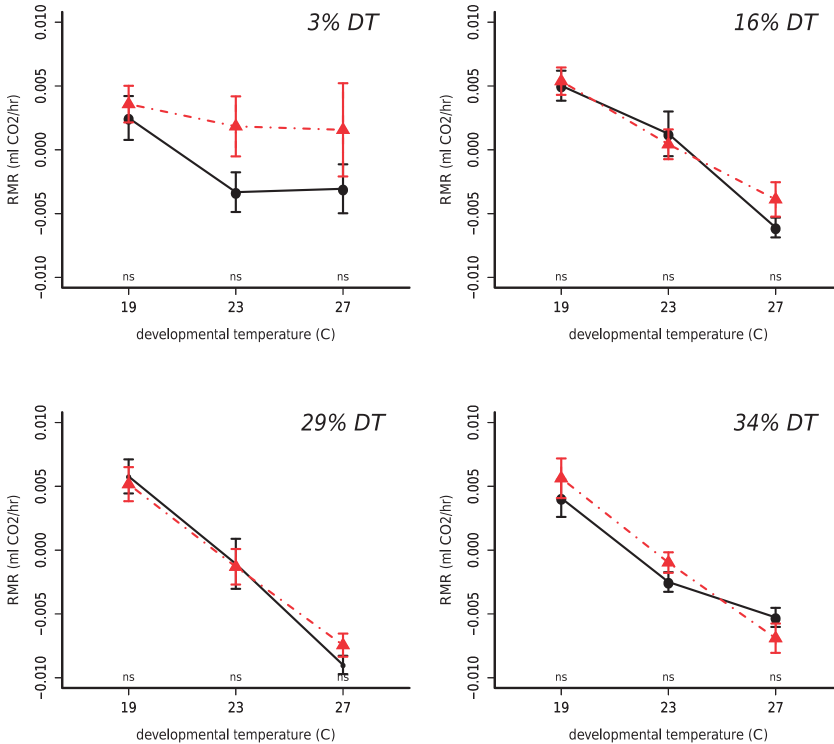

Figure 2. Developmental temperature imprint on adult resting metabolic rate (RMR) is not affected by pupal Ecdysteroids. Mass-corrected RMR (ml CO_2 hr^{-1}; see Table S1) is significantly affected by developmental temperature with individuals reared at lower temperature having higher RMR (two-way ANOVA $p < 0.05$). However, 20E treatment in the pupal stage has no significant effect on RMR at any of the four injection time points (compare black and red reaction norms). See also Table S2. For legend see Fig. 1.

However, we observed no significant effect of 20E treatment on size-corrected RMR for any of the four injection time points at any of the three temperatures (Fig. 2; Table S2).

Pupae show a limited window of sensitivity to Ecdysteroid manipulation

Pupal sensitivity to 20E treatment was not constant in time. Pupal development rate, abdomen dry weight and fat content were most strongly affected by injections at the two earlier time points (3 and 16% DT; Figs. 1, 3, 4), when natural Ecdysone titres are rising. In contrast, later in the pupal stage (29 and 34% DT), when natural Ecdysone titres are decreasing (Oostra *et al.* 2011), these traits showed little if any response to injections. Furthermore, this window of hormone sensitivity was affected by the temperature at which the pupae had developed. Pupae from 19°C or 23°C developed an enlarged abdomen and accelerated pupal development rate in response to 20E injections at both 3 and 16% DT. However, those reared at the wet season temperature of 27°C only developed an enlarged

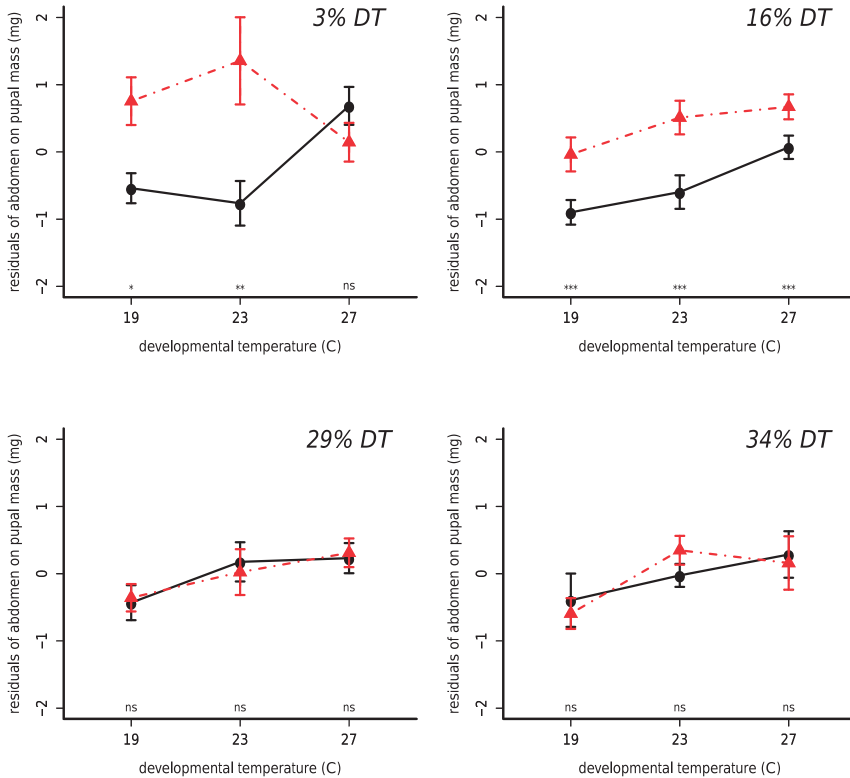

Figure 3. Pupal Ecdysteroids induce high, wet-season like allocation to abdomen mass. Mass-corrected abdomen dry weight (mg; see Table S1) is significantly affected by developmental temperature with females reared at high temperatures (wet season conditions) having a larger abdomen (two-way ANOVA $p < 0.05$). In addition, pupae injected with 20E (red) at 3 or 16, but not at 29 or 34% DT, show a substantial increase in abdomen mass compared to controls (black), similar in magnitude and direction to the temperature effect (two-way ANOVA $p < 0.001$). The earliest injection only affects females reared at 19 or 23, not at 27°C (two-way ANOVA $p < 0.05$ for temperature x treatment interaction). See also Table S2 and Fig. S1. For legend see Fig 1.

abdomen when injected at 16, not 3% DT, and accelerated development when injected at 3, not 16% DT. In the same 27°C cohort (and not at 19 or 23°C), late injections at 34% DT had the reverse effect on rate of development compared to injections at 3 and 16% DT: development was slowed rather than accelerated.

Pupal Ecdysteroids affect reproductive schedule, lifespan and starvation resistance

To assess whether the observed induction of relatively larger, wet season-like abdomens by pupal Ecdysteroid levels has fitness consequences for the adult life history, we reared an independent cohort of larvae at 23°C, injected females at 16% of pupal development time,

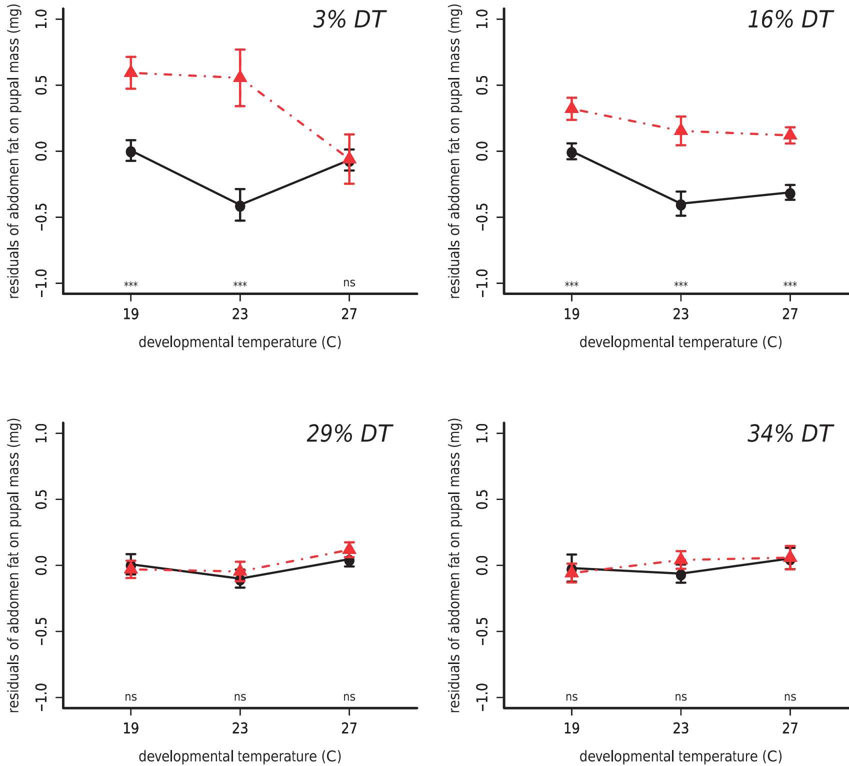

Figure 4. Pupal Ecdysteroids induce higher abdominal fat content in adult females. Mass-corrected abdomen fat content (mg; see Table S1) is significantly affected by temperature (two-way ANOVA $p < 0.05$), except in the cohorts injected at 34% DT. In addition, pupae injected with 20E (red) at 3 or 16, but not at 29 or 34% DT, show a substantial increase in fat content compared to controls (black; two-way ANOVA $p < 0.001$). Similar to the pattern observed for abdomen mass, the earliest injection only affects females reared at 19 or 23, not 27°C (two-way ANOVA $p < 0.01$ for temperature x treatment interaction). See also Table S2 and Fig. S1. For legend see Fig 1.

and measured effects on adult performance. We focused on this temperature and time point because they revealed the largest effects of Ecdysteroids on abdomen size in the first set of experiments (Fig. 3).

One day after eclosion, females were mated and allowed to oviposit for four consecutive periods of three days. In the first period of oviposition (age 2-4 days), not all females had started laying eggs. Among the control treated females, 35% had not laid their first egg during this period, while this percentage was less than half (17%) among the 20E treated individuals (Fig. 5B). Thus, 20E treatment during the early pupal stage significantly accelerated the onset of first egg laying (χ^2 $p < 0.05$; Table S3), resulting in a *ca.* 31% increase in mean number of eggs produced in this period (Fig. 5A). Among those females laid eggs in this period, there was no significant difference in mean number of eggs between the 20E and control treated

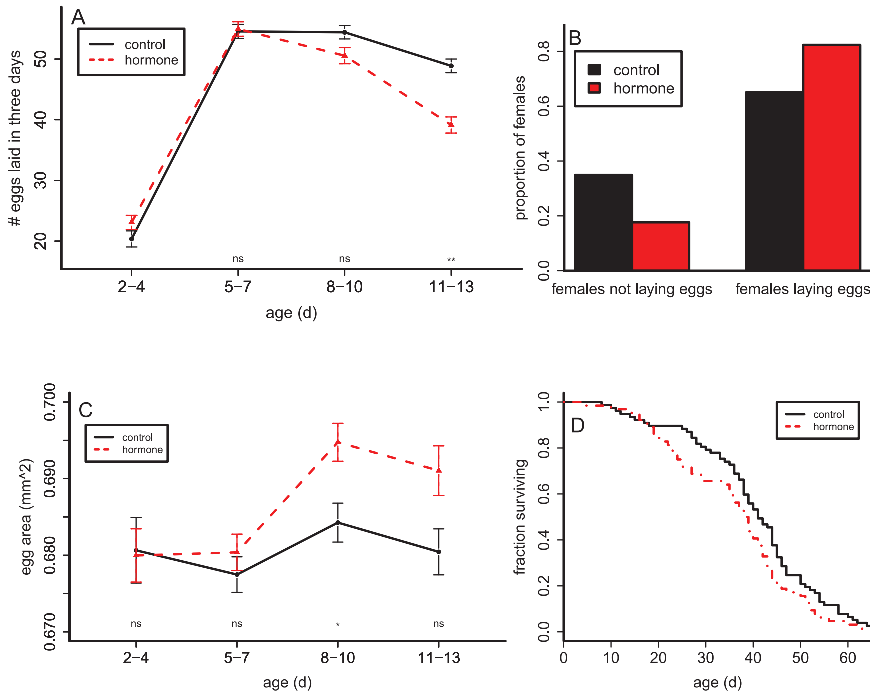

Figure 5. Pupal Ecdysteroids affect reproductive schedule and lifespan. A) Female fecundity (number of eggs laid) is highly affected by female age ($p < 0.00001$ for likelihood ratio test (LRT) between model with and without age). In addition, adult females injected as pupa with 20E (red) had lower fecundity compared to controls (black), but only later in life ($p < 0.001$ for LRT with and without treatment x age interaction). B) Pupal Ecdysteroids accelerate onset of oviposition. Proportion of females that have already started laying eggs at age 4 days is significantly higher when injected as pupa with 20E (red bars) than when injected with control solution (black bars; χ^2 $p < 0.05$). All females not laying eggs at age 4 days did lay eggs later in life. C) Pupal Ecdysteroids induce increased egg size. Egg area (mm^2) is significantly affected by female age ($p < 0.00001$ for likelihood ratio test (LRT) between model with and without age), and females injected as pupa with 20E (red) lay larger eggs than control females (black), but only at age 8-10 days ($p < 0.05$ for LRT with and without treatment x age interaction). D) Pupal Ecdysteroids reduce adult lifespan of mated females. Daily adult survival under *ad libitum* food is reduced in mated females injected as pupa with 20E (red) compared to controls (black; Cox proportional hazard $p = 0.05$; hazard ratio = 1.38). Lifespan reduction was stronger for females that had started laying eggs before age 4 d (Cox proportional hazard $p < 0.05$; hazard ratio = 1.58) than for those that did not lay eggs before age 4 d. See also Table S3.

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group (Table S3). This indicates that Ecdysteroids probably do not increase the rate of egg production once it has started, but instead bring forward the onset of oviposition.

Later in life, after the peak in egg laying, the 20E treated females laid fewer eggs compared to control females (Fig. 5A; Table S3); at age 8-10 days the reduction was 9% (MCMC $p = 0.19$, see Materials and Methods) but in the final oviposition period that was monitored

(age 11-13 days) the difference was more substantial (23%, MCMC $p < 0.005$). Although the total number of eggs produced in all four oviposition periods combined was 7% lower in the 20E treated females compared to controls, this effect was not significant (Table S3). Thus, it appears that pupal 20E treatment, while accelerating the onset of oviposition, inflicts a fecundity cost later in life by accelerating the normal age-related decline in fecundity.

Since females can adjust their egg size and number (Fischer *et al.* 2003), we wanted to know whether the decrease in later-life fecundity was offset by an increase in egg size. This was indeed the case: eggs of the 20E treated females were larger compared to control treated females (Fig. 5C; Table S3). However, this was only observed at age 8-10 days (MCMC $p < 0.05$) and to a lesser extent at age 11-13 days (MCMC $p = 0.07$).

After the final fecundity measurements (age 13 days), we monitored individual daily survival. Females treated with 20E as pupa lived, on average, 4.7 days (12%) shorter than control females (Fig. 5D; Cox proportional hazard $p = 0.05$; hazard ratio = 1.38; Table S3). Splitting the females into two groups according to early reproductive status revealed that the negative effect of 20E treatment on lifespan was only significant for those females that had reproduced before the age of 4 days; the females that showed accelerated egg laying in response to 20E showed reduced lifespan (Cox proportional hazard $p < 0.05$; hazard ratio = 1.58; Table S3), while those that did not lay eggs in that period showed the same lifespan as control females. It appears that, in addition to reducing fecundity later in life (Fig. 5A), Ecdysteroid-induced acceleration in onset of oviposition (Fig. 5B) inflicts a fitness cost on lifespan (Fig. 5D).

The increased allocation to abdomen mass in the Ecdysteroid-injected females observed in the first experiment (Fig. 3) could also have been related to aspects of adult performance other than fecundity. In particular, both non-fat and fat mass were increased in these females (Fig. 4, Fig. S1) which could contribute to survival under starvation (Zwaan *et al.* 1991). To test this hypothesis, we measured starvation resistance (SR) in adult females from the cohort of larvae reared at 23°C and injected at 16% pupal DT. We found that 20E treated females survived, on average, *ca.* 1 day (8%) longer without food compared to the control treated females (Fig. 6; Cox proportional hazard $p < 0.01$; hazard ratio = 0.68). In addition, smaller females showed the largest increase in adult SR when injected with 20E

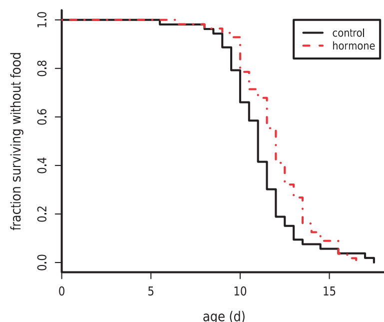

Figure 6. Pupal Ecdysteroids enhance adult starvation resistance in virgin females. Daily adult survival without food is increased in virgin females injected as pupa with 20E (red) compared to controls (black; Cox proportional hazard $p < 0.01$; hazard ratio = 0.68). See also Table S3.

(Cox proportional hazard $p < 0.05$ for mass x treatment interaction; Table S3). This suggests that virgin females with an Ecdysteroid-induced increased abdomen mass are able to use the increased abdominal resources to live longer when confronted with food stress.

Discussion

Our hormonal manipulations of pupae reared at a range of seasonal conditions revealed that pupal Ecdysteroid hormone titres provide the crucial link between temperature during development and adult abdomen size. Manipulation of Ecdysteroid levels during pupal development induces similar changes in allocation of adult mass to reproductive function normally induced by developmental temperature (Fig. 3; Table S2). By injecting at four different time points, we found that the window of sensitivity is open earlier (relative to the total duration of the pupal stage) for pupae reared at 19 and 23°C compared to 27°C. However, at all temperatures the window of sensitivity was restricted to the early pupal period, when natural hormone concentrations in wet season pupae are already high, but those of dry season pupae are still low (Oostra *et al.* 2011).

To probe the ecological relevance of the hormonal link between developmental temperature and abdomen size, we measured performance traits in adult females injected as pupa with Ecdysteroids. We found that the enlarged abdomens, typical of the reproductive wet season form and which we show to be induced by hormone treatment, indeed translate to the fitness level: injected females accelerate the onset of oviposition at the expense of fecundity later in life, but partly offset this decrease in fecundity by an increase in egg size. They also show (slightly) reduced lifespan (Fig. 5; Table S3). Thus, Ecdysteroids after pupation mediate strategic adult reproductive investment decisions in response to variation in the quality of the environment.

As reported previously for *B. anynana* (Koch *et al.* 1996; Zijlstra *et al.* 2004), exogenous Ecdysteroids applied early in the pupal stage accelerate pupal development. In the present study, we included two additional, later injection time points and found no such hormone-induced acceleration later in the pupal stage (Fig. 1; Table S2). Thus, as was the case for abdomen size, we observed a restricted window of sensitivity to hormone manipulations. In both cases, sensitivity was limited to the earliest 16% of the pupal stage. Pupal development time and timing of Ecdysteroid pulses in the pupal stage are genetically correlated (Zijlstra *et al.* 2004), and discrete variation in timing of Ecdysteroid pulses in the pupal stage is phenotypically correlated with adult reproductive allocation (Oostra *et al.* 2011). These observations suggest changes in timing of developmental events as a mechanism by which Ecdysteroids induce the alternate seasonal morphs in *B. anynana*. In the wet season, an early Ecdysteroid pulse would accelerate development, resulting in an increased abdomen size and accelerated onset of oviposition. This is consistent with the well-known function of Ecdysteroids as a developmental timer during the larval stage (Klowden 2007). The increased egg size and lowered fecundity later in life, as well as the decreased lifespan we observed in the Ecdysteroid injected females could be indirect consequences, mediated

by mechanisms other than the pupal Ecdysteroid signal. Developmental plasticity in *B. anynana* might also share components of its regulatory mechanisms with larval and pupal diapause expression in other insects, which has been linked to Ecdysteroids (Denlinger 2002). In some cases, Ecdysteroid titres are lower in diapausing larvae or pupae (e.g. Koch 1996; Munyiri & Ishikawa 2004), and in other cases exogenous Ecdysteroid applications terminate diapause and induce the continuation of normal development (Arpagaus *et al.* 1986; Singtripop *et al.* 1999).

In adult insects, Ecdysteroids interplay with other hormones (in particular Juvenile hormones) to regulate several aspects of female reproduction (Klowden 2007). For example, ovarian growth in young *Gryllus firmus* adults is positively correlated with Ecdysteroid titres (Zera 2009). Mutant *Drosophila melanogaster* females with reduced Ecdysteroid signalling show reduced rates of oocyte maturation or oviposition, as well as increased lifespan (Schwedes & Carney 2012). Adult reproductive diapause in *D. melanogaster* females, characterised by arrested reproductive development and increased lifespan (Schmidt 2011), can be terminated by Ecdysteroid injection (Richard *et al.* 2001). Such a reproductive function of Ecdysteroids in adult females is consistent with the increased abdomen size and accelerated onset of oviposition we observed in Ecdysteroid-injected *B. anynana* females, suggesting some overlap in function between Ecdysteroid signalling in the pupal and adult stages. Pupal Ecdysteroids might affect adult reproductive function in *B. anynana* by accelerating the maturation of developing ovarioles as in some other Lepidoptera where larval or pupal Ecdysteroids are required for oocyte maturation and vitellogenesis (e.g. Tsuchida *et al.* 1987). As *B. anynana* belongs to a group of Lepidoptera in which oocytes mature after eclosion (Ramaswamy *et al.* 1997) and no vitellogenins are yet detectable in pupae or freshly eclosed females (Geister *et al.* 2008), the Ecdysteroid signal is probably transduced to accelerated oocyte maturation and vitellogenesis via an intermediate developmental cascade. The lack of phenotypic response to our late injections (Fig 3) indicates that only the earlier part of this cascade is sensitive to Ecdysteroids. In this scenario, during a transient sensitive stage of pupal development, Ecdysteroids would act as a switch between alternate developmental cascades that ultimately lead to the observed adult reproductive strategies and trade-offs later in life (*cf*. Nijhout 2003). It remains to be tested whether other traits that commonly trade off with reproductive investment, such as flight ability (Zera 2009), are also integrated into this hormone-mediated seasonal syndrome. One indication that this might indeed be the case is the observation that larval food stress-induced allocation to thorax at the expense of abdomen increases flight endurance in adults (Saastamoinen *et al.* 2010), which a modelling approach shows to be an adaptive response (van den Heuvel *et al.* 2013).

In contrast to their effects on abdomen size, development time and adult reproductive strategy, exogenously applied Ecdysteroids did not affect adult RMR. Previous studies in *B. anynana* and other insects reported a negative effect of developmental temperature on adult RMR (Berrigan 1997; Le Lann *et al.* 2011; Pijpe *et al.* 2007), and in the opposite direction to the positive effect of adult acclimation temperature (Oostra *et al.* 2011). We

confirmed the developmental imprint of temperature on adult RMR, but showed that hormone manipulations did not induce changes in RMR at any of the tested time points or rearing temperatures (Fig. 2; Table S2). This reveals that, despite a correlated response with developmental temperature, RMR and pupal Ecdysteroid signalling are not functionally linked. Thus, the developmental temperature imprint is independent of pupal Ecdysteroid signalling and probably originates during the larval stage (*cf*. Pijpe *et al.* 2007).

Adult RMR and SR show a negative phenotypic correlation in *B. anynana*, responding in opposite directions to developmental temperature (Pijpe *et al.* 2007). Nevertheless, here we uncovered independent variation between RMR and SR; virgin females injected with Ecdysteroids live longer under starvation despite having unchanged RMR (Fig. 8, 2). The proximate cause of the increased SR probably lies in the observed increase in abdominal fat content in response to pupal Ecdysteroids injections (Fig. 4). This strongly suggests that under stressful conditions, females can re-allocate these abdominal resources, and in particular fat (*cf*. Zwaan *et al.* 1991), to survival rather than reproduction.

Our findings reveal that not all traits involved in the seasonal syndrome (and responding to developmental temperature) are regulated by pupal Ecdysteroids. This underscores the idea that, even when traits are correlated and co-vary with hormonal patterns, a functional study is needed to ascertain whether a particular hormone is indeed mediating these relationships, including potential trade-offs (Zera & Harshman 2001).

Conclusions

Seasonal phenotypic plasticity in *B. anynana* involves a whole suite of morphological, physiological and life history traits co-varying across the seasons in response to developmental temperature. Previously, we observed a correlation between expression of some of these adult traits and Ecdysteroid dynamics during the pupal stage. Here, we functionally test the involvement of these hormones in the developmental regulation of the seasonal syndrome. We manipulate Ecdysteroids at various time points during pupal development, and observe significant shifts in reproductive resource allocation in response to early, but not late injections. Crucially, these allocation changes are accompanied by changes in ecologically relevant fitness traits, including timing of reproduction, egg size, and lifespan. This does not apply to RMR, which probably responds to temperature by mechanisms independent of pupal Ecdysteroids. Together, our results support a functional role for Ecdysteroids during development of *B. anynana* in translating information on environmental quality into adaptive alterations in the adult. This illustrates how organisms can use systemic hormones and their time- and tissue-specific sensitivity to respond to predictive indicators of environmental quality and make strategic life history decisions that enable them to cope with fluctuating environments.

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Supplementary information

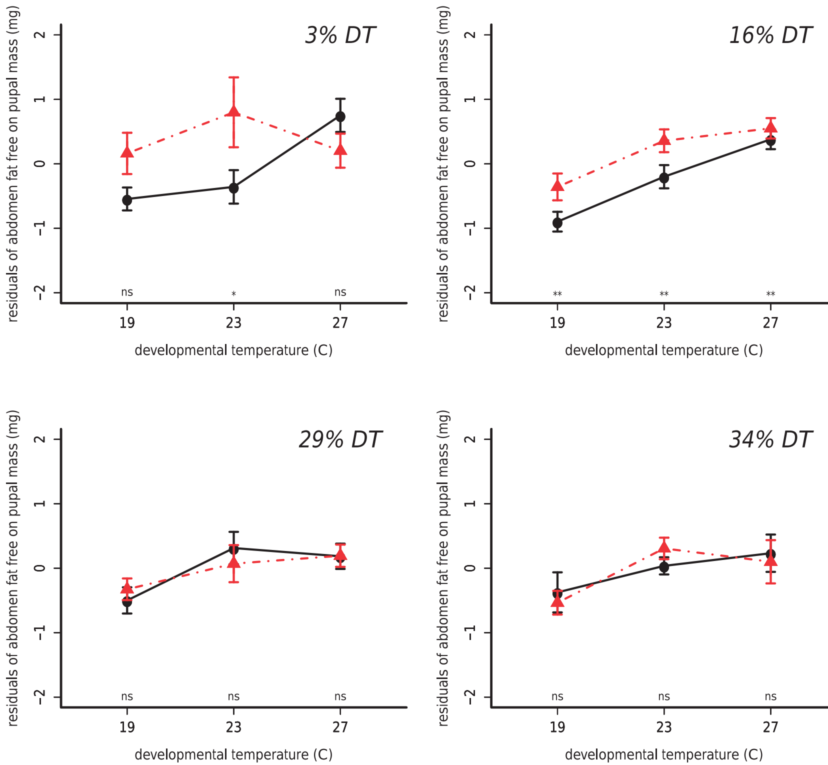

Figure S1. Pupal Ecdysteroids induce increase in fat-free abdomen mass. Mass-corrected abdomen fat fat-free dry weight (mg; see Table S1) is significantly affected by temperature (two-way ANOVA $p < 0.01$). In addition, pupae injected with 20E (red) at 16, but not at 29 or 34% DT, show a substantial increase in fat fat-free dry weight (two-way ANOVA $p < 0.001$) compared to controls (black). The earliest injection (3% DT) only significantly affects females reared at 19 or 23C (two-way ANOVA $p = 0.06$ for temperature x treatment interaction). See also Figures 3 and 4, and Table S2. For legend see Fig 1.

Table S1. Linear regression models of resting metabolic rate (RMR), abdomen mass, abdomen fat content and fat-free abdomen mass on pupal mass for cohorts injected at 3, 16, 29 or 34% of pupal development, related to Figures 2 to 4 and Table S2. The residuals of each model were used as size-corrected measure of each trait in subsequent analyses.

dependent variable	injection time point	covariate	F	df	p
RMR	3%	pupal mass	101.39	1, 157	<0.00001
RMR	16%	pupal mass	265.34	1, 305	<0.00001
RMR	29%	pupal mass	129.18	1, 292	<0.00001
RMR	34%	pupal mass	246.46	1, 272	<0.00001
abdomen dry mass	3%	pupal mass	234.43	1, 156	<0.00001
abdomen dry mass	16%	pupal mass	561.94	1, 305	<0.00001
abdomen dry mass	29%	pupal mass	445.68	1, 247	<0.00001
abdomen dry mass	34%	pupal mass	337.71	1, 224	<0.00001
fat-free abdomen dry mass	3%	pupal mass	174.13	1, 156	<0.00001
fat-free abdomen dry mass	16%	pupal mass	427.81	1, 305	<0.00001
fat-free abdomen dry mass	29%	pupal mass	422.71	1, 246	<0.00001
fat-free abdomen dry mass	34%	pupal mass	341.31	1, 224	<0.00001
abdomen fat content	3%	pupal mass	152.97	1, 156	<0.00001
abdomen fat content	16%	pupal mass	314.15	1, 305	<0.00001
abdomen fat content	29%	pupal mass	228.26	1, 246	<0.00001
abdomen fat content	34%	pupal mass	151.53	1, 224	<0.00001

Table S2. Minimum adequate models of developmental, morphological, and physiological traits at 3, 16, 29 or 34% of pupal development, related to Figures 1 to 4 and Table S1. For each injection time point separately, we analysed each trait as dependent variable in a two-way ANOVA with temperature and injection treatment (Ecdysteroid or control injection) as fixed effects.

dependent variable	injection time point	fixed effects	F	df	p
log (pupal time)	3%	temperature	2036.50	2, 57	<0.00001
		treatment	78.33	1, 57	<0.00001
		temperature x treatment	2.54	2, 57	0.08754
log (pupal time)	16%	temperature	3629.10	2, 99	<0.00001
		treatment	43.86	1, 99	<0.00001
		temperature x treatment	5.71	2, 99	0.00449
log (pupal time)	29%	temperature	4080.5	2, 100	<0.00001
log (pupal time)	34%	temperature	3432.21	2, 77	<0.00001
		treatment	6.98	1, 77	0.00998
		temperature x treatment	4.52	2, 77	0.01396
size-corrected RMR	3%	temperature	4.37	2, 156	0.01423
size-corrected RMR	16%	temperature	42.35	2, 304	<0.00001
size-corrected RMR	29%	temperature	49.29	2, 291	<0.00001
size-corrected RMR	34%	temperature	36.61	2, 270	<0.00001
size-corrected abdomen mass	3%	temperature	3.70	2, 152	0.02708
		treatment	11.68	1, 152	0.00081
		temperature x treatment	4.54	2, 152	0.01213
size-corrected abdomen mass	16%	temperature	8.97	2, 303	0.00016
		treatment	21.55	1, 303	0.00001
size-corrected abdomen mass	29%	temperature	3.45	2, 246	0.03330
size-corrected abdomen mass	34%	temperature	3.69	2, 223	0.02643
size-corrected fat-free abdomen mass	3%	temperature	6.85	2, 152	0.00162
		treatment	4.05	1, 152	0.04845
		temperature x treatment	2.75	2, 152	0.06720
size-corrected fat-free abdomen mass	16%	temperature	22.96	2, 303	<0.00001
		treatment	7.41	1, 303	0.00687
size-corrected fat-free abdomen mass	29%	temperature	4.91	2, 245	0.00810
size-corrected fat-free abdomen mass	34%	temperature	4.82	2, 223	0.00891
size-corrected fat content	3%	temperature	5.64	2, 152	0.00433
		treatment	25.91	1, 152	<0.00001
		temperature x treatment	4.87	2, 152	0.00890
size-corrected fat content	16%	temperature	7.84	2, 303	0.00048
		treatment	47.68	1, 303	<0.00001
size-corrected fat content	29%	temperature	3.06	2, 245	0.04846
size-corrected fat content	34%	temperature	0.14 *	2, 223	0.54330 *

* None of the fixed terms in the model had a significant effect.

Table S3. Statistical models of life history traits in response to Ecdysteroid treatment, related to Figures 5 and 6. All butterflies were reared at 23C and injected at 16% of pupal development. See Methods for details on data analysis.

dependent variable	**fixed effects**	**covariates**	**random factors**	**test statistic**	**df**	**p**
early reproductive status (age 2-4 d)	treatment	-	-	$\chi^2 = 5.65$	1	0.01745
egg number age 2-4 d among egg-laying females	treatment	-	-	t = 0.24	1, 108	0.8104
egg number age 5-13 d	treatment age treatment x age	-	individual	F = 4.55 F = 33.30 F = 7.80	NA[*] NA[*] NA[*]	0.00018[†] <0.00001[†] 0.00046[†]
total egg number	treatment	-	-	F = 2.05	1, 149	0.1539
egg size	treatment age treatment x age	-	individual	F = 1.32 F = 14.62 F = 3.46	NA[*] NA[*] NA[*]	0.019[†] <0.00001[†] 0.01553[†]
lifespan	treatment	 adult mass	-	Wald z = 1.88 Wald z = -3.62	1 1	0.05983 0.00030
lifespan (early reproducing females only)	treatment	 adult mass	-	Wald z = 2.26 z = -2.05	1 1	0.0409 0.0241
starvation resistance	treatment	 adult mass treatment x adult mass	-	Wald z = -2.77 Wald z = -5.57 Wald z = 2.37	1 1 1	0.00567 <0.00001 0.01798

[*] In these general linear mixed effects models, degrees of freedom could not be estimated (see Bates *et al.* 2011).

[†] P values for each main effect are based on a comparison between a mixed model without the main effect and the full mixed model, using a likelihood-ratio test.

3

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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 ►►

Manuscript in preparation

►► 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 correleted 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

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_20 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.

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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).

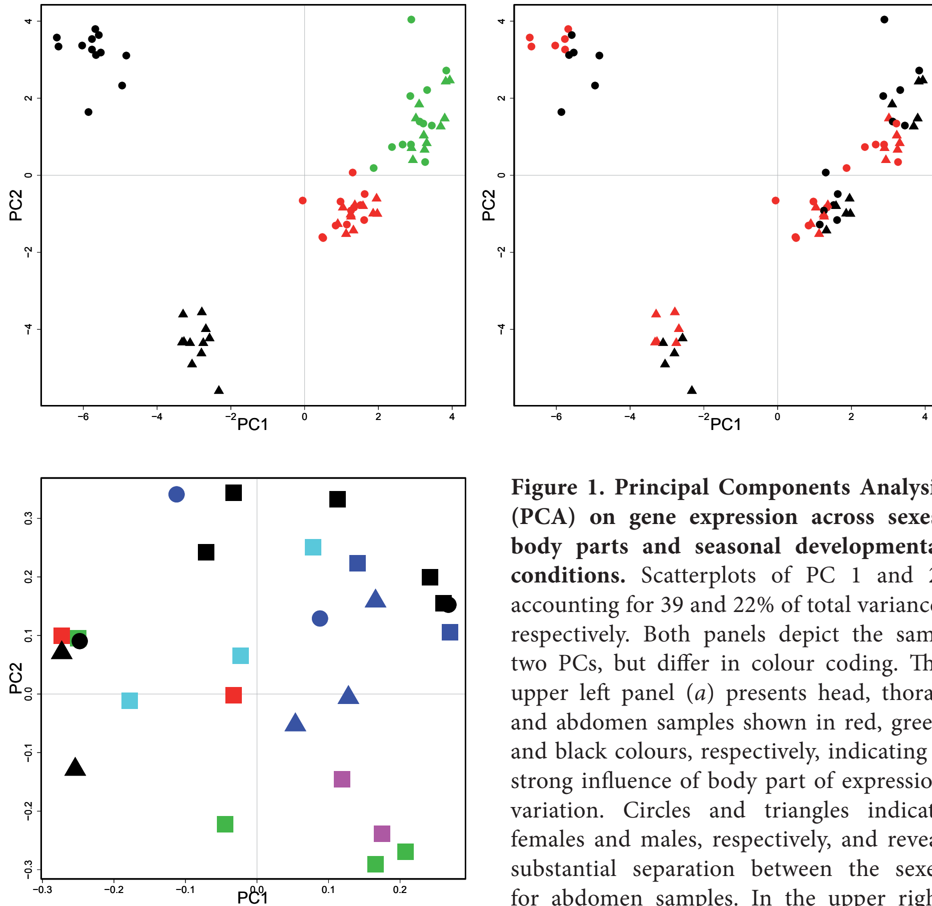


Figure 1. Principal Components Analysis (PCA) on gene expression across sexes, body parts and seasonal developmental conditions. Scatterplots of PC 1 and 2, accounting for 39 and 22% of total variance, respectively. Both panels depict the same two PCs, but differ in colour coding. The upper left panel (*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.

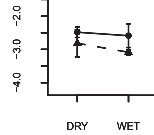
Including the exact same dilution series of five samples on all nine arrays allowed us to correct for technical variation in expression across arrays. The regression of expression (C_t) on the (base 2) logarithm of the dilution factor for the five samples in the dilution series varied both in intercept 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.

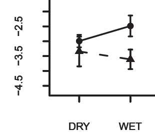
The four most stable reference genes tested in the single array pilot were used in the nine experimental arrays. To examine whether these genes indeed showed stable expression across all experimental treatments, stability of all 32 genes was evaluated and ranked using the internal control gene stability 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.

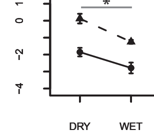
Figure 2 (next three pages). Expression of 27 candidate life history genes as measured by qPCR. Each row depicts expression for a single gene as a function of seasonal developmental condition (DSF: dry season form; WSF: wet season form) for females (solid lines) and males (dotted lines) in head (left), 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. ►►

Biological process | **Gene** | **HEAD** | **THORAX** | **ABDOMEN**

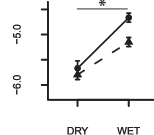
innate immunity
pathogen recognition
BGRP

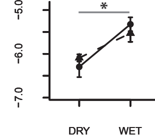


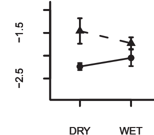




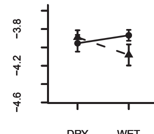
innate immunity
pathogen recognition
Pgrp-1

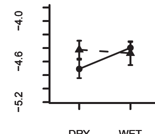


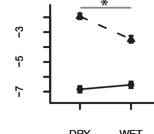




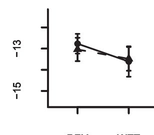
innate immunity
Toll signalling pathway
Spz

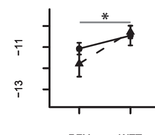


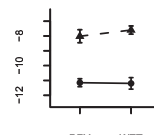




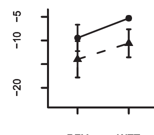
innate immunity
Toll signalling pathway
TLR-2

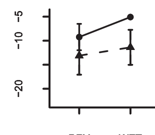


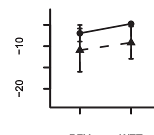




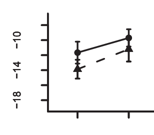
innate immunity
Antimicrobial peptide
Att

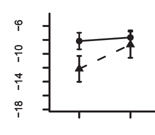


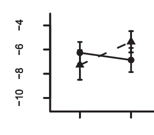




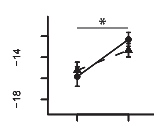
innate immunity
Antimicrobial peptide
Cec

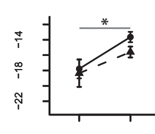


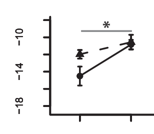




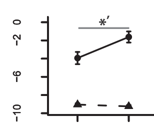
innate immunity
Antimicrobial peptide
Glov

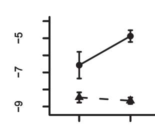


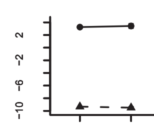




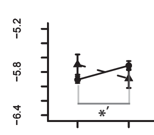
reproduction
Vg

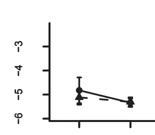


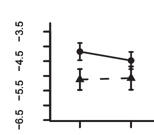




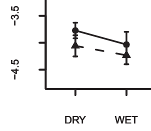
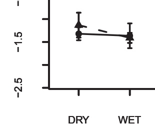
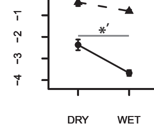
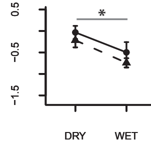
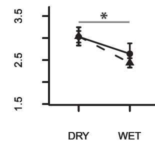
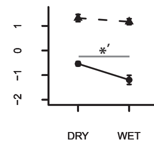
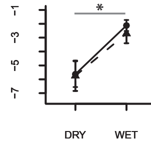
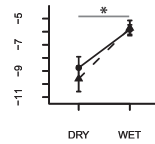
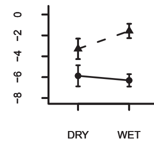
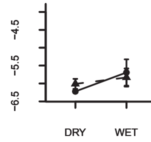
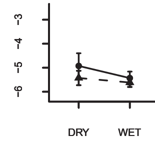
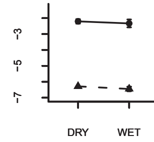
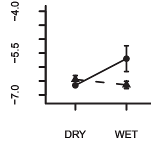
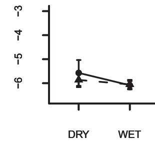
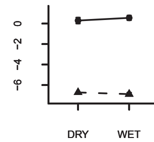
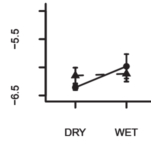
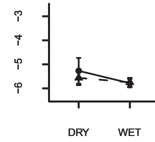
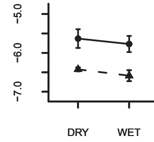
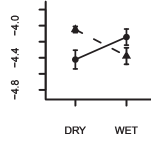
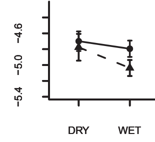
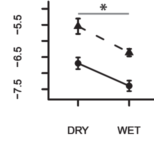
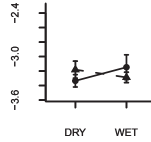
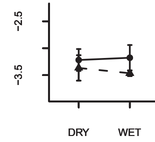
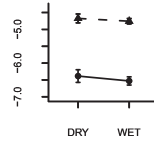
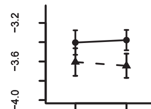
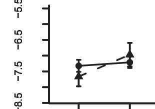
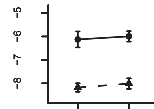
reproduction
VgR

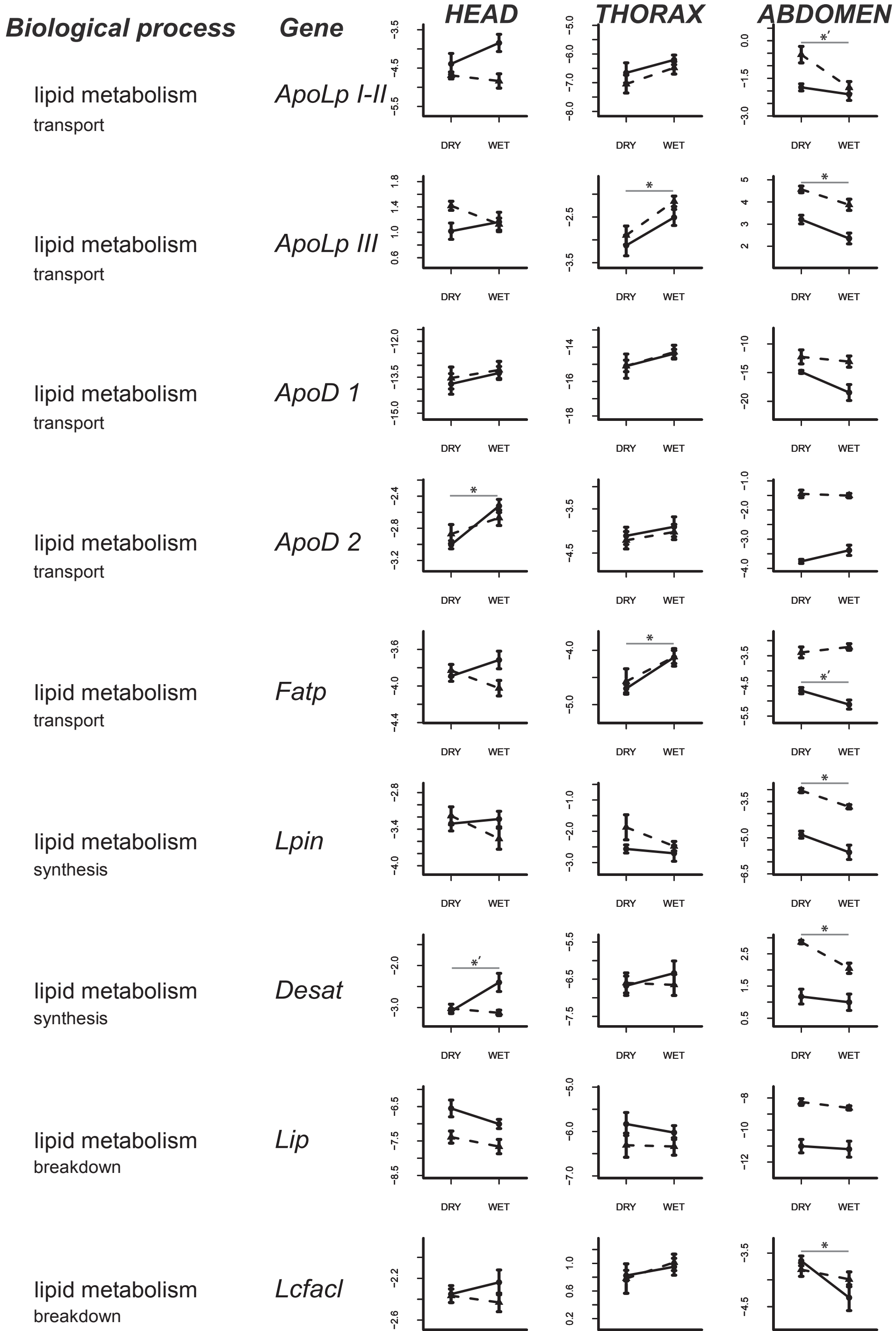






▶▶

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*			


Biological process
Gene
HEAD
THORAX
ABDOMEN
lipid metabolism
transport
ApoLp I-II
lipid metabolism
transport
ApoLp III
lipid metabolism
transport
ApoD 1
lipid metabolism
transport
ApoD 2
lipid metabolism
transport
Fatp
lipid metabolism
synthesis
Lpin
lipid metabolism
synthesis
Desat
lipid metabolism
breakdown
Lip
lipid metabolism
breakdown
Lcfacl
DRY
WET

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 between 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 negative 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

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.

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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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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

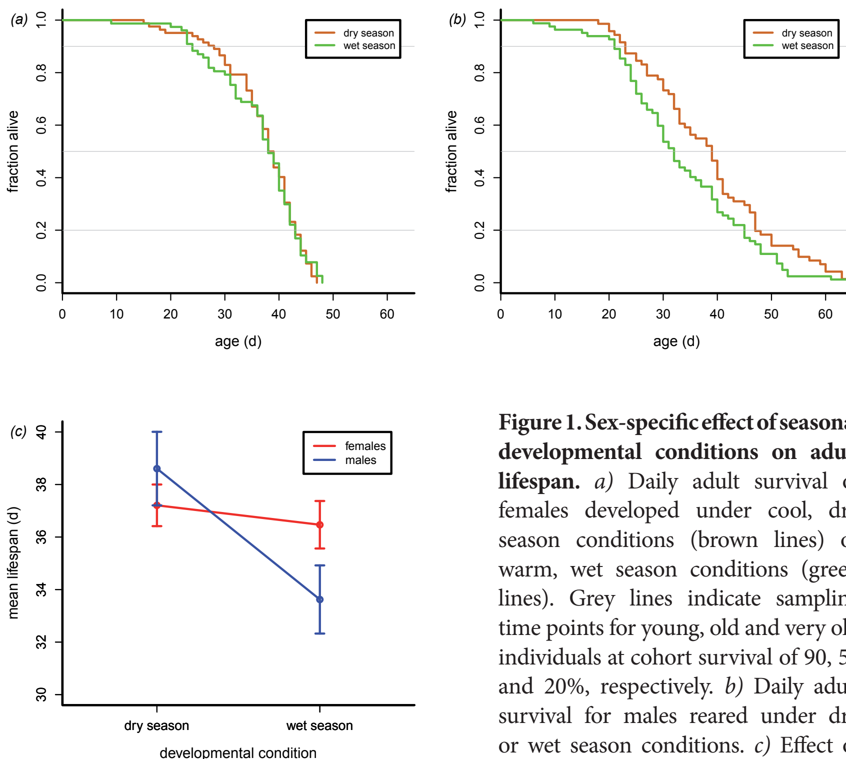


Figure 1. Sex-specific effect of seasonal developmental conditions on adult lifespan. *a)* Daily adult survival of females developed under cool, dry season conditions (brown lines) or warm, wet season conditions (green lines). Grey lines indicate sampling time points for young, old and very old individuals 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

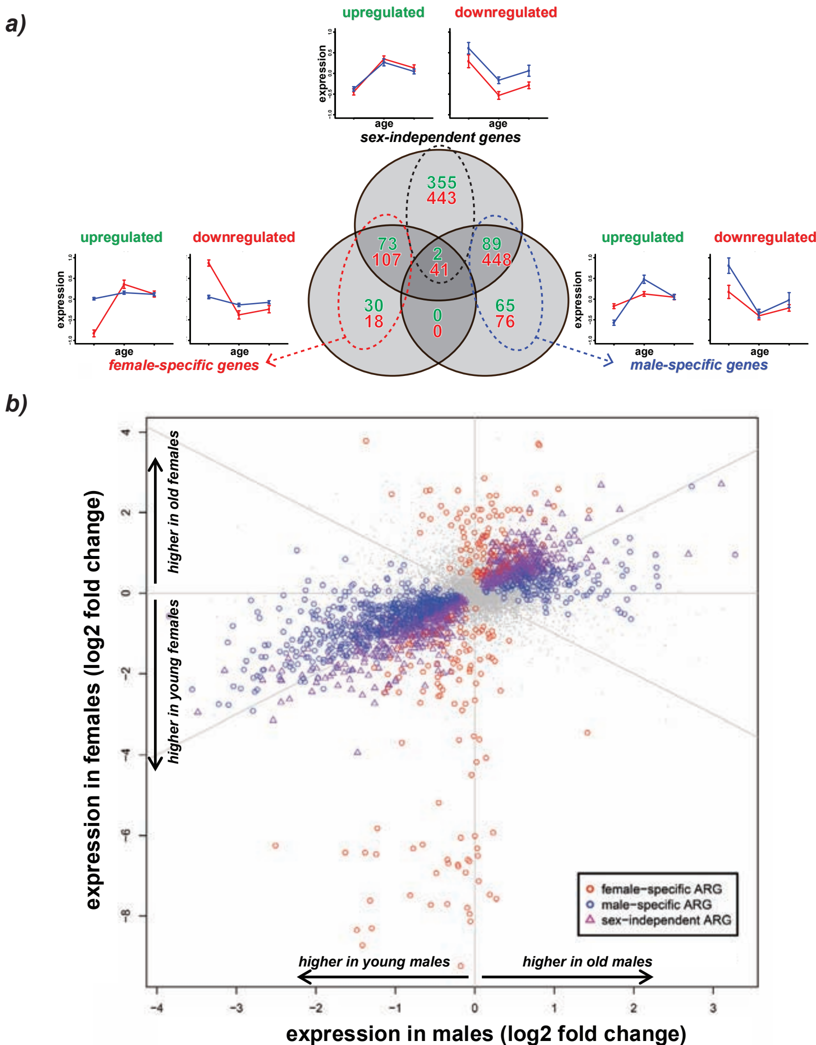


Figure 2. Sex-specificity in transcriptional response to ageing. *a)* Venn diagram showing the groups of genes significantly up- and down-regulated with age in a pooled-sex (top), a female-only (left) and a male-only (right) analysis, as well as overlap between these groups. The six smaller plots show 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 *endonucluease-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*, *endonucluease-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	-	-	-	-	-	-	-	

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

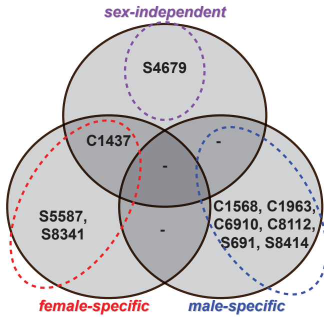
5

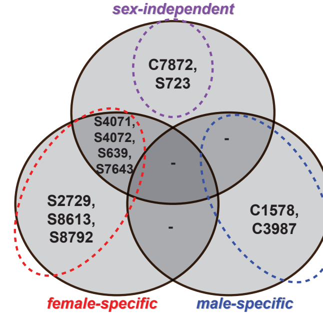
▶▶

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.





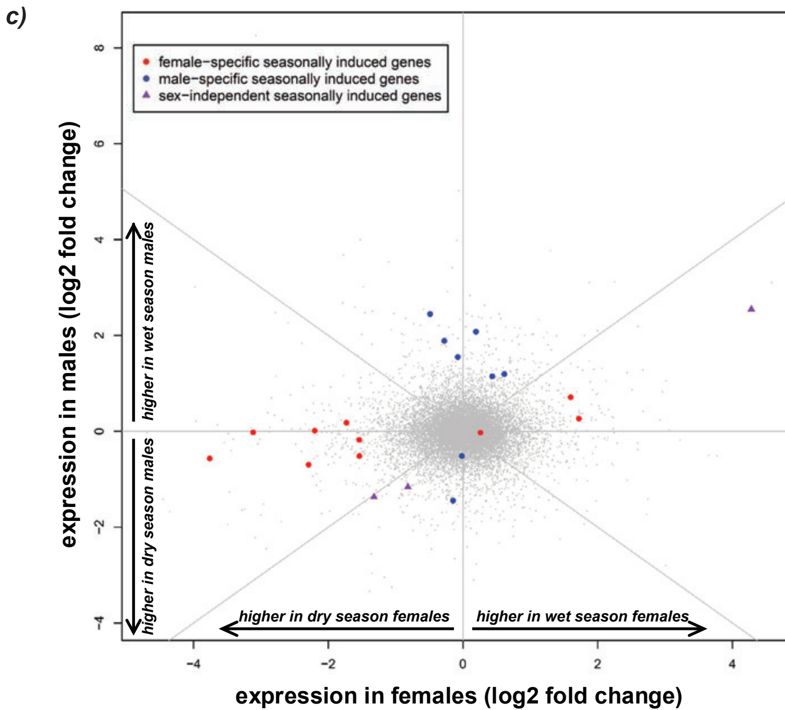


Figure 3. Season-biased gene expression in female and male adults. *a*) Venn diagram showing genes overexpressed in adults developed in warm, wet season conditions, in a pooled-sex (top), a female-only (left) and a male-only (right) analysis, as well as overlap between these groups. Names of genes correspond 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).

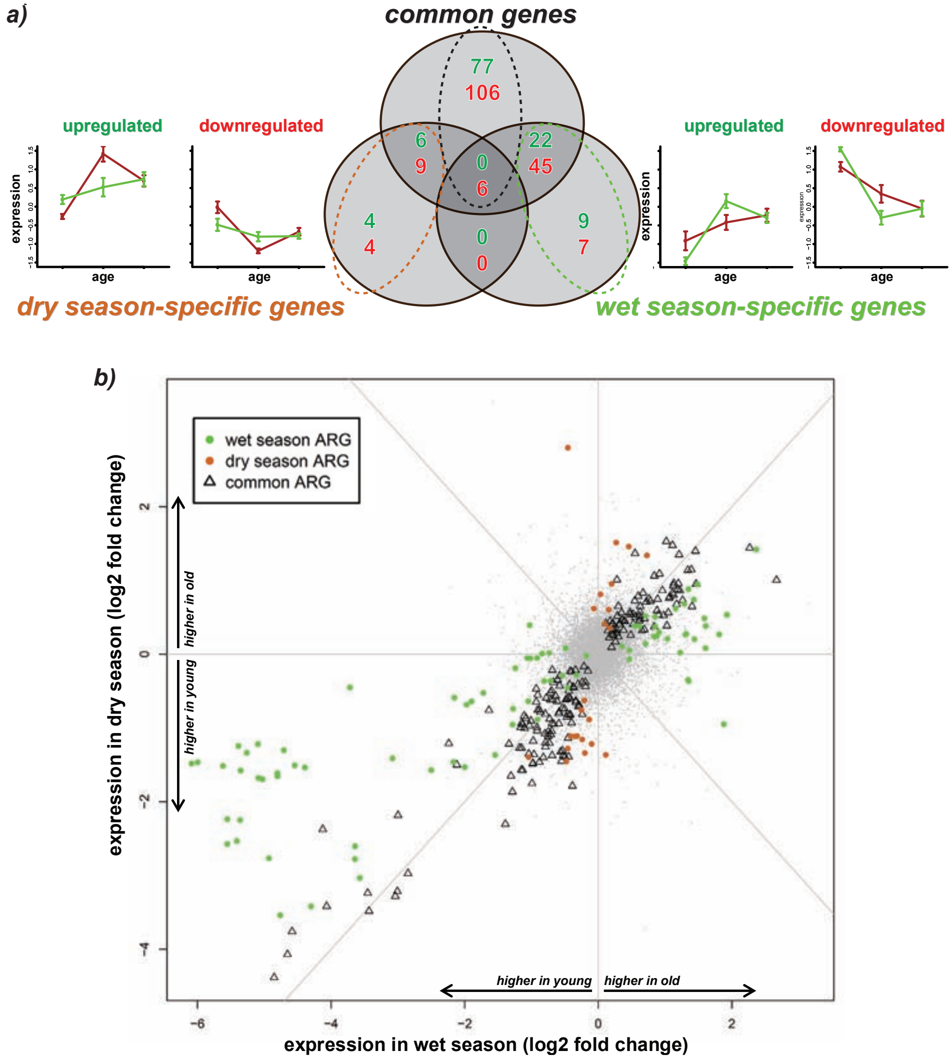


Figure 4. Effect of seasonal condition on transcriptional response to ageing in females. *a)* Venn diagram showing the groups of genes significantly up- and down-regulated with age in an analysis pooling all females (top), for females reared in dry season conditions (left), or for females reared in wet 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

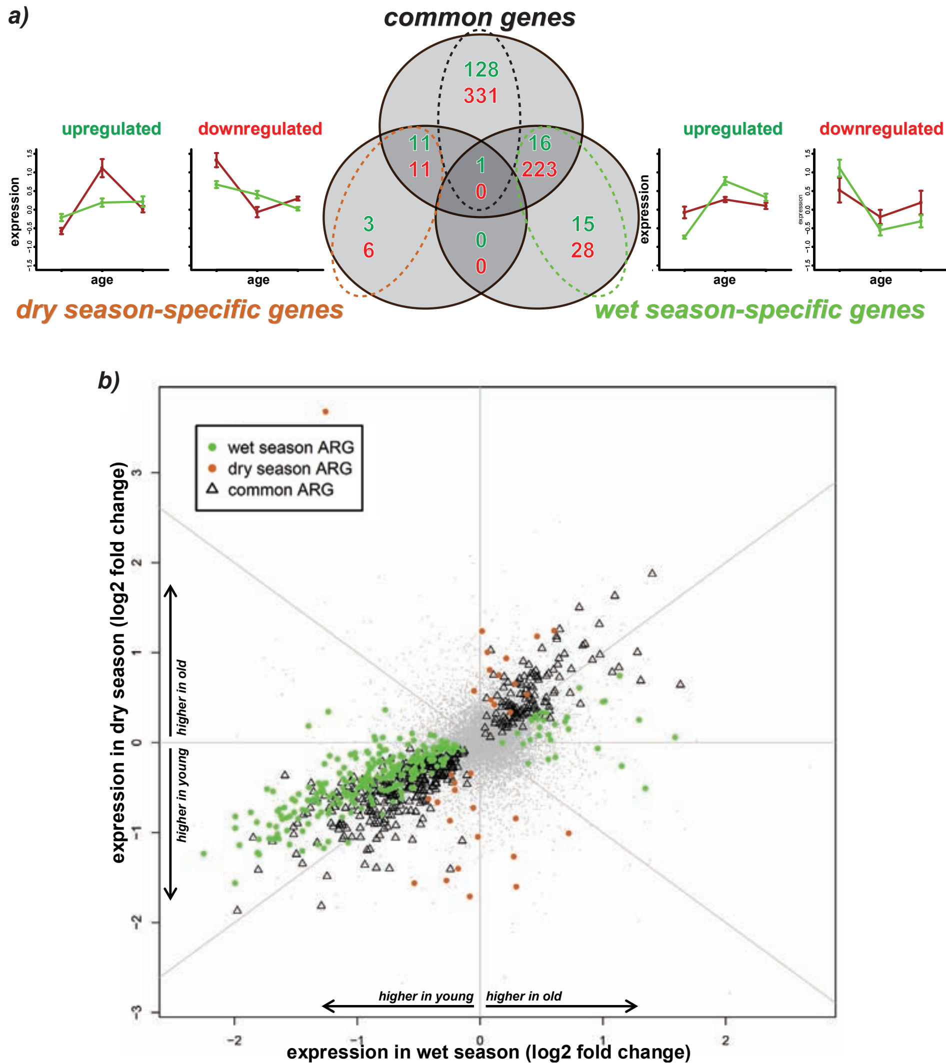


Figure 5. Effect of seasonal condition on transcriptional response to ageing in males. *a)* Venn diagram showing the groups of genes significantly up- and down-regulated with age in an analysis pooling all males (top), for males reared in dry season conditions (left), or for males reared in wet season 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.

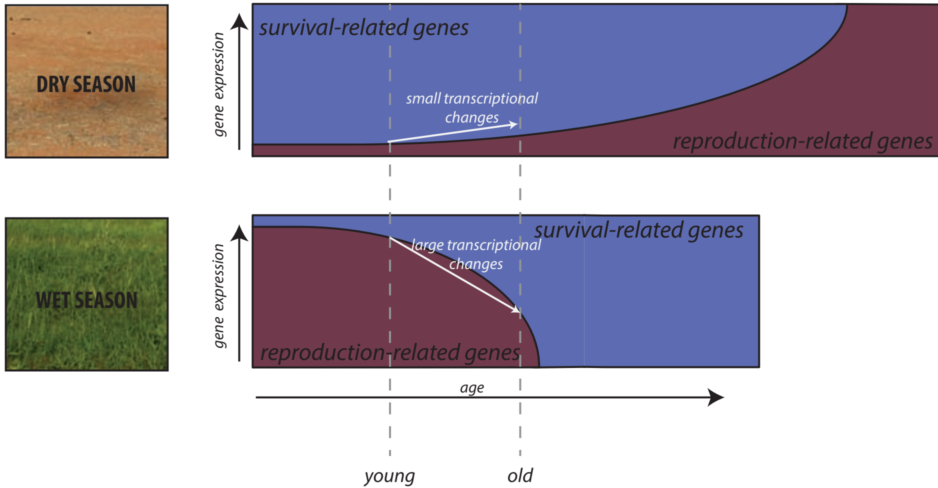


Figure 6. Graphical representation of season-specificity in age-related transcriptional changes. In this model, individuals reared in the wet season start their life as adults with high expression of reproduction-related genes (indicated in blue), which show a relatively rapid down-regulation with age. 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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On the fate of seasonally plastic traits in a rainforest butterfly under relaxed selection

6

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Abstract

Many organisms display phenotypic plasticity as adaptation to seasonal environmental fluctuations. Often, such seasonal responses entails plasticity of a whole suite of morphological and life history traits that together contribute to the adaptive phenotypes in the alternative environments. Hormonal systems are crucial mediators of plasticity, and their central regulation in response to environmental cues allows the organism to mount an integrated and coordinated response to environmental variation. However, little is known about the evolutionary fate of plastic responses if natural selection on plasticity is relaxed. This may occur when the environment becomes more constant, and part of the organism's phenotypic range is no longer expressed. In particular, it has rarely been studied whether plastic responses are lost or retained when selective pressures on different plastic traits sharing a hormonal regulator diverge. Such shared regulation may constrain evolutionary decoupling of plastic traits that are under relaxed selection for some traits but not all. Here we study whether a phylogenetically widespread and likely ►►

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►► ancestral capability to respond to seasonal environmental variation is still retained in a species that inhabits a less seasonal habitat, where natural selection on plastic responses is assumed to be relaxed. Exposing the aseasonal rainforest butterfly *Bicyclus martius* to an unnatural range of temperatures in the laboratory revealed hidden reaction norms for several traits, including wing pattern. In contrast, allocation of adult mass to the abdomen, as a proxy for early-life reproductive investment, was not affected by developmental temperatures. In the savannah butterfly *Bicyclus anynana* these traits show strong developmental plasticity as an adaptation to the contrasting environments of its seasonal habitat. In that species, wing pattern and allocation to abdomen respond to developmental temperature via a common hormonal system active during pupal development. Our results for *B. martius* indicate that such shared hormonal regulation does not preclude decoupling of temperature responses between traits over evolutionary time. There is likely strong natural selection against plasticity in fecundity in the rainforest, but for wing pattern, such selective forces are likely much weaker. This indicates that hormonal integration between plastic traits—as a result of past selection on expressing a coordinated environmental response—can be broken when the optimal reaction norms for those traits diverge in a new environment.

Introduction

Phenotypic plasticity is the ability of a particular genotype to express different phenotypes in response to environmental variation (Schlichting & Pigliucci 1998; West-Eberhard 2003). Although not necessarily adaptive, many instances of adaptive phenotypic plasticity have been documented. In these cases, organisms expressing distinct phenotypes in alternative environments have their highest relative fitness in the environment in which they typically occur (see Beldade *et al.* 2011; Simpson *et al.* 2011; Stearns 1989). In seasonal habitats, phenotypic plasticity may evolve as a result of contrasting but predictable seasonal selection pressures, resulting in different morphologies or life history strategies being expressed in each season (Brakefield & Zwaan 2011; Shapiro 1976).

It has rarely been studied what the evolutionary fate of plasticity would be in an ancestrally plastic species that no longer inhabits such a seasonal environment and the traits are no longer exposed to environmental variation previously associated with their plasticity (Lahti *et al.* 2009; Snell-Rood *et al.* 2010; but see Aalberg Haugen *et al.* 2012). This could occur when a species adapted to a seasonally fluctuating habitat establishes itself in a new, aseasonal habitat, or when the environmental conditions within a habitat change such that it becomes less seasonal. In such circumstances, part of the previous phenotypic range of this trait is no longer expressed on a regular or predictable basis and therefore not exposed to natural selection. Such relaxed selection also applies to plasticity for the trait, i.e. the ability to express other mean trait values in response to environmental variation.

The question whether relaxed selection will result in loss or retention of seasonal plasticity for a particular trait will depend on the interplay between benefits, costs (both direct and indirect via pleiotropy), and neutral processes such as mutation accumulation and genetic drift (Lahti *et al.* 2009). Retaining plasticity in a less seasonal environment might be favoured through direct benefits if the environment still shows limited variability, but of a less regular nature. Indirect benefits might be due to pleiotropic effects of seasonal plasticity on correlated traits that are still under strong selection. This might be the case if genetic or developmental mechanisms regulating seasonal plasticity are also involved in other, non-seasonal environmental responses such as responses to diurnal temperature fluctuations. If there is no such pleiotropy, mutations in genes responsible for seasonal plasticity can accumulate, potentially, but not necessarily, leading to reduction or loss of the seasonal response (Aalberg Haugen *et al.* 2012; Lahti *et al.* 2009). Finally, plasticity, when under relaxed selection, might be reduced or lost due to two proposed types of costs of plasticity. First, there can be direct 'production' costs of expressing a particular trait, as in the case of energetic costs of inducible anti predatory defences. Second, theoretical considerations suggest the existence of inherent costs associated with the capacity to produce different phenotypes under different conditions. The costs of this capacity presumably lie in the maintenance of sensory and regulatory systems needed to sense environmental conditions and translate them into phenotypic alterations. However, empirical studies suggest that such plasticity costs are generally weak, making it unclear whether they would play a large role in evolutionary loss of plasticity under relaxed selection (Auld *et al.* 2010;

Callahan *et al.* 2008; Van Buskirk & Steiner 2009). In general, empirical studies on the evolutionary consequences of relaxed selection on plasticity are relatively rare (Lahti *et al.* 2009; Schwander & Leimar 2011; Snell-Rood *et al.* 2010), especially in the context of plasticity as adaptation to seasonally fluctuating environments (but see Aalberg Haugen *et al.* 2012 for a notable example to the contrary). Here we study whether a phylogenetically widespread and likely ancestral capability to respond to seasonal environmental variation is still retained in a species that inhabits a less seasonal habitat, where natural selection on plastic responses is assumed to be relaxed.

Seasonal plasticity of wing pattern occurs frequently in the Nymphalidae family, for example in *Melanitis leda* (Brakefield & Larsen 1984), *Junonia coenia* (Rountree & Nijhout 1995), and *Araschnia levana* (Windig & Lammar 1999). Seasonal plasticity, often with discrete phenotypes in the wet and dry season, is also well documented in the Tribe Mycalesina, which inhabit a multitude of habitats in the old world tropics (Braby 1994; Brakefield & Frankino 2009; Brakefield & Reitsma 1991). Particularly well studied is the genus *Bicyclus* in this Tribe, where seasonal plasticity in wing pattern is very widespread. The genus comprises of *ca*. 90 butterfly species distributed throughout sub-Saharan Africa, inhabiting savannah-woodland as well as rainforest habitats (Brakefield & Frankino 2009; Condamin 1973). In the dry season in seasonal habitats, adult *Bicyclus* butterflies express a cryptic wing pattern allowing them to rest undetected among the dried out vegetation. In the wet season, vegetation is green and abundant and the adults express prominent concentric eyespots along the distal margin of their wings (see Fig. 6). The eyespots are probably involved in deflecting vertebrate predator attacks away from the vulnerable body towards the margin of the wing (Brakefield & Frankino 2009; Lyytinen *et al.* 2004; Lyytinen *et al.* 2003). The most studied species is *Bicyclus anynana*, a savannah -woodland butterfly distributed throughout East Africa (Brakefield *et al.* 2009). In addition to wing pattern, *B. anynana* adults of the wet and dry seasons differ markedly in their life history strategies. In the field, adults spend the harsh dry season being relatively inactive and delay reproduction until the beginning of the wet season, when larval food plants reappear. The relatively short-lived adults of the wet season morph are more active and reproduce rapidly. In the laboratory, wet season females allocate relatively more mass to the abdomen and lay more (albeit smaller) eggs (Brakefield & Zwaan 2011).

At a proximate level, the major cue for the induction of adult dry or wet season phenotypes is the temperature at which the larvae have been reared. At high temperatures, corresponding to wet season conditions in the field, *B. anynana* larvae develop into wet season adults, whereas low temperatures, indicative of an approaching dry season in the field, induce development of the dry season form (see Oostra *et al.* 2011). Both adult wing pattern and life history are determined by developmental temperature, although life history traits retain the ability to acclimatise when environmental conditions change during adult life (e.g. Fischer *et al.* 2003). Recently, we showed that Ecdysteroid hormones during the pupal stage play a functional role in regulating developmental plasticity of adult reproductive strategy, a role for this hormone that had already been established for wing pattern plasticity (see Chapter 2, Oostra *et al.* 2011; and Chapter 3). This indicates that,

at least in *B. anynana*, developmental plasticity of wing pattern and of life history share developmental-physiological mechanisms. Such developmental integration of both forms of plasticity may have been driven by the correlated fluctuations in selection pressures on both wing pattern and life history between the seasons.

To examine the effects of relaxed selection on developmental plasticity of wing pattern and life history, we used *Bicyclus martius*, a species which would only rarely be exposed to dry season like conditions in the field. It inhabits the wet forests of equatorial central and western Africa (Condamin 1973; Larsen 2005), a generally wet season-like environment, with limited fluctuation in temperature, larval food availability or reproductive opportunities (see Fig. 1 and Methods). This lack of seasonal exposure to harsh dry season conditions is likely to reflect a situation of relaxed selective pressures, both on dry season-specific adaptations and on plasticity itself.

Figure 1. Lowland rainforest habitat of the *B. martius* population used in this study (Ologbo Forest, southern Nigeria). *a)* Late in the dry season (end of March). *b)* Early in the wet season (mid June). Photos by Oskar Brattström.

The aim of the present study was to establish the extent to *B. martius* has retained the ability to express alternative phenotypes when exposed in the laboratory to a range of 'seasonal' temperatures not normally encountered in the field. We established a laboratory population from wild-caught females, and, on parallel cohorts of developing larvae, imposed a range of temperatures that in other *Bicyclus* species induce plasticity (Roskam & Brakefield 1996). Subsequently, we measured thermal responses for a suite of plastic traits that in *B. anynana* are involved in the seasonal adaptation. These traits included life history, physiological traits, and wing patterns. We then compared thermal responses to those observed in previous reaction norm experiments in *B. anynana*. Our experiment allowed us to determine not only the thermal plasticity of each individual trait, but also the extent to which these traits show an integrated response to the environment comparable to that in *B. anynana*. Such knowledge on levels of phenotypic integration can subsequently be used to discern patterns of pleiotropy between traits and their role in non-seasonal environments.

Materials and methods

B. martius habitat and population

B. martius, in several previous publications called *Bicyclus sanaos* (Larsen 2003), has been observed by one of us (O.B.) on several occasions during fieldwork in Nigeria, Ghana and Liberia, where it is fairly common in rainforest but never found in open savannah habitats. The laboratory stock was established in the laboratory in Leiden from gravid females collected in Ologbo Forest (N 6.02, E 5.55, 20 m.a.s.l.) in southern Nigeria. Here, temperature varies very little throughout the year (25 to 28°C mean monthly temperature) but precipitation shows marked seasonality (30-440 mm per month; as measured at a weather station *ca.* 60 km away (National Climatic Data Center). Despite the variation in precipitation, the soil remains wet during the whole dry season, the vegetation in the forest interior remains green and the humidity stays high throughout the year (Fig. 1). Thus, larval food plants (grasses) are likely to be continuously available during the whole annual cycle. Furthermore, *B. martius* adults of all ages can be observed at any given time, from recently eclosed individuals with no visible wing wear through to old individuals with extensive wing damage (O.B. personal observation). This indicates that females breed throughout the year and show no seasonal reduction in reproductive activity in accordance with the availability of larval food plants. Occasionally, individuals with small ventral eyespots, resembling a typical *Bicyclus* dry season form, have been observed in the field. However, the majority of individuals have large, wet season-like eyespots including during the dry season (O.B. personal observation; Roskam & Brakefield 1999). Around 25 females were collected in December 2009 to establish an initial population, and approximately 35 additional females were collected in April 2010 (both by O.B.) and introduced to the laboratory population.

Overall, the butterfly rearing setup in the laboratory was comparable to that used in *B. anynana* (see Brakefield *et al.* 2009), with a slightly higher temperature (28°C) and relative humidity (RH; 85%). The only major difference with *B. anynana* was the type of larval food plants. To collect eggs, we provided ovipositing females with young pot-grown *Oplismenus sp*. and *Triticum sp*. (wheat) plants (both Poaceae). Larvae grew readily on both plants, but for the first few generations we exclusively reared them on *Oplismenus*. In later generations, when the population was well-established, *Triticum* was used in addition to *Oplismenus*. Each generation, about 400 larvae were reared, of which between 40 to 60% normally survived through to adulthood. Females generally started ovipositing at 2-3 weeks after eclosion, and continued to do so at a relatively constant rate for several weeks.

Pilot experiment

We performed a pilot experiment prior to the main experiment to optimize rearing and experimental protocols, and to collect preliminary data on developmental plasticity in *B. martius*. Comparatively high mortality in the pilot led to the use for the main experiment of small environmental climate chambers (Sanyo Versatile Environmental Test Chamber model MLR-351H), at higher humidity than during the pilot.

Experimental design and measurement of phenotypic responses

We assessed developmental plasticity in *B. martius* by rearing separate cohorts of larvae at three different temperatures and measuring phenotypic responses for life history and wing pattern. We collected eggs from the stock population and let the larvae hatch on *Triticum* (wheat) plants. The freshly hatched larvae were collected on a daily basis and transferred in batches of 20 larvae onto separate one week old *Triticum* plants kept in net sleeves, which were each placed in one of three environmental climate chambers set at 19, 23 or 27°C (and 85% RH with a 12:12 L:D photoperiod). We placed a total of 10 such sleeves in each climate chamber, rearing 200 larvae per temperature. Plants were monitored daily and watered or replaced as necessary. Pre-pupae were collected daily and placed in petri dishes. One day old pupae were weighed to the nearest 0.01 mg using a Sartorius Research RC 210D scale, and then placed in individual pots until eclosion. Larval and pupal development times were recorded in days and each adult was weighed alive one day after eclosion. Subsequently, resting metabolic rate (RMR) was measured as the individual rate of CO_2 respiration (ml hr^{-1}) over a period of 20 min, at 27°C during the dark phase of the diurnal cycle (following Pijpe *et al.* 2007). Butterflies were then frozen at -20°C until further processing. Abdomens and thoraces (removing head, wings, antennae and legs) were then dried to constant mass for 48 h at 60°C before being weighed separately. One ventral hind wing of each adult was imaged using a Leica M125 stereo microscope coupled to a Leica DFC495 digital camera. In *B. martius*, the basic wing pattern elements on the ventral wing surfaces are similar to those in *B. anynana*: a series of concentric eyespots along the distal margins of the fore and hind wing (Roskam & Brakefield 1996). We used ImageJ software v1.46 r (Abramoff *et al.* 2004) to measure three characteristics of the ventral wing pattern on the photo of each hind wing: radius of the second eyespot (starting from anterior), radius of the fifth eyespot, and distance between the centre of the second and the fifth eyespot (see Fig. 6). Eyespot radii were measured from the centre of each eyespot's white focus to the most proximal point on the outer boundary of the golden ring.

Comparison with B. anynana

We compared the temperature responses in *B. martius* to those of *B. anynana* by using data on developmental plasticity in *B. anynana* from two previous reaction norm experiments: 1) a dataset published previously (Oostra *et al.* 2011), and 2) part of a data set from a hormone manipulation experiment, using the uninjected individuals (Chapter 3). In both experiments, *B. anynana* larvae were reared at 19, 23 and 27°C and phenotypic responses were measured in the adults. The major difference with the current experiment was the food plant: *B. anynana* larvae were fed *Zea mays* plants (see Brakefield *et al.* 2009). In addition, in the first experiment, adult fresh weight was not recorded (only pupal weight and adult dry weight). As a measure of wing size the distance between the centres of the first and fifth eyespot (rather than the second and fifth) was used, and as a measure of eyespot size the radius of the black disc excluding the golden ring. In both species the wing pattern measurements were taken from the ventral hind wing. Finally, in the second *B. anynana* data set, we only measured females. All other phenotypic traits were measured in the same way in all experiments.

Statistical analyses

Two-way ANOVAs were used to analyse the effect of developmental temperature and sex on each phenotypic trait of interest, initially fitting full models including temperature, sex, and their interaction as fixed factors and removing non-significant terms successively. Simplified models were evaluated using a likelihood ratio test (LRT) until the minimum adequate model was found (non-significant terms presented in the Results are based on the full models). Subsequently, Tukey's Honest Significant Differences (HSD) test was used to statistically compare specific temperatures or combinations of sex and temperatures. RMR, abdomen mass and eyespot size were first corrected for body or wing size prior to analysis in the two-way ANOVAs. This was done by first fitting separate linear regression models on each of those traits with adult dry mass (in the case of RMR), adult fresh mass (in the case of abdomen mass) or wing size (in the case of eyespot size) as sole predictor variables. The residuals of each of these models were analysed as dependent variables in the two-way ANOVAs. In addition to the two-way ANOVAs, the scaling of adult body mass on pupal mass was analysed by fitting a general linear model (GLM) with temperature and sex as fixed factors and pupal mass as covariate. A similar approach was used for the scaling of (uncorrected) RMR and abdomen mass on adult mass, using adult mass as the covariate. In a direct comparison between *B. martius* and *B. anynana* in relative abdomen size, we combined both data sets (including only females), and calculated, for each individual, the ratio between abdomen and thorax dry weight. After arctangent transformation this ratio was analysed in a two-way ANOVA using temperature, species and their interaction as fixed factors, followed by Tukey's HSD tests. Mortality was compared between the three temperature treatments by analysing egg to adult survival using a chi square test for each sex separately. Finally, a Principal Components Analysis (PCA) was performed on a combined data set of *B. martius* and *B. anynana* to visualise the temperature responses of both species in pupal mass, larval development time, time, pupal development time, thorax dry weight, abdomen dry weight, RMR, radius of eyespot 5 and interfocal distance. For the PCA as well as for the between-species comparison of coefficients of variance (standard deviation / mean) in eyespot size (Fig. 6*d*), we first randomly excluded samples from the *B. anynana* data (separately for each combination of temperature and sex) so that sample sizes (per treatment group) were equal to those in *B. martius*. All analyses were performed in the R statistical environment (R Development Core Team 2010).

Results

Low survival at lowest temperature

Larvae and pupae of both sexes performed poorly at the lowest temperature. In females, egg to adult survival rate was 18% at 19°C, 37% at 23°C, and 34% at 27°C ($\chi^2 = 7.04$, df = 2, $p = 0.03$). In males, survival was also lowest at 19°C with 22% and higher at 23°C and 27°C, where it was 47% and 49%, respectively ($\chi^2 = 11.51$, df = 2, $p = 0.003$). Females survival was slightly lower but at none of the three temperatures was this difference significant

(χ^2 = 0.4-2.7, df = 2, p = 0.5-0.1). At 23°C and 27°C, survival rates were comparable to survival rates during the general breeding of the stock population (at 28°C). In the initial pilot experiment, we observed the same pattern of highest mortality at 19°C.

Development time and lack of protandry

Total egg to adult development time in *B. martius* was strongly affected by developmental temperature, as indicated by the shape of reaction norms and wide differences between extreme temperatures (solid lines in Fig. 2*c*). Both females and males developed faster at higher temperatures but there was no evidence for protandry in *B. martius*. The sexes developed at the same rate and showed the same temperature response (see Supplementary Table 1 for ANOVA results). This contrasts sharply with the protandry shown by *B. anynana* across all temperatures, especially at the higher temperatures (dashed lines in Fig. 2*c*). Overall, *B. martius* developed much slower than *B. anynana*. Examining larval and pupal development time separately revealed that the lack of protandry in *B. martius* originated in the larval stage. The average duration of the larval stage was equal for females and males (Fig. 2*a*), while during the pupal stage females developed faster, as they do in *B. anynana* (Fig. 2*b*).

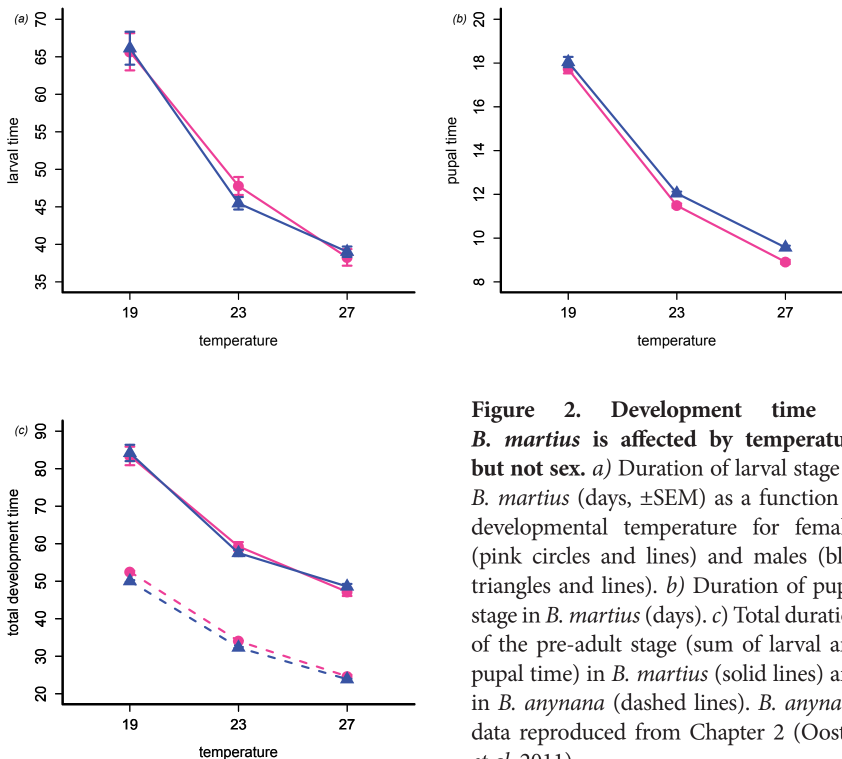

Figure 2. Development time in *B. martius* is affected by temperature but not sex. *a)* Duration of larval stage in *B. martius* (days, ±SEM) as a function of developmental temperature for females (pink circles and lines) and males (blue triangles and lines). *b)* Duration of pupal stage in *B. martius* (days). *c)* Total duration of the pre-adult stage (sum of larval and pupal time) in *B. martius* (solid lines) and in *B. anynana* (dashed lines). *B. anynana* data reproduced from Chapter 2 (Oostra *et al.* 2011).

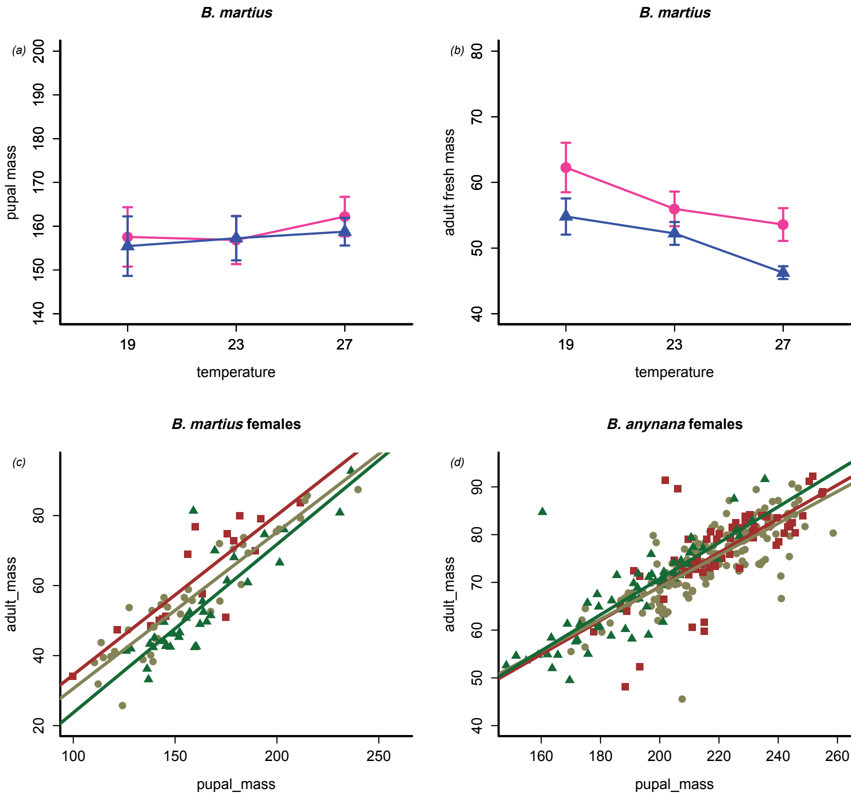

Figure 3. Adult but not pupal size in *B. martius* is affected by developmental temperature and sex. *a)* Pupal mass in *B. martius* (mg) as a function of developmental temperature for females and males. *b*) Adult fresh mass in *B. martius* as a function of developmental temperature. *c*) Scaling of adult fresh mass on pupal mass in *B. martius* females for individuals developed at 19, 23 and 27°C indicated by red squares, grey circles and green triangles, respectively. *d*) Scaling of adult fresh mass on pupal mass in *B. anynana* females. *B. anynana* data from Chapter 3. For legend see Fig. 2.

Plasticity and sexual dimorphism in body size

Despite large changes in development time in response to temperature, pupal mass did not differ across developmental temperatures. Furthermore, there was no sexual dimorphism in this trait (Fig. 3*a*). In contrast, adult mass showed a significant temperature response, with a larger size at lower temperatures, and females were consistently larger than males (Fig. 3*b*). Analysing adult fresh mass as a function of pupal mass (Fig. 3*c*) confirmed the developmental temperature imprint on adult mass, such that pupae of similar size become relatively large adults when reared at lower temperatures. *B. anynana*, where both pupal and adult mass are affected by developmental temperature (Chapter 2), does not show such temperature plasticity in scaling of adult on pupal mass (Fig. 3*d*). Furthermore, female

B. martius pupae developed to become larger adults than male pupae of similar mass. Thus in *B. martius*, in contrast to *B. anynana*, temperature plasticity of body size and sexual size dimorphism are both expressed only in adults, and, therefore, originate in the pupal stage. Although the same average weight is accumulated during the larval stage, male pupae lose more weight than female pupae during metamorphosis and end up as smaller adults. Pupae developed at higher temperatures lose more mass during the pupal stage than those developed at lower temperatures and do so in a shorter time period (*cf*. Fig. 2*b*). Analysing adult dry mass instead of fresh mass yielded the same results (data not shown), indicating that sex- and temperature-specific mass loss during the pupal stage is not due to water loss.

Imprint of developmental temperature on adult RMR

Developmental temperature had a significant effect on mass-corrected adult RMR: females and males reared at low temperatures expressed higher RMR as adult. Furthermore, males had a higher RMR across all temperatures (Fig. 4*a*). The scaling of uncorrected RMR on adult body mass confirmed the negative imprint of developmental temperature in both females (GLM $p = 0.0057$ for temperature effect) and males (GLM $p < 0.00001$). In addition, body mass and temperature in females interacted in such a way that larger individuals showed a stronger temperature response (GLM $p = 0.030$), but in males this was not the case (GLM $p = 0.36$; Fig. 4*b*, *c*). Previous work on *B. anynana* showed a similar temperature imprint: size-corrected adult RMR was higher in individuals reared at lower temperatures. Females have a lower RMR than males, but only at 27°C (Fig. 4*d*). Finally, an analysis of the scaling of uncorrected RMR on body mass in *B. anynana* showed that body mass affected RMR in the same way for all developmental temperatures in both females (GLM $p = 0.97$ for interaction term) and males (GLM $p = 0.69$; Fig. 4*e*, *f*). Taken together, our findings indicate that, as observed previously for *B. anynana*, developmental temperature has a significant imprint on RMR in the adult stage: increased developmental temperature decreases RMR.

Reduced plasticity in reproductive body allocation

We measured allocation of adult body mass to abdomen as a measure of reproductive investment (*cf*. Chapter 2). In *B. martius*, size-corrected abdomen mass was not affected by developmental temperature (Fig. 5*a*). To examine the relationship between total adult mass and uncorrected abdomen mass in more detailed, we analysed the former as a function of the latter in each sex. In females, we found no evidence for an effect of temperature on the scaling of uncorrected abdomen mass on total body size (GLM $p = 0.33$ for temperature effect; Fig. 5*b*). However, in males a small but significant effect occurred of temperature on the scaling of abdomen mass on body size (GLM $p = 0.015$). Examining the initial one-way ANOVA on size-corrected abdomen mass in males revealed that this was due to males at 23°C having a slightly (but not significantly) larger abdomen than at 19°C (Tukey's HSD $p = 0.13$) or 27°C (Tukey's HSD $p = 0.20$). As a comparison, we again analysed *B. anynana* females in the same manner and found that size-corrected abdomen mass was strongly affected by developmental temperature, with more mass allocated to the abdomen at higher

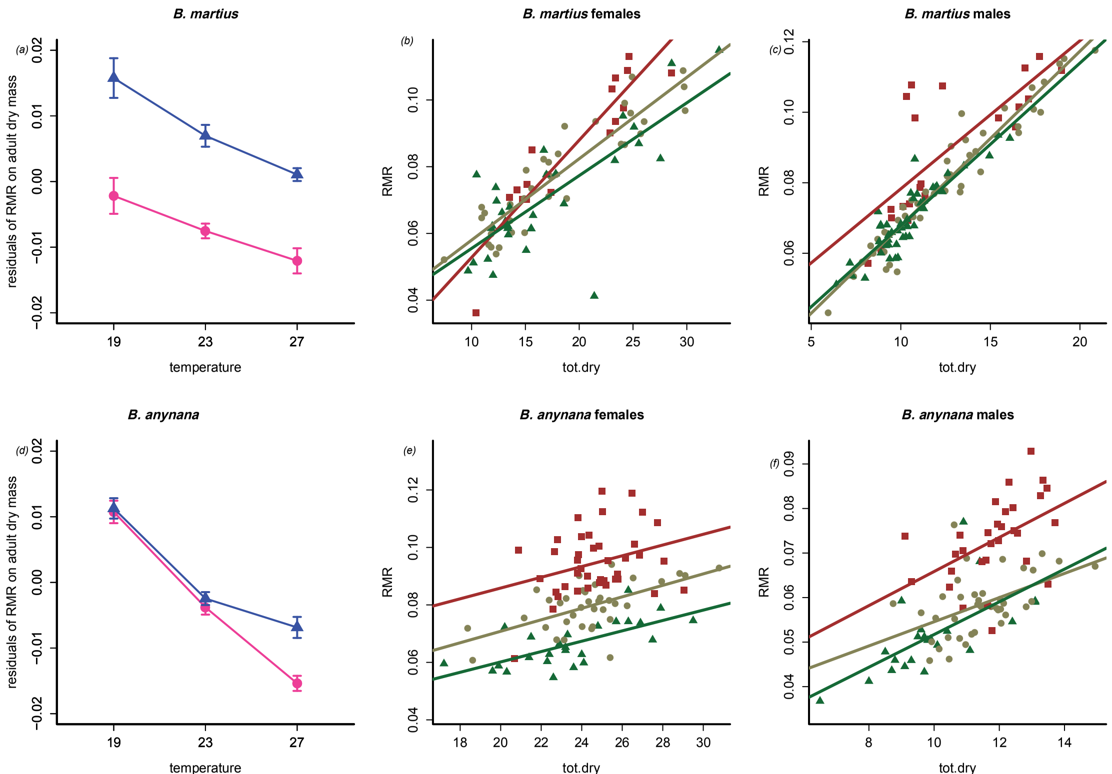

Figure 4 (next page). Adult resting metabolic rate (RMR) in *B. martius* shows imprint of developmental temperature. *a)* Mass-corrected adult RMR in *B. martius* (ml CO_2 hr^{-1}; see Methods) as a function of developmental temperature for females and males. *b)* Scaling of uncorrected RMR on adult mass for *B. martius* females developed at 19, 23 and 27°C. *c)* Scaling of uncorrected RMR on mass for *B. martius* males. *d)* Mass-corrected adult RMR in *B. anynana* as a function of developmental temperature. *e)* Scaling of uncorrected RMR on adult mass for *B. anynana* females developed at 19, 23 and 27°C. *f)* Scaling of uncorrected RMR on mass for *B. anynana* males. *B. anynana* data reproduced from Chapter 2 (Oostra *et al.* 2011). For legend see Figures 2, 3.)

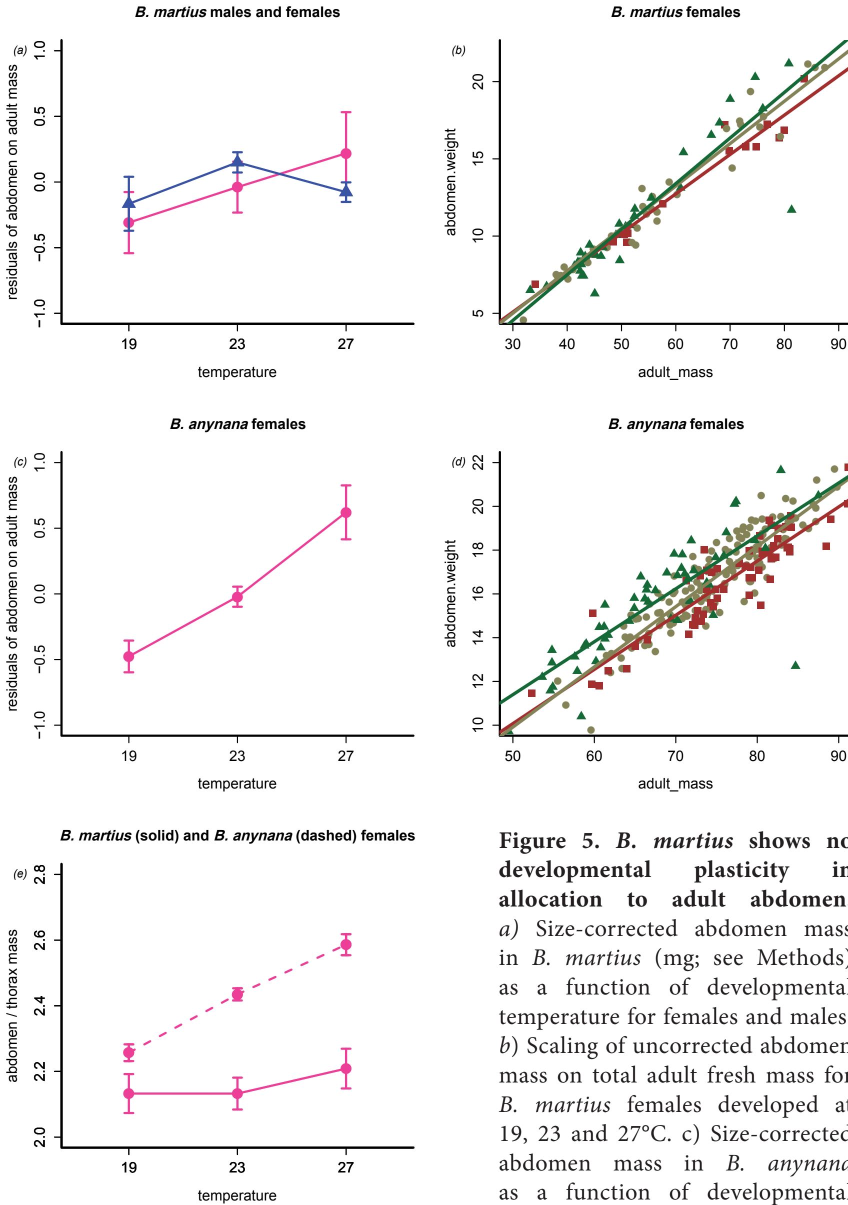

Figure 5. *B. martius* shows no developmental plasticity in allocation to adult abdomen. *a)* Size-corrected abdomen mass in *B. martius* (mg; see Methods) as a function of developmental temperature for females and males. *b*) Scaling of uncorrected abdomen mass on total adult fresh mass for *B. martius* females developed at 19, 23 and 27°C. c) Size-corrected abdomen mass in *B. anynana* as a function of developmental temperature (only female data available). *d*) Scaling of uncorrected abdomen mass on total adult fresh mass for *B. anynana* females developed at 19, 23 and 27°C. *e*) Ratio of abdomen on thorax mass as a function of developmental temperature for *B. martius* and *B. anynana* females. *B. anynana* data from Chapter 3. For legend see Figures 2,3.

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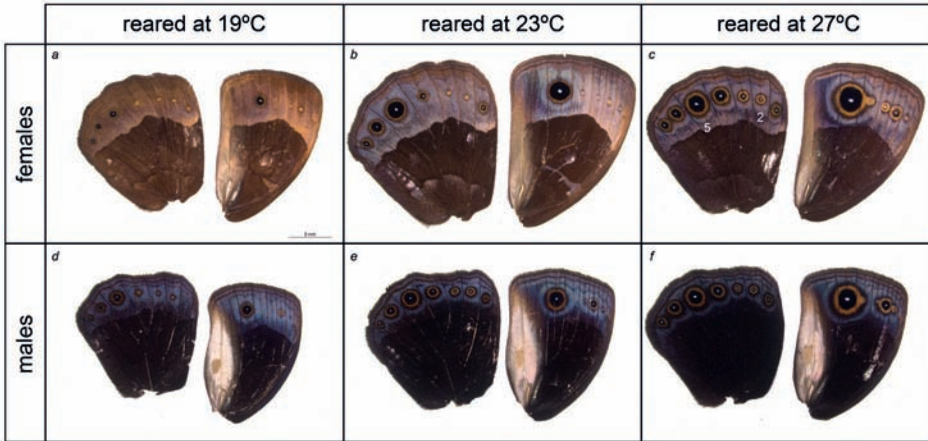

Figure 6. Wing patterns in *B. martius* at different temperatures. The ventral surfaces of fore and hind wings of *B. martius* females (upper panel) and males (lower panel) reared at 19 (*a*, *d*), 23 (*b*, *e*) or 27°C (*c*, *f*). Latin numbers 2 and 5 in *c* indicate the second and fifth eyespots of the hind wing, respectively. The distance between these eyespots and their sizes were quantified (see Methods) and are depicted in Fig. 7.

temperatures (Fig. 5*c*). Correspondingly, the scaling of uncorrected abdomen mass on total body mass was strongly affected by developmental temperature in *B. anynana* females (GLM $p < 0.\ 00001$ for temperature effect; Fig. 5*d*). Finally, a direct comparison between the two *Bicyclus* species in their ratio of abdomen on thorax dry weight showed a significant difference in the response of each species to temperature (two-way ANOVA $p = 0.015$ for interaction term; Supplementary Table 1), with a steep reaction norm for abdomen/thorax ratio in *B. anynana* and a relatively flat reaction norm in *B. martius* (Fig. 5*e*). Together, these data indicate a lack of developmental plasticity of allocation to abdomen in *B. martius*. When reared at higher temperatures, *B. martius* adults do not allocate relatively more mass to the abdomen, which is the case for *B. anynana* adults.

Phenotypic plasticity of wing pattern

The ventral wing patterns of both females and males showed a marked response to developmental temperature. Both the sizes of the eyespots as well as colouration of the wing differed substantially between cohorts reared at different temperatures (Fig. 6). We quantified these differences for two eyespots and found that the size of the fifth eyespot on the ventral hind wing, corrected for wing size, was strongly affected by developmental temperature in *B. martius.* Both males and females had smaller eyespots when reared at lower temperatures but there was no sexual dimorphism in eyespot size (Fig. 7*a*). Likewise, the size of the second eyespot on the same wing surface was significantly smaller in adults reared at lower temperatures. Furthermore, this eyespot was larger in females compared to males, but there was no evidence for a different temperature response among the sexes

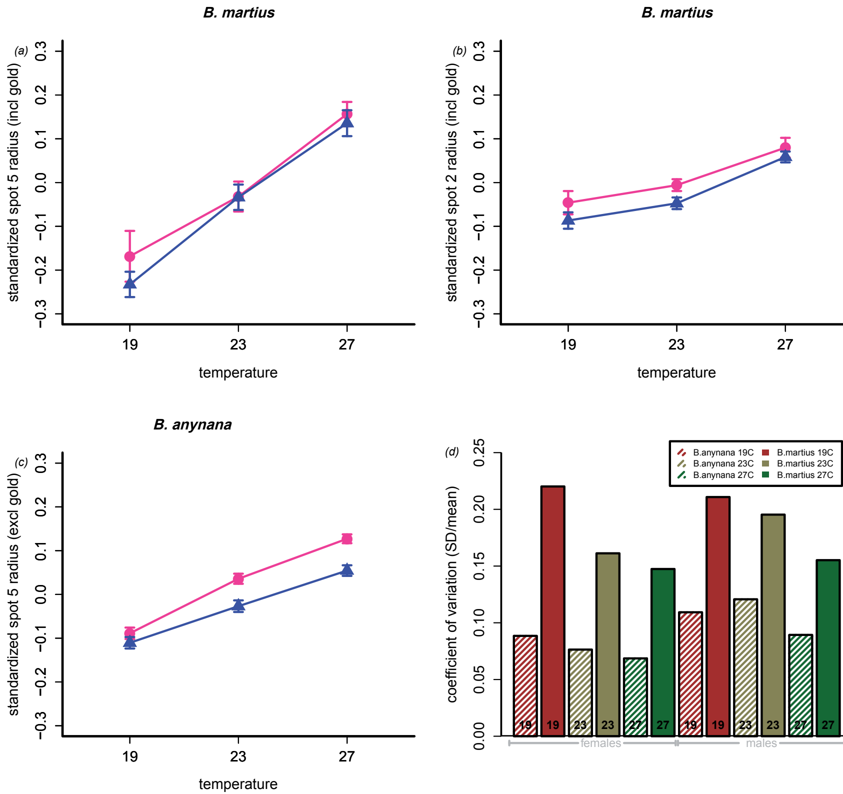

Figure 7. *B. martius* has a phenotypically plastic wing pattern. *a*) The relative size of the fifth eyespot on the ventral hind wing (mm; see Methods) as a function of developmental temperature, for females and males. *b*) The relative size of the second eyespot on the ventral hind wing as a function of developmental temperature. *c*) The relative size of the fifth eyespot on the ventral hind wing as a function of developmental temperature in *B. anynana*. *d*) Coefficients of variation (standard deviation / mean) in the size of the fifth eyespot (mm) in *B. martius* (filled bars) and *B. anynana* (shaded bars) in females (left) and males (right) at all three developmental temperatures (equal sample sizes for both species; see Methods), with 19, 23 and 27°C represented by red, grey and green, respectively. *B. anynana* data reproduced from Chapter 2 (Oostra *et al.* 2011).

(Fig. 7*b*). As a comparison, we re-analysed wing pattern data for *B. anynana*. In particular, we analysed the radius of the fifth eyespot (corrected for wing size) and found a significant effect of developmental temperature and sex. Individuals reared at lower temperatures had substantially smaller eyespots and females had larger eyespots than males. The interaction between sex and temperature was not significant (two-way ANOVA p = 0.093; Supplementary Table 1) but did suggest that at 19°C the sexual dimorphism might have disappeared. Indeed, comparing these two groups directly (i.e. females *vs*. males at 19°C) revealed that they did not differ statistically from one another (Tukey's HSD p = 0.35). As

we measured the size of the fifth eyespot in both *Bicyclus* species, we were able to compare variation in eyespot size between the species. The coefficients of variation (standard deviation divided by the mean per species per sex per temperature) were on average roughly twice as high in *B. martius* compared to *B. anynana*, and at all temperatures variation was highest in *B. martius* in each sex. In addition, in *B. martius* variation was substantially higher at 19°C compared to 27, while in *B. anynana* this difference was smaller (Fig. 7*d*). Taken together, we showed that eyespot size is a phenotypically plastic trait in *B. martius*. It responds in the same direction and to the same extent to developmental temperature as in its seasonal congener *B. anynana*: a lower temperature during development induces the expression of smaller eyespots in adults. Although both species show plasticity in wing pattern, variation in eyespot size is consistently higher in *B. martius* compared to *B. anynana*.

Plasticity across species along axes of phenotypic variance

We combined data from both species and used a Principal Components Analysis (PCA) on eight phenotypic traits (larval development time, pupal development time, pupal mass, abdomen dry weight, thorax dry weight, RMR, interfocal distance and size of eyespot 5) to separate and visualise variation along different major axes. Principal Components (PCs) 2 and 3 separate the different temperature cohorts in both species, and reflect the common phenotypic plasticity (Fig. 8*a*). In contrast, PCs 1 and 6 separate the temperature cohorts mainly in *B. anynana*, and reflect the reduced plasticity in *B. martius* (Fig. 8*b*).

PC2 (x axis in Fig. 8*a*), explained 23% of total variance and was mainly a measure of development time (see Supplementary Table 2 for loadings of each trait along the PCs). It was strongly affected by developmental temperature, revealing the large effect of temperature on rate of development. Both species responded to the same extent to developmental temperature along this axis, but for each temperature the species were clearly separated, reflecting the much slower development of *B. martius* compared to *B. anynana*. Furthermore, this axis separates females and males at each temperature in *B. anynana* but not in *B. martius*, showing the protandry in the former species. PC3 (y axis in Fig. 8*a*), explained 17% of total variance and was mainly determined by the eyespot size. Again, in both species this axis separates temperature cohorts, reflecting wing common pattern plasticity. We interpreted PC1, explaining 31% of variance, as a measure of body size, separating females from males, in particular in *B. anynana* (x axis in Fig. 8*b*). In addition, cohorts reared at different temperatures also separated along this axis, but this was again mainly the case in *B. anynana*. Thus, this axis reflects the reduced sexual size dimorphism as well as the weaker body size plasticity in *B. martius* (*cf*. Fig. 3). Finally, PC6 has loadings with opposite signs for thorax and pupal mass on the one hand and abdomen mass on the other hand (Supplementary Table 2). It explained 5% of total variance and separated the cohorts reared at different temperatures, in particular 19°C from the rest, but almost exclusively in *B. anynana* and only very weakly in *B. martius* (y axis in Fig. 8*b*). This axis thus reflects the reduced developmental plasticity of abdomen size in *B. martius* (*cf*. Fig. 5).

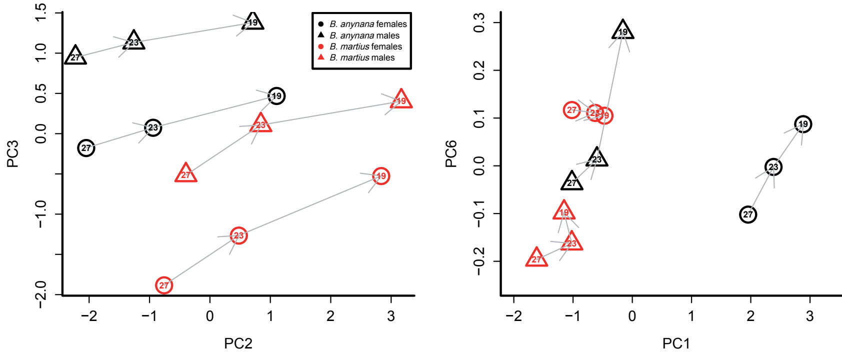

Figure 8. Plasticity in *B. martius* compared to *B. anynana* along major axes of phenotypic variance. Principal Components Analyses (PCA) on eight phenotypic traits in *B. martius* (red) and *B. anynana* (black) females (circles) and males (triangles) for cohorts reared at three different developmental temperatures (numbers plotted in graph), with grey arrows representing reaction norms. *a*) Second and third Principal Components (PCs), explaining 23 and 17% of phenotypic variance, respectively, and showing temperature plasticity in both species. *b*) First and sixth PC, explaining 31 and 5% of phenotypic variance, respectively, and showing reduced temperature plasticity in *B. martius* compared to *B. anynana*. *B. anynana* data reproduced from Chapter 2 (Oostra *et al.* 2011). See Methods and Results for details on phenotypic traits and Supplementary Table 2 for loadings of traits on each PC.

Together, the PCA visualises how the two species respond to developmental temperature along several important phenotypic axes. In particular, it is clear that *B. martius* responds to a lesser extent to developmental temperature along some aspects of life history variation (PC1 and 6 in Fig. 8*b*) compared to *B. anynana*, while the responses to developmental temperature along other axes (PC2 and 3 in Fig. 8*a*) were much more similar between the species and indicated extensive plasticity.

Discussion

The rainforest butterfly *B. martius* showed striking differences in thermal responses among traits that in *B. anynana* are all highly responsive to developmental temperature and are involved in seasonal adaptation. Most traits (development time, adult mass, RMR and ventral eyespot size), responded readily to temperature, albeit not always in exactly the same way as in *B. anynana* (Fig. 2-4 and 6-7). For example, male and female *B. martius* larvae developed on average equally fast, while *B. anynana* larvae show consistent protandry. The observed temperature plasticity in these traits was in stark contrast with the lack of response to temperature for relative abdomen size. In *B. anynana*, adults develop a relatively larger abdomen when reared under warm, wet season conditions, reflecting the higher early reproductive investment in this season (see Chapter 2, Oostra *et al.* 2011). However, in *B. martius* this was not the case: neither females nor males showed evidence of increased

mass allocation to abdomen when reared in warm conditions, with the slope of thermal reaction norms not deviating from zero (Fig. 5*a*). Thus, exposing *B. martius* in the laboratory to an unnatural range of temperatures reveals plasticity for some traits, but not all.

In many animals, plastic traits are integrated into functional suites that co-vary in response to environmental cues (Brakefield & Zwaan 2011; Pigliucci 2003; Schlichting & Pigliucci 1998; Simpson *et al.* 2011). For example, diapause phenotypes in insects involve many physiological and morphological traits that need to be adjusted in a coordinated and timely fashion (e.g. Gotthard & Berger 2010). Such phenotypic integration is often accomplished by shared endocrine regulation of these traits (Denlinger 2002; Ketterson *et al.* 2009). It has been hypothesised that central hormonal regulation of suites of traits is the result of past selection on tight integration of traits that need to work well together. Such selection may lead to depletion of trait-specific genetic variation, which in turn could constrain the short-term independent evolution of these traits (Ketterson & Nolan 1999; McGlothlin & Ketterson 2008). However, if there is enough trait-specific genetic variation left or if there is enough time for new mutational variation to accumulate, there is scope for antagonistic selection to decouple traits sharing a hormonal regulator (e.g. Zijlstra *et al.* 2003).

Several seasonally plastic traits in *B. anynana* have been found to be controlled by the same hormonal system. Artificial selection and hormone manipulation experiments established that Ecdysteroid hormones active during the pupal stage mediate developmental plasticity of ventral wing pattern (Brakefield *et al.* 1998; Koch *et al.* 1996). Subsequently, it was discovered that the same hormone also controls pupal development time, explaining why selection on development time affected wing pattern and vice versa. Antagonistic selection on these traits revealed that Ecdysteroids are more tightly associated with development time than with wing pattern (Zijlstra *et al.* 2003; Zijlstra *et al.* 2004). Examining the thermal reaction norm at finer detail showed that hormone dynamics respond in a threshold-like manner to developmental temperature, while wing pattern responds linearly. This suggested some additional level of regulation between hormone signalling and trait response (Oostra *et al.* 2011). In the same experiment, it was found that relative abdomen size shows the same threshold-like response as the hormone dynamics. Manipulative experiments confirmed that pupal Ecdysteroids provide the functional link between juvenile environment and adult reproductive strategy, although not all life history traits seem to fall under this control (Chapter 3). Together, these studies point to pupal Ecdysteroids as a general (though not sole) regulator of developmental plasticity in a suite of seasonally plastic traits in *B. anynana* (a 'developmental switch' *sensu* Nijhout 2003).

Our current results for *B. martius* indicate that this hormonally mediated co-variation across temperatures between wing pattern and allocation to abdomen has been broken: the former still responds to developmental temperature while the latter does not. At the mechanistic level, two alternative routes could lead to the observed difference in temperature responses between the traits. First, the upstream Ecdysteroid switch may have lost (some) sensitivity to developmental temperature in *B. martius*. With systemic hormone titres no longer responding to temperature, the developing abdomen would receive no temperature

signal and plasticity would be reduced. However, temperature-dependent hormone signalling to the developing wing would be reduced as well. This would imply that an unknown, Ecdysteroid-independent mechanism would be responsible for the observed temperature plasticity in wing pattern. In the alternative scenario, the upstream Ecdysteroid switch is still temperature sensitive, but the developing abdomen has evolved reduced sensitivity to circulating Ecdysteroids. This would lead to reduced plasticity of abdomen allocation, but not of wing pattern, as the developing wings would retain their hormone sensitivity. This hypothesis could be tested by measuring systemic hormone concentrations in *B. martius* pupae reared at different temperatures in conjunction with measuring the effects of hormone manipulations on wing pattern and abdomen allocation (*cf.* Chapters 2 and 3). A likely mechanism for abdomen-specific reduction in Ecdysteroid sensitivity is reduced expression of Ecdysone receptor (EcR) or other elements of the Ecdysteroid signalling pathway in the abdomen, but not in the wings. Finally, measuring genetic correlations between abdomen size and wing pattern in different environments in a species that is plastic for both traits, such as *B. anynana*, will indicate how much trait-independent genetic variation is available for evolutionary decoupling of temperature responses (*cf.* Aalberg Haugen *et al.* 2012; McGlothlin & Ketterson 2008; Zijlstra *et al.* 2003)).

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The allocation of body mass to the abdomen is an important determinant of female reproductive investment and early fecundity in many insects, including Lepidoptera (Boggs 1981; Jervis *et al.* 2005; Kivela *et al.* 2012). Any (plastic) reduction in female abdomen size would thus likely inflict a strong cost on fecundity for species under time constraints. For *B. anynana*, the dry season in the savannah is a period of severely limited reproductive opportunities, due to lack of larval food plants. In addition, food is limited for adults as well (Brakefield & Zwaan 2011). Under such circumstances, selective pressures in the dry season likely drive the seasonally plastic re-allocation of mass away from the abdomen, as observed in the laboratory. This increases availability of resources for survival until the end of the dry season. The large abdomen at eclosion expressed in the wet season is probably particularly important for early life fecundity. This is a major component of life-time fecundity in this species, as it can only reproduce during a limited period of the year (Brakefield *et al.* 2001). In contrast, the rainforest species *B. martius* does not experience a seasonal reduction in food availability, as green larval food plants continue to be abundant even at the end of the dry season (see Fig. 1). Therefore, it has the potential to breed continuously throughout the year, with overlapping generations. This would relax the need to invest larval-derived resources in a large abdomen already at eclosion in order to be able to start ovipositing very early in life (potentially at the expense of lifetime fecundity; *cf.* Chapter 3). Indeed, *B. martius* females in the laboratory start ovipositing late, continue to do so for a long time and live relatively long lives (pers. obs.). In addition, a relatively heavy abdomen could potentially constrain adult flight ability, increasing susceptibility to predation (*cf.* Srygley & Chai 1990). Together, this could explain why the ratio of abdomen to thorax mass at eclosion was similar to that in *B. anynana* in the dry, not the wet season (Fig. 5*e*). If *B. martius* females would still have the developmental machinery for reduced mass allocation to abdomen in response to temperature, occasional colder periods or even the limited seasonal fluctuations

in temperature could inflict a serious cost on fecundity by further reducing an already decreased allocation to the abdomen. This loss of temperature sensitivity in abdominal allocation might therefore have evolved via selection for maintaining a stable abdomen size despite occasional temperature fluctuations.

Plasticity of eyespot size in *Bicyclus* butterflies is maintained by opposing forces of natural selection in the dry and wet season savannah environments. In the dry season, when plants are brown and dried out, there are strong survival benefits of having a cryptic wing pattern with no or only small eyespots (Brakefield & Frankino 2009). As a rainforest species from a constant, green environment, *B. martius* is never or at most very rarely exposed to this selection pressure for enhanced crypsis. In the wet season on the savannah, adults express large eyespots as part of an alternative anti-predatory strategy. These eyespots probably deflect vertebrate predator attacks away from the vulnerable body towards the margin of the wing, allowing butterflies to be more active than if they would have to rely on crypsis (Brakefield & Frankino 2009; Kodandaramaiah 2011). This type of selection pressures is likely to be relevant for *B. martius*, as the rainforest habitat it inhabits is more comparable to the savannah wet season environment. In the absence of selection for small, cryptic eyespots in a dry season environment, *B. martius* would only experience natural selection for large, deflective eyespots. Such on-going selection would presumably lead to reduced phenotypic plasticity, provided that no other major selective forces act on eyespot size. However, this is contrary to the observed plasticity in the current experiment. One explanation for the evolutionary maintenance of eyespot size plasticity in *B. martius* could be that temperature sensitivity is still beneficial within the rainforest environment. Higher temperatures permit higher flight activity (e.g. via increased metabolic rate; Niitepold *et al.* 2009) but this may carry enhanced predation risks (*cf*. Bonte *et al.* 2012). If large eyespots are more effective in deflecting predatory attacks than small ones, expressing large eyespots might permit higher levels of activity at higher temperatures. On the other hand, when activity is restricted at lower temperatures, having large eyespots would be less beneficial and may even attract predators. Testing this hypothesis would require experimentally quantifying predation risk as a function of eyespot size and activity levels. In several *Bicyclus* species, eyespot size variation associated with temperature fluctuations within the wet season has been found, but how this relates to selection is unknown (Roskam & Brakefield 1999; Windig *et al.* 1994). To date, empirical support for the selective advantage of wet season-like eyespots as deflectors of predator attacks in *Bicyclus* remains weak. Field studies on the savannah showed that compared to the strong selection for crypsis in the dry season, selection for large eyespots in the wet season is too weak to detect (Brakefield & Frankino 2009). Laboratory studies indicated some selective advantage of eyespots when the predators were naïve birds, but not with lizards or experienced birds (Lyytinen *et al.* 2004; Lyytinen *et al.* 2003). Within the rainforest (or during the wet season on the savannah), any temperature-dependent fitness consequences of variation in eyespot size are likely even smaller and probably very difficult to detect.

If indeed there is no strong selection acting on wing pattern plasticity within the rainforest environment, constraints on evolutionary change towards reduced plasticity might be more important. In this hypothesis, the evolutionary retention of eyespot

size plasticity in *B. martius* is best explained as a legacy of past selection in a seasonal environment. In *B. anynana*, it has been shown that a lack of temperature-independent genetic variation in eyespot size can constrain the evolution of reduced plasticity, at least in the short term. Artificial selection experiments targeting the slope of the reaction norm for wing pattern failed to produce lines with reduced plasticity. This was due to high, positive genetic correlations across temperatures (Wijngaarden & Brakefield 2001; Wijngaarden *et al.* 2002). The observed retention of wing pattern plasticity in *B. martius* indicates that such strong developmental constraints may also be relevant over longer evolutionary time scales, especially if selective benefits of wing pattern plasticity in the rainforest environment are weak. Although we did not measure genetic variation, phenotypic variation for eyespot size was substantially higher in *B. martius* compared to *B. anynana*, and more so at lower temperatures (Fig. 7*d*), consistent with the view of relaxed selection on eyespot size and / or increased mutational variation.

Taken together, our results suggest that costs of plasticity in *Bicyclus* butterflies mainly stem from mismatch costs, i.e. expressing a suboptimal phenotype in a particular environment (Auld *et al.* 2010), which likely differ between phenotypically plastic traits. We hypothesise that this has caused some traits (allocation to abdomen) to evolve reduced environmental sensitivity and other traits (eyespot size) to retain plasticity, despite sharing a hormonal regulatory mechanism underlying the temperature responses. Our understanding of the extent to which plasticity in wing pattern and in life history can evolve independently would greatly benefit from studying these traits systematically in a phylogenetic context. Many *Bicyclus* species, including species closely related to *B. martius*, show plasticity in wing pattern (Brakefield & Frankino 2009; Roskam & Brakefield 1999), but it is unknown how plastic they are in their life history, in particular reproductive investment. Combining such knowledge with data on seasonal variation in reproductive opportunities and food levels in the natural habitat of each species will provide a powerful framework for testing hypotheses of trait-specific loss and retention of plasticity in this genus.

In seasonal insects, time constraints on development can promote the evolution of protandry while continuous breeding opportunities with overlapping generations are associated with absence of protandry (Allen *et al.* 2011; Blanckenhorn *et al.* 2007; Nylin *et al.* 1993). *B. martius*, inhabiting an environment with limited seasonal variation in reproductive opportunities, showed no evidence of protandry: males did not develop faster than females at any temperature (Fig. 2). This contrasts sharply with the significant protandry observed in a number of previous experiments in *B. anynana* (e.g. Chapter 2, Oostra *et al.* 2011), which only has a limited time period in the wet season to reproduce. In this species, artificial selection experiments showed that strong genetic correlations in development time between the sexes preclude all but a small short term evolutionary response to selection for increased or decreased protandry (Zwaan *et al.* 2008). This is likely the results of strong selection in the past on protandry, reducing sex-independent genetic variation for development time (Allen *et al.* 2011). The lack of protandry observed in *B. martius* suggests that such a genetic constraint can be broken over longer evolutionary time scales.

As in the majority of insects (Stillwell *et al.* 2010), *B. martius* adults showed female-biased sexual size dimorphism (Fig. 3*b*). However, this was not yet the case in the pupal stage (Fig. 3*a*), when males and females were equally large. This indicates that, in contrast to many insects (Allen *et al.* 2011), female and male *B. martius* larvae grow equally fast, reaching the same pupal mass at the same time (Fig. 2*a*). Subsequently, during the pupal stage, female pupae lose less mass and end up being larger as adult than males. In several Lepidoptera males lose more mass than females, which is probably related to a sex-specific trade-off between water allocation to mature eggs and flight ability (Molleman *et al.* 2011). However, adult dry and fresh weight in *B. martius* showed the same sex-specific relation with pupal mass, indicating that the sex-specificity in pupal mass loss was not related to water content. Instead, the higher RMR observed in adult males compared to females (Fig. 4) together with the longer pupal development time (Fig. 2*b*) suggests that pupal development consumes more resources in males compared to females.

A similar pattern was observed for thermal plasticity of pupal and adult mass: individuals reared at lower temperatures were larger as adult, but not as pupa (Fig. 3). The negative effect of developmental temperature on adult size observed in many ectotherms (Atkinson 1994) is usually explained by the effect of temperature on duration and rate of growth (Edgar 2006; Davidowitz & Nijhout 2004). However, *B. martius* larvae developing at low temperatures reached the same average pupal mass as those developing at high temperatures, despite taking much longer (Fig. 2*a*). During the pupal stage, these pupae lost less mass than those that had developed at higher temperatures, ending up as larger adults. Intriguingly, these larger adults had a higher mass-specific RMR than the smaller ones developed at lower temperature (Fig. 4). Thus, independent of its effects on larval growth, temperature can affect metamorphosis and utilisation of larval-derived resources to the adult body.

Previous studies in *B. anynana* (e.g. Pijpe *et al.* 2007) and other insects (Berrigan 1997; Le Lann *et al.* 2011) reported a negative effect of development temperature on adult RMR, suggesting a general mechanism for coping with lower temperatures rather than a specific seasonal adaptation. We observed the same general pattern in *B. martius*, but there are interesting differences between the sexes. In *B. anynana*, RMR is higher in males compared to females, but only at 27 C. This might relate to the higher reproductive activity in wet season conditions, when males are actively searching for females and fighting for territories. *B. martius* males have a higher RMR compared to females regardless of developmental temperature, suggesting that *B. martius* males do not alter breeding activity in response to temperature, similar to the response of female abdominal allocation (see above).

Conclusions

Exposing the aseasonal rainforest butterfly *Bicyclus martius* in the laboratory to a range of temperatures not normally encountered in the field revealed hidden reaction norms for several traits, including wing pattern and adult size. In contrast, allocation of adult mass to the abdomen, as a proxy for early-life reproductive investment, was not affected by

developmental temperatures. In the savannah butterfly *Bicyclus anynana* these traits show developmental plasticity as an adaptation to the contrasting environments of its seasonal habitat. In that species, wing pattern and allocation to abdomen respond to developmental temperature via a common hormonal system active during pupal development. Our results for *B. martius* indicate that such shared hormonal regulation does not preclude decoupling of temperature responses between traits over evolutionary time. We hypothesise that the loss of plasticity in abdomen allocation is the result of strong natural selection against temperature-induced fecundity reduction in the rainforest, combined with selection for more continuous breeding. For wing pattern, such selective forces are likely much weaker, resulting in retention of developmental plasticity. Thus, hormonal integration between plastic traits—as a result of past selection on expressing a coordinated environmental response—can be broken when the optimal reaction norms for those traits diverge in a new environment.

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Supplementary table 1. Minimum adequate models of the effect of developmental temperature and sex on a suite of phenotypic traits in *B. martius* and *B. anynana*, related to Figures 2 to 7. In case none of the fixed effects was significant ($p < 0.05$) in a particular model, the full model is shown (in grey). For *B. anynana*, previously collected data was used (see Methods), from 1) Chapter 2 (Oostra *et al.* 2011), or 2) Chapter 3.

species	dependent variable	fixed effects	F	df	p
B. martius	larval development time	temperature	201.54	2, 202	<0.00001
B. martius	pupal development time	temperature	2212.965	2, 201	<0.00001
		sex	34.689	1, 201	<0.00001
B. martius	total development time	temperature	356.69	2, 202	<0.00001
B. anynana[1]	total development time	temperature	6095.9699	2, 516	<0.00001
		sex	77.4916	1, 516	<0.00001
		temperature x sex	5.0336	2, 516	0.006839
B. martius	pupal mass	temperature	0.3135	2, 200	0.7312
		sex	0.1495	1, 200	0.6994
		temperature x sex	0.0879	2, 200	0.9159
B. martius	adult fresh mass	temperature	6.4465	2, 196	0.001943
		sex	10.5795	1, 196	0.001346
B. martius	size-corrected RMR	temperature	20.209	2, 191	<0.00001
		sex	106.269	1, 191	<0.00001
B. anynana[1]	size-corrected RMR	temperature	121.7973	2, 186	<0.00001
		sex	6.0362	1, 186	0.01493
		temperature x sex	4.0784	2, 186	0.01847
B. martius (females)	size-corrected abdomen mass	temperature	0.8475	2, 84	0.4321
B. martius (males)	size-corrected abdomen mass	temperature	2.4629	2, 110	0.08988
B. martius (pooled sexes)	size-corrected abdomen mass	temperature	0.4635	1, 194	0.4968
		sex	1.3876	2, 194	0.2522
		temperature x sex	1.7990	2, 194	0.1682
B. anynana[2] (females)	size-corrected abdomen mass	temperature	14.923	2, 260	<0.00001
both species[1] (females)	ratio of abdomen on thorax mass	temperature	13.0584	2, 347	<0.00001
		species	99.9531	1, 347	<0.00001
		temperature x species	4.2408	2, 347	0.01515
B. martius	size corrected fifth eyespot radius	temperature	45.202	2, 188	<0.00001
B. martius	size corrected second eyespot radius	temperature	33.2006	2, 187	<0.00001
		sex	6.1256	1, 187	0.01421
B. anynana[1]	size corrected fifth eyespot radius	temperature	120.6144	2, 298	<0.00001
		sex	27.9619	2, 298	<0.00001
		temperature x sex	2.3951	1, 298	0.09291

Supplementary table 2. Loadings of phenotypic traits on first six Principal Components (PC), as shown in Figure 8, with explained variance of each PC as percentage of total variance in parentheses. For *B. anynana*, previously collected data (Chapter 2; Oostra *et al.* 2011) was used (see Methods).

trait	**loading on PC1 (31%)**	**loading on PC2 (23%)**	**loading on PC3 (17%)**	**loading on PC4 (9%)**	**loading on PC5 (7%)**	**loading on PC6 (5%)**
larval development time	-0.171	0.584	-0.249	0.308	-0.060	-0.571
pupal development time	-0.009	0.638	0.165	0.219	0.454	0.414
pupal mass	0.488	-0.081	0.024	-0.108	0.291	-0.404
abdomen dry weight	0.431	0.030	-0.303	0.515	-0.583	0.223
thorax dry weight	0.487	0.097	-0.008	-0.117	0.121	-0.365
RMR	0.320	0.418	-0.218	-0.667	-0.215	0.296
interfocal distance	0.452	-0.133	0.234	0.348	0.304	0.218
size of eyespot 5	-0.054	-0.206	-0.846	0.034	0.461	0.145

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7 Summary, discussion and perspective

Hormones linking the environment with life history syndromes

Hormones play central regulatory roles linking environmental cues to alternative life history strategies in many animals (discussed in Chapter 1). Previous work in *Bicyclus anynana* established Ecdysteroid hormones as mediators of developmental plasticity in adult wing pattern, and identified the early pupal stage as the critical period for hormone signalling (Brakefield *et al.* 1998; Koch *et al.* 1996). Developmental plasticity in *B. anynana* not only entails wing pattern, but a much broader suite of life history traits involved in the adaptation to alternative seasonal environments (reviewed in Brakefield *et al.* 2007; Brakefield & Zwaan 2011). Experiments by Zijlstra and colleagues (2004) revealed that wing pattern and Ecdysteroids are both tightly linked to development time (Zijlstra *et al.* 2004), suggesting additional roles for these hormones. Thus, the question that motivated the experiments described in Chapters 2 and 3 was whether these developmental hormones are specialised wing pattern plasticity regulators, or actually play a broader role and mediate plasticity in the full life history syndrome, beyond rate of development.

In **Chapter 2**, we approached this question by characterising fine scale reaction norms for adult phenotypic traits involved in the seasonal adaptation, in conjunction with reaction norms for pupal hormones putatively regulating plasticity in these traits. We reared cohorts of larvae at five temperatures spanning the natural range of seasonal conditions and measured pupal hormone dynamics for Juvenile Hormone (JH) I, II and III as well as for the Ecdysteroids 20-hydroxyecdysone and Ecdysone. For both Ecdysteroids, we discovered a threshold response in timing of peak titres to the linear environmental gradient. This demonstrates that hormone dynamics can translate a linear environmental gradient into a discrete signal and, thus, that the dichotomy between adult phenotypic morphs can already be programmed at the stage of hormone signalling during development. In contrast, none of the JHs showed any association with seasonal temperature and thus likely play no role in regulating the developmental plasticity. Crucially, some adult traits, most notably relative abdomen mass and resting metabolic rate (RMR), showed the same binary response to developmental temperature, providing a testable hypothesis regarding the role of Ecdysteroids in mediating the temperature response of these traits. Interestingly, wing pattern—known from injection and genetic studies to be regulated by Ecdysteroids—showed a linear response to the temperature gradient, contrasting with the dimorphic hormonal response. This suggests additional layers of regulation between the hormone signal and the response of the developmental pathways patterning the developing pupal wings. Such variation in hormone sensitivity could be achieved by variation in a number of mechanisms, including in overall Ecdysone Receptor (EcR) expression, in isoform-specific EcR expression, in EcR/USP binding affinity for Ecdysteroids, or in chromatin binding of the EcR/USP/Ecdysteroid complex (Klowden 2007). Together, the range of phenotypic responses suggests both shared regulation among traits as well as independent, trait-specific sensitivity to the systemic hormone signal.

Chapter 3 presents the results of a manipulative study that followed up on the correlative evidence implicating pupal Ecdysteroids in the regulation of developmental plasticity in adult life history strategy. Exogenous Ecdysteroids were applied to pupae reared at three separate temperatures, ranging from dry to wet season conditions, and phenotypic effects were monitored for a suite of seasonally plastic traits. Hormones were injected at one of four separate time points during pupal development, representing different stages of the natural dynamics in Ecdysteroid titres as measured in Chapter 2. In addition to accelerating pupal development, injections during the two earliest (but not the two later) time points induced increased allocation of adult body mass to the abdomen—a hallmark of the temperature-induced reproductive wet season morph. This demonstrates that pupal Ecdysteroids link developmental temperatures to adult reproductive body allocation. In contrast, RMR was not affected by exogenous Ecdysteroids, indicating that the imprint of developmental temperature on adult RMR is likely mediated by mechanisms independent of Ecdysteroid signalling early in the pupal stage. A subsequent follow-up experiment showed that the shift in reproductive body allocation is accompanied by changes in ecologically relevant traits such as timing of reproduction, lifespan and starvation resistance. Females injected with Ecdysteroids started egg laying earlier, with a faster decrease in later life egg output but an increased egg size compared to those injected with control solution. In addition, the earlier reproducing females had a shorter lifespan. Together, these findings support a functional role for pupal Ecdysteroids in mediating strategic reproductive investment decisions in response to variation in the quality of the environment experienced during development.

Initially it was hypothesised that similarity in the shape of reaction norms between traits would indicate shared underlying regulation. In particular, both RMR and relative abdomen size showed a threshold-like response to the linear temperature gradient, as did the pupal Ecdysteroids (Fig. 1*d*, 2 and 4 in Chapter 2). However, the prediction that both traits would thus be regulated by these hormones was falsified by a functional test: only abdomen size, not RMR was affected by pupal Ecdysteroids (Fig. 2 and 3 in Chapter 3). This could be explained if the effect of developmental temperature on adult RMR were determined prior to metamorphosis and the pupal Ecdysteroid cascade (*cf*. Pijpe *et al.* 2007). This is likely the case for pupal mass, which, at least in females, also showed a threshold response to temperature (Fig. 1*b* in Chapter 2), similar to the Ecdysteroid response. Pupal mass is determined by larval growth rate and by the duration of the growth period, both of which can be affected by temperature via several hormonal systems including Ecdysteroids, Insulin signalling and PTTH (Davidowitz & Nijhout 2004; Edgar 2006; Mirth & Riddiford 2007; Shingleton *et al.* 2007). It is tempting to speculate that temperature plasticity in *B. anynana* ultimately stems from temperature sensitivity of larval growth, which in turn affects pupal mass, RMR and pupal Ecdysteroid dynamics. Variation in the latter then induces alternative phenotypes for wing pattern, reproductive allocation decisions and life history strategy. Such a scenario could be tested by combining environmental manipulations with detailed measurements of larval growth as well as measurements on hormonal regulators (i.e. Ecdysteroids, Insulin and PTTH) during larval development. An

Figure 1. *a)* Experimental setup for the experiments described in Chapters 4 and 5. *b)* Female # 6.57 from the experiments described in Chapter 3, at the age of 80 days, 13 days before she died of natural causes. She outlived all her contemporaries, and laid 77 eggs during the first 2 weeks of her life. *c) B. martius* male in Ologbo Forest, Nigeria during the late dry season. Photo by Oskar Brattström. *d) B. martius* females ovipositing on *Oplismenus* grasses in the laboratory.

intriguing result in this context is expression data in *B. anynana* larvae, showing that *EcR*, coding for the Ecdysone Receptor, is more highly expressed in last instar larvae at high temperatures compared to larvae at low temperatures. However, this difference disappears 24-48 hours before pupation (K. van der Burg and V. Oostra, unpubl. data).

Together, the experiments described in Chapters 2 and 3 establish pupal Ecdysteroids as an important regulator of developmental plasticity in *B. anynana*. This role is not restricted to regulating wing pattern plasticity but encompasses a full suite of plastic traits that together contribute to the alternative seasonal life history syndromes. Although shared hormonal regulation of traits can on the one hand constrain the evolution of independent environmental responses, the modular nature of hormonal systems may on the other hand contribute to trait-specificity in responses (Ketterson *et al.* 2009). This could explain the shape differences in reaction norms between traits functionally regulated by Ecdysteroids, as observed here for *B. anynana*.

Transcriptional patterns underlying life history plasticity

Studying mechanisms of phenotypic plasticity provides a unique window into developmental processes that translate genotypes into phenotypes (Gilbert 2005; West-Eberhard 2003). Plasticity occurs when these processes show environmental sensitivity, resulting in alternative phenotypes. Because these phenotypes develop from the same genetic background, regulation of gene expression is a critical aspect of plasticity (Beldade *et al.* 2011). Furthermore, hormonal signalling pathways involved in regulating aspects of plasticity often converge on transcription factors, which can regulate expression of myriads of genes (e.g. McElwee *et al.* 2007). In a variety of animals, expression variation associated with alternative, environmentally induced phenotypes has been characterised. For example, in the honey bee *Apis mellifera*, Corona and colleagues (2005) studied expression of genes encoding antioxidant and mitochondrial metabolic enzymes, comparing short-lived workers with long-lived queens (Corona *et al.* 2005). Other studies have compared expression between alternative phenotypic morphs at the whole-genome level, for example between short- and long-lived morphs of the parasitic nematode *Strongyloides ratti* (Thompson *et al.* 2009).

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In *B. anynana*, environmental regulation of gene expression has received very little empirical attention. Gene expression associated with the alternative seasonal morphs has only been analysed in the context of wing pattern plasticity in developing pupal wings, and only for Distal-less (Brakefield *et al.* 1996) and EcR (P.B. Koch, unpubl. data). Interestingly, it was shown that EcR expression in developing wings is upregulated upon Ecdysteroid injection, revealing positive feedback between hormone levels and expression of its receptor (P.B. Koch, unpubl. data). Life history-related gene expression has been studied in lines artificially selected for starvation resistance, both under benign and starvation conditions, for three candidate genes involved in response to oxidative stress: *Indy*, *sod2* and *catalase* (Pijpe *et al.* 2011). However, this was not done in the context of seasonal plasticity. In Chapters 4 and 5, I studied gene expression variation associated with the alternative seasonal life history strategies, taking a candidate gene approach in parallel to an unbiased screen.

In **Chapter 4**, we analysed transcriptional variation in young, recently eclosed adults that differ in life history strategy as a result of development under alternative seasonal conditions. Using qPCR, expression of 27 life history-related genes was measured, as putative molecular effectors underlying the two phenotypes. These genes are associated with biological processes involved in the seasonal adaptation in *B. anynana* (e.g. lipid metabolism, Ecdysteroid signalling) or are associated with life history variation in other species (e.g. innate immunity, Insulin signalling). We found the clearest evidence for a developmental signature on adult expression in innate immune and metabolic genes, effector genes likely to be tightly linked to observed life history phenotypes. Immune genes were generally more highly expressed in the wet season, potentially reflecting a higher immune risk due to higher temperatures and reproduction-related immune challenges in the wet season. If the immune risk is indeed lower for dry season adults, they would thus be able to afford down-regulating innate immunity, avoiding the harmful 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 only a limited number of genes in this pathway could be sampled, Insulin signalling appears to be higher in the dry season. This is contrary to expectations, as high Insulin signalling is generally associated with increased reproduction and short lifespan. To reproduce successfully, adults of the dry season form in the field must survive many months of inactivity and down-regulated reproduction before the rains come. It would thus be interesting to analyse how expression in this and other pathways is affected by altered reproductive status and seasonal conditions during adult life.

In **Chapter 5**, we used custom-designed microarrays to probe whole-genome transcriptional profiles of young and old butterflies that developed in dry or wet season conditions, but lived as adults in the same wet season environment. Expression of *ca*. 10% of all genes was affected by age, the majority of which was down-regulated in older individuals. Strikingly, we observed extensive sex-specificity in the transcriptional response to aging, with half of all aging-related genes only being affected in a single sex. Females up-regulated stress response genes and down-regulated 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 were 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 may play additional roles in different life stages or environments.

The results from Chapters 4 and 5 on Insulin signalling are strikingly contrasting. In recently eclosed virgin adults (Chapter 4) developed in dry season conditions, *Pk61C*, a repressor of FoxO, was up-regulated and *Pepck*, a FoxO target, was down-regulated. Both results indicate low FoxO activity and thus high Insulin signalling in the dry season. In contrast, in mated adults of young and old age (Chapter 5), we observed up-regulation in the dry season of *PkC53E*, an activator of FoxO, indicating low Insulin signalling in the dry season. These seemingly conflicting results may be due to the somewhat different experimental design. Although in both experiments larvae were reared under the two different seasonal conditions, in Chapter 4 they were sampled as virgins, one day after eclosion when the developmental signature is likely the strongest. The higher Insulin signalling in dry season-reared adults might also be related to the similar developmental imprint on RMR, needed in the larval stage to sustain growth at the cooler temperatures of the dry season (discussed below). The adults sampled for the microarrays in Chapter 5 were older, mated and had all lived as adults in the same (wet season) conditions. Looking up the two Insulin-related genes measured using qPCR (Chapter 4) in the microarray data (Chapter 5) revealed that *Pk61C* and *Pepck* both showed lowest expression in the dry season,

although this was not statistically significant (t test, unadjusted p = 0.10 to 0.14). Low *Pepck* would indicate high Insulin signalling, but low *Pk61C* would indicate low Insulin signalling in the dry season. Clearly, adult conditions such as age, temperature and reproductive status can substantially affect expression of Insulin signalling-related genes. For a clearer understanding of the role of Insulin signalling in the seasonal adaptation in *B. anynana* it would be important to sample more genes involved in this pathway, which was currently not possible due to technical limitations (see Discussion in Chapter 5). As part of the same practical effort as the experiment described in Chapter 4, we sampled adults at various time points in adult life under virgin and reproductive conditions. Analysing these data and comparing with the results for young virgin adults will likely shed more light on this issue. It would also be important to be able to mimic more closely the full extent of dry season field conditions in the laboratory, so that the full natural progression of physiological and life history events in adults of the dry season form could be tracked effectively.

An evolutionary perspective on plasticity

The hormonal and transcriptional mechanisms analysed in Chapters 2 through 5 have likely evolved in a context of strong, contrasting selective pressures in the alternative environments, in combination with selection on environmental sensitivity to be able to switch between life history modes (Brakefield & Zwaan 2011). Understanding these selective pressures and their consequences for the evolution of plasticity would require a more detailed picture of the natural ecology of *Bicyclus* butterflies than we currently have. Similarly, comparative analyses of different species inhabiting environments with different degrees of seasonality would greatly enhance understanding of selective pressures driving the evolution of plasticity. The last data chapter of this thesis presents an analysis that shows the potential of such an approach.

In **Chapter 6**, we studied whether seasonal plasticity is still retained in *Bicyclus martius*, a butterfly species that inhabits the less seasonal rainforest in West Africa, where natural selection on plastic responses is assumed to be less strong or even absent. Little is known about the evolutionary fate of such responses when natural selection on plasticity is relaxed. Even less well studied are the consequences for plastic traits sharing a hormonal regulator when selective pressures on those traits diverge. In *B. anynana*, wing pattern and allocation to the abdomen respond to developmental temperature via a common hormonal system active during pupal development (Chapters 2 and 3). Such shared regulation may constrain evolutionary decoupling of plastic traits of which some, but not all, are under relaxed selection. Exposing the rainforest butterfly *B. martius* to an unnatural range of temperatures in the laboratory revealed hidden reaction norms for several traits, including wing pattern. Larval and pupal survival was lowest at the cool temperature, which in the field is experienced only very rarely or not all. In contrast, allocation of adult mass to the abdomen, as a proxy for early-life reproductive investment, was not affected by developmental temperatures. This indicates that shared hormonal regulation does not preclude decoupling of temperature responses between traits over evolutionary time. There is likely strong natural selection against plasticity in fecundity in the rainforest. However, for wing pattern such selective

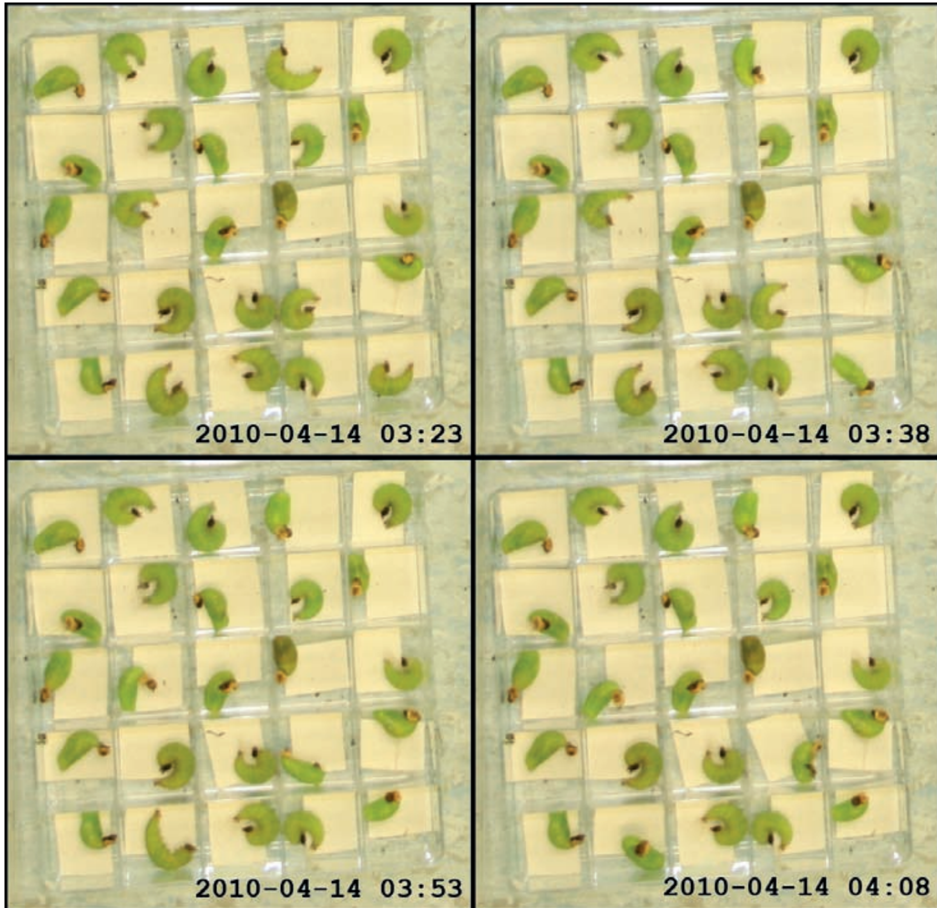

Figure 2. Timing of pupations. For the experiments described in Chapters 2 and 3, it was necessary to time the moment of pupation accurately in order to establish time series of hormone concentrations or to inject at the right moment during development. This was done by using time-lapse photography of larvae that were close to pupation. The four photos in this panel were taken at 15 minutes interval from one another during the daily peak of pupations.

forces are probably much weaker, as in *B. anynana* wing pattern is under much stronger natural selection in the dry *versus* the wet season (Brakefield & Frankino 2009). Thus, hormonal integration between plastic traits—as a result of past selection on expressing a coordinated environmental response—can be broken when the optimal reaction norms for those traits diverge in a new environment.

The molecular mechanisms of plasticity in *B. martius* are unknown, but it seems likely that Ecdysteroids are involved, as they are in *B. anynana* (see Chapters 2 and 3). A simple scenario for how plasticity of abdomen size has been lost could be abdomen-specific

reduction in sensitivity to circulating Ecdysteroids during the critical part of the pupal stage (see also Discussion in Chapter 6). Retention of wing pattern plasticity would, in this scenario, be explained by retention of environment-sensitivity in hormone signalling as well as hormone-sensitivity of wing pattern development. Such hypotheses could be tested by combining measurements and manipulations of Ecdysteroids with analyses of gene expression for genes involved in Ecdysteroid signalling, in particular Ecdysone Receptor.

Interestingly, *B. martius* showed the same temperature plasticity in resting metabolic rate (RMR) as observed previously in *B. anynana* (e.g. Chapters 2 and 3). In particular, young adults reared in cool, dry season conditions as larvae had a higher RMR as adult than those reared in warm, wet season conditions (when measured at the same adult temperature). Although it is tempting to interpret this developmental imprint in the context of seasonal developmental plasticity, it is also worth noting that such an imprint has been described previously for *D. melanogaster* (Berrigan 1997) and for the parasitic wasp *Aphidius rhopalosiphi* (Le Lann *et al.* 2011). These studies were interpreted in the light of benefits of thermal compensation at low temperatures during larval growth (Clarke 1993). Likewise in *B. anynana*, the effect of developmental temperature on adult RMR has been interpreted as a consequence of increased larval metabolism, needed to sustain growth at these low temperatures, but non-adaptive in adults (Pijpe *et al.* 2007). An additional potential benefit of increasing metabolic rate is to produce additional metabolic water during the cool dry season. However, an adaptive reason why altered RMR during the larval stage should affect adult RMR has not been proposed, nor has any mechanism linking RMR in the two life stages. In any case, the developmental imprint on adult RMR is much smaller than the opposing direct effect of ambient adult temperature, which indicates that the imprint is not so important for adult performance (Pijpe *et al.* 2007). This highlights the need for a comprehensive study on RMR in relation to temperature manipulations in both the larval and adult stage.

Perspective

This thesis aims to contribute to a better mechanistic understanding of plastic responses as adaptation to environmental fluctuations, in particular in seasonal environments. One major outcome emerging from these studies was the involvement of highly conserved hormone signalling pathways in specific ecological adaptations in *B. anynana* butterflies. This fits with findings on phenotypic plasticity in other animals, where the same hormonal systems have been co-opted over and over again for the regulation of a surprising variety of highly lineage-specific phenomena, ranging from beetle horn polymorphisms to reproductive diapause in fruit flies to social behaviour in Hymenoptera. This might seem less surprising if we interpret these hormonal systems as performing a more general function of linking information on the internal or external environment to the tuning of organismal functions that together make up an animal's life history (see also Fig. 2 in Chapter 1). A pathway already performing a function such as regulating growth rate under variable nutritional levels, might be co-opted relatively easily for phenotypic plasticity in other traits. The

modular nature of hormone systems, both in space (across separate body parts) and time (across life stages), likely contributes to this versatility (Heyland *et al.* 2005).

One aim of this thesis was to use *B. anynana* as a model of developmental plasticity in an effort to contribute knowledge on mechanisms linking development and aging in humans. Observations that events during early embryonic development can have profound effects on adult health and lifespan have fuelled hypotheses such as the 'thrifty phenotype hypothesis' and the 'predictive adaptive response' (discussed in Chapter 1). Using model organisms in an experimental setting can be a powerful approach in uncovering mechanistic links between development and adult health span. Furthermore, as the adaptive significance of the effects of fetal events on adult health in humans is far from clear, it is particularly useful to use models for which the ecological and evolutionary background is well studied. In *B. anynana*, the links between the developmental environment and the adult phenotype form an integral part of the life history, and are relatively well understood in ecological terms. The most relevant results in this context are likely those for transcriptional variation associated with the two seasonal morphs (Chapters 4 and 5). In both cases, we found indications that the Insulin signalling pathway shows a transcriptional signature in adults of events experienced during development. Although these observations are not easily interpreted in the light of human health, they do provide starting points for additional experimental work in model organisms. For example, quantifying the extent to which transcriptional signatures of developmental events may be reversible during adult life is an interesting avenue for follow-up research, and would provide information on the feasibility of counteracting or reversing developmental imprints in gene expression that negatively affect health at old age.

These are exciting times, as biologists are increasingly bringing together traditions of molecular and developmental biology with those of ecology and evolutionary biology, and linking understanding of mechanistic function with ecological function (Breuker *et al.* 2006; Ellers & Stuefer 2010; Flatt & Heyland 2011; Partridge 2008; Pavey *et al.* 2012; Sultan 2007; Zera *et al.* 2007). By combining an ecological and evolutionary perspective with the ambition to understand developmental, physiological and molecular genetic mechanisms underlying environmental sensitivity of life history strategies, this thesis has hopefully contributed to an integrative understanding of mechanisms underlying life history variation in variable environments.

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8 Nederlandse Samenvatting – Summary in Dutch

Flexibele vlinders – een hormonale schakelaar tussen kort of lang leven

De tropische savanne-vlinder *Bicyclus anynana* kan zijn voortplanting en veroudering omgooien als de omstandigheden daarom vragen. De hormonen en genen die dit voor elkaar krijgen bieden een inkijk in veroudering, ook bij mensen.

Zomba, Malawi – Het is half april, midden in de droge tijd. Al vier maanden heeft het nauwelijks geregend op deze stoffige, bruine vlakte. De bomen en struiken zijn verdord, de grassen zijn bijna verdwenen en de meeste dieren zijn hongerig weggetrokken naar betere streken om pas weer over twee maanden met de regens terug te keren. Toch zie je hier en daar wat kleine, bruine vlinders zitten. Die houden het uit door heel rustig aan te doen, vooral niet te veel te vliegen en al helemaal niet aan voortplanting te denken. Degene die het zuinigst aan kunnen doen, redden het tot het begin van de regentijd. De aanhoudende tropische buien die dan losbarsten zorgen voor een explosie van leven. Overal schieten planten uit de grond en binnen enkele weken ziet het groen tot aan de horizon. En de vlinders? Die bloeien nog een laatste keer op voordat ze het loodje leggen. Ze gaan na de eerste regens meteen voortplanten en leggen hun eitjes op het verse gras voordat ze sterven. Inmiddels zijn ze dan zes maanden oud. Maar wat doet de volgende generatie? Etend van het sappige gras ontwikkelen de jonge rupsen zich snel en komen uit als volwassen vlinders in een totaal andere omgeving dan waar hun ouders uitkwamen. Hun favoriete maaltijd, rottend fruit, ligt overal onder de bomen. Snel gaan ze op zoek naar een partner om mee te paren en leggen dan zo veel mogelijk eitjes. Uitgeput van dit snelle leven sterven ze als ze nog geen drie maanden oud zijn.

Omgaan met omgevingsvariatie

Niet alleen vlinders op de savanne staan voor de uitdaging het beste te maken van deze totaal verschillende omstandigheden. Voor bijna alle dieren en planten geldt dat hun mogelijkheden te overleven en succesvol te reproduceren diepgaand beïnvloed worden door variatie in temperatuur, neerslag, voedselhoeveelheid of roofdieractiviteit. Een belangrijke vraag in evolutiebiologie is hoe organismen omgaan met dit soort variatie. Zoals de vlinder *Bicyclus anynana* in de verschillende seizoenen op de savanne een totaal andere levensstijl vertoont (zie **Figuur 3 van Hoofdstuk 1**), zo zijn veel dieren opmerkelijk flexibel, en kunnen hun gedrag, fysiologie of morfologie aanpassen aan nieuwe omstandigheden. Een bekend voorbeeld zijn sprinkhanen, die bij het aanbreken van voedselschaarste en overbevolking van solitair, rustig gedrag omschakelen naar massaal en verwoestend zwermgedrag. Een aantal andere voorbeelden staan beschreven in **Box 1 van Hoofdstuk 1**.

De algemene term voor het vermogen om verschillende fenotypes (gedrag, fysiologie, morfologie) te produceren als reactie op veranderingen in de omgeving is *fenotypische plasticiteit*. Cruciaal hierbij is dat er geen genetische variatie ten grondslag ligt aan de fenotypische veranderingen—ze worden door de omgeving geïnduceerd. Er zijn verschillende vormen van fenotypische plasticiteit te onderscheiden. In dit proefschrift

onderzoek ik *ontwikkelingsplasticiteit*, waarbij de fenotypische veranderingen die door de omgeving geïnduceerd worden tijdens de ontwikkeling ontstaan. Het proces van ontwikkeling—het onstaan van een volwassen dier uit een bevruchte eicel—is in dit geval gevoelig voor omgevingsfactoren, en het uiteindelijke fenotype van de adult hangt af van de specifieke omgeving die het ontwikkelende embryo ervaren heeft. Een bekend voorbeeld is geslachtsdeterminatie in veel soorten reptielen. Afhankelijk van de temperatuur tijdens de incubatieperiode van het ei ontwikkelt het embryo zich in een mannetje of een vrouwtje. Het bestuderen van ontwikkelingsplasticiteit biedt dus inkijk in de ontwikkelingsprocesses die het genotype in het fenotype vertalen, en in hoe gevoeligheid van deze processen voor omgevingsfactoren een bron van fenotypische variatie kan zijn.

Van de savanne naar het lab

Leidse evolutiebiologen op veldwerk in Malawi raakten geïnteresseerd in *Bicyclus anynana*, en ontdekten al gauw dat de generaties vlinders verschillen al van elkaar wanneer ze uit de pop komen en hun ontwikkeling klaar is. De kortlevende vlinders van het natte seizoen baginnen hun leven actief en gaan vlug paren en eitjes leggen. Die van het droge seizoen komen ter wereld met grotere vetreserves, die ze als rups aangelegd hebben, en stellen de voortplanting uit. Ook vertonen de twee seizoensvormen opmerkelijke verschillen in vleugelpatroon. Er moet dus al tijdens de ontwikkeling, voordat de rupsen volgroeid en als vlinder uit de pop komen, iets in die vlinders anders zijn tussen de twee seizoenen. De biologen besloten een honderdtal levende vlinders mee te nemen naar het laboratorium en daar een populatie op te kweken, zodat ze er meer gecontroleerde experimenten mee konden doen. In het lab in Leiden deden ze een belangrijke ontdekking. Het bleek dat een jonge rups de potentie heeft om zowel droog- als natseizoensvlinder te worden. Er zijn geen genetische (overerfbare) verschillen tussen de twee seizoensvormen, en een opgroeiende rups kan, tot een bepaald moment, nog 'kiezen' tot wat voor volwassen vlinder hij zich zal ontwikkelen. Er is dus sprake van ontwikkelingsplasticiteit, waarbij omgevingsfactoren tijdens de ontwikkeling bepalen of een rups droog- of als natseizoensvlinder wordt. Zijn DNA is dus in staat om twee programma's af te draaien: één om kortlevende, snel voortplantende natseizoensvlinder te worden, en één om langlevende, stress-resistente droogseizoensvlinder te worden. Maar hoe weet een opgroeiende rups welk programma hij aan moet zetten, dus in wat voor vlinder hij zich moet ontwikkelen?

Op de savanne van Malawi is regen is niet het enige verschil tussen het droge en natte seizoen. In het droge seizoen is het ook een stuk kouder dan in het natte. Vlak voordat de regentijd aanbreekt, begint het warmer te worden (zie ook F**iguur 3 van Hoofdstuk 1**). Het blijkt dat dit temperatuurverschil voor opgroeiende rupsen het cruciale signaal is om te weten welk seizoen het gaat worden. Als het warm is, betekent dat op de savanne dat de regentijd er aan zit te komen. Een rups die dan groeit zal als vlinder in de regentijd uitkomen, en zet zijn regentijdprogramma aan. Als hij als volwassen vlinder uitkomt is hij meteen klaar om snel en veel te reproduceren. Kou is juist de voorbode voor de droge tijd. De rups zet bij lage temperatuur zijn drogeseizoensprogramma aan en ontwikkelt tot langlevende droogseizoensvlinder, die het goed volhoudt met weinig voedsel. Natuurlijk

zijn er altijd fluctuaties in temperatuur in het wild. Soms daalt de temperatuur een beetje in de warme tijd, soms stijgt hij een beetje in de droge tijd. Het is voor een opgroeiende rups dus van levensbelang om onderscheid maken tussen die kleine fluctuaties binnen een seizoen en de kritieke temperatuurwisseling tussen de seizoenen. Eigenlijk zou het handig zijn om een soort schakelaar te hebben die óf het natseizoensprogramma aanzet, óf het recept om droogseizoensvlinder te worden. Die schakelaar zou dan alleen 'om' moeten gaan bij een drempelwaarde van temperatuur die in het wild indicatief is voor de wisseling van de seizoenen. Precies zo'n schakelaar is nu ontdekt—een hormoon met de naam Ecdyson.

Hormonen als schakelaar

In insecten sturen hormonen allerlei fundamentele processen aan zoals groei, metamorfose, voortplanting en veroudering. Ook fenotypische plasticiteit wordt gereguleerd door hormonen, die prikkels uit de omgeving vertalen naar expressie van alternative fenotypes. Eén belangrijk insectenhormoon is Ecdyson, een steroïdhormoon dat als receptor een transcriptiefactor heeft die de activiteit van honderden genen beïnvloedt. Het was al van eerder onderzoek in de vlinder *Bicyclus anynana* bekend dat dit hormoon belangrijk is voor het reguleren van plasticiteit in vleugelpatroon. In poppen stijgt de concentratie Ecdyson in het bloed eerder als ze zijn opgegroeid op hoge temperatuur zijn opgegroeid—wat indicatief is voor het regenseizoen—dan als ze zijn opgegroeid op lage temperatuur—de omstandigheden van het droge seizoen. Met hormooninjecties op precies het juiste moment tijdens de ontwikkeling kan het vleugelpatroon veranderd worden, zodat droog seizoensvlinders nat seizoensvlinders worden. Maar de verschillen tussen de seizoensvormen gaan veel verder dan vleugelpatronen, en behelzen een heel scala aan eigenschappen, zoals voortplanting en levensduur. De vraag of Ecdyson ook betrokken is bij het reguleren van dit brede pallet aan verschillen in levensstijlen tussen de seizoenen, en dus een meer algemene regulator van plasticiteit is, is het eerste onderwerp van dit proefschrift.

In **Hoofstuk 2** onderzoek ik niet alleen poppen die óf op lage, óf op hoge temperatuur opgroeiden, maar ook op drie andere temperaturen tussen de twee extremen in. Door Ecdysonconcentraties te meten in poppen langs de hele gradient van seizoenstemperaturen was ik in staat te onderzoeken of de hormonale veranderingen tussen droog en nat seizoen geleidelijk zijn of juist in een sprong verlopen. Het laatste bleek het geval te zijn. Dit betekent dat dit hormoon precies de rol van schakelaar vervult tussen kleine en grote temperatuurfluctuaties in de omgeving, en zo zorgt voor het kiezen van het juiste programma voor het droge of natte seizoen. Als de temperatuur laag is, hebben kleine fluctuaties geen invloed op dit hormoon. Ook als de temperatuur hoog is wordt dit hormoon niet beïnvloed. Alleen bij een drempelwaarde van temperatuur—die in het wild de wisseling van de seizoenen aankondigt—schakelt dit hormoon tussen hoge en lage concentraties en zet op die manier het programma voor het droge of natte seizoen aan. Dit gebeurt allemaal terwijl de rups net in een pop veranderd is, en er dus nog tijd is om het ontwikkelingsprogramma bij te stellen voordat de vlinder uit de pop komt. Naast hormonen in de pop heb ik een aantal eigenschappen in de jongvolwassen vlinder gemeten,

waaronder de verdeling van massa tussen thorax en abdomen. Aangezien in de thorax de vleugelspieren zitten en in het abdomen de reproductieve organen betekent veel massa naar de thorax dat de vlinder relatief veel energie investeert in vliegvermogen. Veel massa naar het abdomen betekent dat de vlinder relatief veel energie investeert in reproductie. Het bleek dat volwassen vlinders die als rups op hoge, nat seizoenstemperatuur zijn opgegroeid een zwaarder abdomen hebben, en vlinders van het droge seizoen juist een lichter abdomen. Belangrijker nog, dit verschil tussen droog en nat seizoen verloopt niet gelijdelijk maar sprongsgewijs, net als de veranderingen in hormoonconcentraties. Deze correlatie suggereert dat Ecdyson niet alleen passief reageert op temperatuur, maar ook tijdens de ontwikkeling direct en actief de processen bijstuurt die ervoor zorgen dat de volwassen vlinder het juiste fenotype krijgt: een groot, voor snelle voortplanting toegerust abdomen voor het natte seizoen, of juist een relatief klein abdomen voor het droge seizoen, waar voortplanting juist uitgesteld wordt.

In **Hoofstuk 3** test ik deze hypothese door middel van hormooninjecties. Poppen die opgegroeid zijn op lage temperatuur ontwikkelen normaal gesproken in droogseizoensvlinders, met een relatief klein abdomen. Injecties van Ecdyson tijdens het popstadium waren voldoende om de volwassen vlinders een fenotype te geven dat ze normaal alleen krijgen als ze op hoge natseizoenstemperatuur opgroeien: een groter abdomen. Nu is een groter abdomen een indicatie voor het investeren van meer energie in snelle voortplanting, maar het is geen directe maat. Daarom heb ik het experiment herhaald en ook eileg en levensduur in vrouwtjes gemeten. Hier bleek dat de morfologische verschillen in abdomengrootte die veroorzaakt worden door Ecdyson inderdaad relevant zijn voor voortplanting en overleving. Vrouwtjes die als pop een Ecdysoninjectie hadden gehad begonnen eerder met eitjes leggen, legden later in het leven juist minder eitjes, en bovendien leefden ze korter.

Genen die veroudering reguleren?

Fenotypische plasticiteit is de expressie van verschillende fenotypen zonder dat er sprake is van genetische verschillen. Hetzelfde DNA is in staat verschillende programma's af te draaien, afhankelijk van de prikkels uit de omgeving. Een belangrijk mechanisme hiervoor is regulatie van genactiviteit. De genen die op het DNA liggen kunnen actief zijn of minder actief, en de gezamenlijke activiteit van de duizenden genen bepaalt het uiteindelijke fenotype. Dit is enigszins vergelijkbaar met hoe verschillende cellen binnen het menselijk lichaam van elkaar kunnen verschillen, ondanks dat ze genetisch identiek zijn. Zo zijn in spiercellen vooral spiergenen actief, die bijvoorbeeld de eiwitten maken waaruit de spieren zijn opgebouwd. In hersencellen zijn vooral hersengenen actief, die coderen voor bijvoorbeeld de eiwitten die van belang zijn voor communicatie tussen zenuwcellen. Om in de twee totaal verschillende seizoenen te kunnen overleven, ligt het voor de hand dat de vlinder *Bicyclus anynana* twee programma's in zich heeft, die afhankelijk van het seizoen aan kunnen worden gezet: één programma om kortlevende, snel voortplantende natseizoensvlinder te worden, en één om langlevende, stress-resistente droogseizoensvlinder te worden. Beide programma's zijn gecodeerd in het DNA dus worden ook beiden elke

generatie doorgegeven aan het nageslacht, zodat ook die tot dezelfde flexibiliteit in staat zijn. De seizoensvormen verschillen niet in welke genen ze hebben maar in de activiteit van de genen in elk van de seizoenen. Het programma voor elk seizoen wordt bepaald door welke genen actief zijn in elk seizoen. Het tweede deel van dit proefschrift gaat over de zoektocht naar deze genen.

In **Hoofdstuk 4** heb ik genactiviteit gemeten van 27 genen waarvan het aannemelijk is dat ze betrokken zijn bij de seizoensadaptatie in *Bicyclus anynana*, of die bij andere dieren van belang zijn voor veroudering en voortplanting. Dit zijn genen betrokken zijn bij vet- en suikermetabolisme, immuungenen, en genen die de hormoonhuishouding regelen. Over het algemeen waren de immuungenen actiever in vlinders van het regenseizoen. Dit kan te maken hebben met verhoogde kans op infecties in dat seizoen, wanneer het warmer is en micro-organismen sneller groeien. Ook is het regenseizoen de periode wanneer vlinders zich voortplanten, en ook dat brengt verhoogde kans op infecties met zich mee. In het droge seizoen waren juist genen actiever die met vet- en suikermetabolisme te maken hebben. Dit geeft aan dat in de droge tijd—wanneer er voedselschaarste heerst—vlinders niet alleen meer investeren in het binnenkrijgen en opslaan van voedsel, maar voor hun energiebehoefte ook meer gebruikmaken van eerder opgeslagen reserves.

In **Hoofdstuk 5** gebruikte ik microarrays om in oude en jonge vlinders van beide seizoensvormen de activiteit van duizenden genen tegelijk te meten. Ten opzichte van jonge vlinders waren in oude vlinders veel genen uitgeschakeld die bij voortplanting betrokken zijn. Genen die met stress-respons te maken hebben vertoonden op hoge leeftijd juist verhoogde activiteit. Opvallend genoeg waren deze leeftijdsgerelateerde veranderingen in genactiviteit vrijwel afwezig bij de langlevende vlinders van het droge seizoen. Deze genen dragen dus potentieel bij aan het langlevende fenotype in het droge seizoen. Veel van deze genen komen bij alle dieren voor. Wellicht zijn ze ook bij andere dieren, inclusief mensen, net zo belangrijk in het reguleren van veroudering. Hiermee biedt deze studie naar tropische vlinders nieuwe aanknopingspunten in het begrijpen van veroudering, ook bij mensen.

De evolutie van fenotypische plasticiteit

Fenotypische plasticiteit in *Bicyclus anynana*—inclusief het hele systeem van hormonen en variatie in genactiviteit dat het reguleert—wordt waarschijnlijk in stand gehouden door sterke natuurlijke selectie in de natuur. In het droge seizoen is het erg voordeling om zuinig te doen met voedsel en energie, en zeer nadelig om voort te planten—er is immers voor de nakomelingen (rupsen) geen voedsel. In het regenseizoen daarentegen is er juist veel haast bij de voortplanting. Vlinders die te lang wachten met voortplanten krijgen geen kans meer, omdat dan immers het droge seizoen weer aanbreekt. Bovendien zal er sterke selectie zijn op het vermogen om signalen van de omgeving op te kunnen pikken en dus flexibel de levensstijl om te gooien wanneer de seizoenen wisselen. Wat zou er gebeuren als deze sterke selectiedruk zou verdwijnen?

In **Hoofdstuk 6** onderzoek ik plasticiteit in een andere vlindersoort, *Bicyclus martius*, die niet van nature op de savanne voorkomt maar in het regenwoud in West-Afrika. Hier

is er geen droog seizoen waar de voedselplanten opdrogen en het beter is de reproductie uit te stellen. Er is ook geen haast met voortplanten en eitjes leggen—dat kan immers het hele jaar door. De vraag is dus in hoeverre *Bicyclus martius* nog steeds gevoelig is voor de temperatuurschommelingen die op de savanne de seizoenswisselingen aanduiden, zoals bijna alle andere *Bicyclus* soorten. Het opkweken van deze rupsen onder lage temperatuur—die ze van nature niet tegenkomen maar op de savanne het droge seizoen aankodigt—resulteerde allereerst in volwassen vlinders met een ander vleugelpatroon, dat erg leek op het patroon van de savannevlinder *Bicyclus anynana* tijdens de droge tijd. De grootte van het abdomen—een maat voor investering van energie in vroege en snelle voortplanting—werd echter niet beïnvloed door temperatuur. Met andere woorden, fenotypische plasticiteit was nog wel aanwezig voor vleugelpatroon, maar niet meer voor reproductie. Dit betekent dat hoewel beide eigenschappen door hetzelfde Ecdyson gereguleerd worden, ze toch losgekoppeld kunnen worden. Waarom dit gebeurd is is hier niet onderzocht, maar het is waarschijnlijk dat plasticiteit in voortplanting nadelig is in een constante omgeving. Als een kortstondige lagere temperatuur in het regenwoud meteen zorgt dat een vrouwtje stopt met voortplanten en eitjes leggen, zal ze meteen minder nakomelingen produceren, terwijl dit niet nodig is. Er is immers in het regenwoud altijd gelegenheid is tot voortplanting en eileg, omdat de voedselplanten nooit uitdrogen. Doordat temperatuur niet meer voorspellend is voor voorplantingsmogelijkeden zal er dus natuurlijke selectie optreden op het verliezen van de koppeling tussen temperatuur en reproductie. Voor vleugelpatronen geldt dit hoogstwaarschijnlijk niet, omdat de selectieve nadelen van het hebben van een 'verkeerd' vleugelpatroon veel kleiner zijn dan voor voortplanting. Dit betekent dat hormonale integratie tussen verschillende eigenschappen gebroken kan worden als natuurlijke selectie niet meer in dezelfde richting werkt voor deze eigenschappen.

Tot slot

In dit proefschrift heb ik gepoogd bij te dragen aan een beter begrip van mechanismen die organismen in staat stellen om te gaan met schommelingen in hun omgeving, en name met de wisseling van de seizoenen. Eén opvallende uitkomst was dat hormonen die zeer geconserveerd zijn en fundamentele processen zoals groei en voortplanting bij heel veel dieren reguleren, ook een zeer specifieke rol kunnen spelen in seizoensadaptatie bij een savanne-vlinder. Het feit dat hormonale systemen sterk modulair zijn zowel in ruimte (tussen lichaamsdelen) als in tijd (tussen levensfasen) draagt waarschijnlijk bij aan deze veelzijdigheid aan rollen die ze kunnen spelen. Een ander doel van dit proefschrift was bij te dragen aan beter begrip van het verband tussen ontwikkeling en veroudering, en hoe gebeurtenissen tijdens de vroege ontwikkelingen langetermijn gevolgen voor gezondheid en levensduur kunnen hebben. Om veroudering bij mensen beter te begrijpen is het noodzakelijk experimenten in modelorganismen te koppelen aan observaties in mensen. Ook wij mensen zijn het product van evolutie, en de processen die veroudering en levensduur reguleren zijn waarschijnlijk onder andere omstandigheden geëvolueerd dan die waar we nu in leven. Daarom is het ook noodzakelijk modelorganismen te gebruiken

waarvan de evolutionaire en ecologische achtergrond beter bekend is, zoals de tropische savannevlinder *Bicyclus anynana*. Dit past in een trend waarbij de tradities van moleculaire biologie en ontwikkelingsbiologie aan de ene kant, en die van evolutiebiologie en ecologie aan de andere kant, meer en meer bij elkaar gebracht worden. Hiermee wordt het begrip van mechanistische functie meer nadrukkelijk gekoppeld aan dat van ecologische functie. Met dit proefschrift hoop ik aan deze integratie een bijdrage te leveren.

8

Addendum

List of Publications

Curriculum Vitae

List of Publications

Peer-reviewed publications

4) **Oostra V.***, de Jong M.A.*, Invergo B.M., Kesbeke F., Wende F., Brakefield P.M. & Zwaan B.J. (2011) Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly. *Proceedings of the Royal Society B 278 (1706): pp. 789-797 (Chapter 2 of this thesis).*
3) Terenius O. *et al*. (2011) RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. *Journal of Insect Physiology 57 (2): pp. 231-245.*
2) **Oostra V.**, Gomes L.G.L. & Nijman V. (2008) Implications of deforestation for the abundance of restricted-range bird species in a Costa Rican cloud forest. *Bird Conservation International 18 (1): pp.11-19.*
1) Gomes L.G.L.*, **Oostra V.***, Nijman V., Cleef A.M. & Kappelle M. (2008) Tolerance of frugivorous birds to habitat disturbance in a tropical cloud forest. *Biological Conservation 141 (3): pp. 860-871.*

** Shared first authorships*

Manuscripts to be submitted

- **Oostra V.**, Mateus A.R.A., van den Burg K.R.L., Piessens T., van Eijk M., Brakefield P.M., Beldade P. and Zwaan B.J. Ecdysteroids link juvenile environment to adult life history in a seasonal insect *(Chapter 3).*
- **Oostra V.**, Pul N., van Eijk M., Brakefield P.M. and Zwaan B.J. Seasonally induced expression variation in life history genes in the butterfly *Bicyclus anynana (Chapter 4).*
- **Oostra V.**, Beldade P., Brakefield P.M., Pul N., van Eijk M. and Zwaan B.J. Developmental signature of the ageing-related transcriptional profile in a seasonal butterfly *(Chapter 5).*
- **Oostra V.**, Brakefield P.M., Hiltemann Y., Zwaan B.J. and Brattström O. On the fate of seasonally plastic traits in a rainforest butterfly under relaxed selection *(Chapter 6).*
- Mateus A.R.A., **Oostra V.**, Marques-Pita M., Lafuente E., Brakefield P.M., Zwaan B.J. and Beldade P. Adaptive developmental plasticity: Fine-scale compartmentalization of ecdysteroid-regulated tissue patterning.
- Mateus A.R.A., **Oostra V.**, Marques-Pita M., Brakefield P.M., Zwaan B.J. and Beldade P. Hormonal regulation of developmental reaction norms.

Curriculum Vitae

Vicencio Oostra was born on 5 January 1981 in Ibagué, Colombia. Shortly thereafter, he moved to The Netherlands. He attended high school in Nijmegen at the Stedelijk Gymnasium Nijmegen, and in Leiden at the Stedelijk Gymnasium Leiden, where he obtained his VWO diploma. In 1999 he enrolled at the University of Amsterdam to study Biology. For the first research internship of his Master's program, he travelled to the Costa Rican cloud forest together with his friend and classmate Laurens. Under the supervision of Prof. Antoine Cleef (Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam), Dr. Maarten Kappelle (The Nature Conservancy, Costa Rica), and Dr. Vincent Nijman (Zoological Museum Amsterdam, University of Amsterdam), Vicencio studied the impact of anthropogenic habitat disturbance on diversity and population densities of cloud forest bird species (2003-2004). He was awarded several small scholarships for the field work, and these studies resulted in two publications. During his years at the University of Amsterdam, Vicencio also worked as a teaching assistant in the Bachelor courses *Genetics* and *Statistics and Mathematical Modeling*, and in *Bètabrug*, a one-year pre-university science program. The second research internship of his Master's progam took Vicencio to Leiden University. In the Evolutionary Biology Group of the Institute of Biology Leiden he studied gene expression during wing pattern development in butterflies, supervised by Dr. Suzanne Saenko and Dr. Patrícia Beldade (2007). He obtained his Master's degree in 2007 with distinction (*cum laude*). Following his graduation, Vicencio had the opportunity to return to the same group in Leiden as a PhD student, with Prof. Bas Zwaan and Prof. Paul Brakefield as advisors (2007-2012). As part of the EU-funded Network of Excellence LifeSpan, he studied developmental plasticity of life histories in the butterfly *Bicyclus anynana*, the results of which are presented in this thesis. Vicencio gave oral presentations of his work at six international conferences, as well as at several annual LifeSpan meetings and smaller seminars. He received two travel grants for lab visits and attendance of conferences (ESF and LifeSpan). In addition, he attended courses on quantitative genetics (University of Liège, Belgium) and on microarray data analysis (Instituto Gulbenkian de Ciência, Portugal). Together with another PhD student and two postdocs, he organized LifeSpan's first Young Investigator Workshop in Dubrovnik, Croatia. Vicencio travelled to the laboratory of Prof. Klaus Hoffmann (University of Bayreuth, Germany) to learn to use liquid chromatography / mass-spectrometry (LC-MS) for hormone quantitations. During the course of his PhD, he supervised the research projects of three MSc and two BSc students. After his time in Leiden, Vicencio followed Prof. Bas Zwaan to the Genetics Department at Wageningen University, where he has started to use RNAseq to better understand the transcriptomic changes underlying seasonal responses.

Photo by Oskar Brattström.

http://www.vicencio-oostra.dds.nl/