# Summary, discussion and perspective

7

# 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).

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

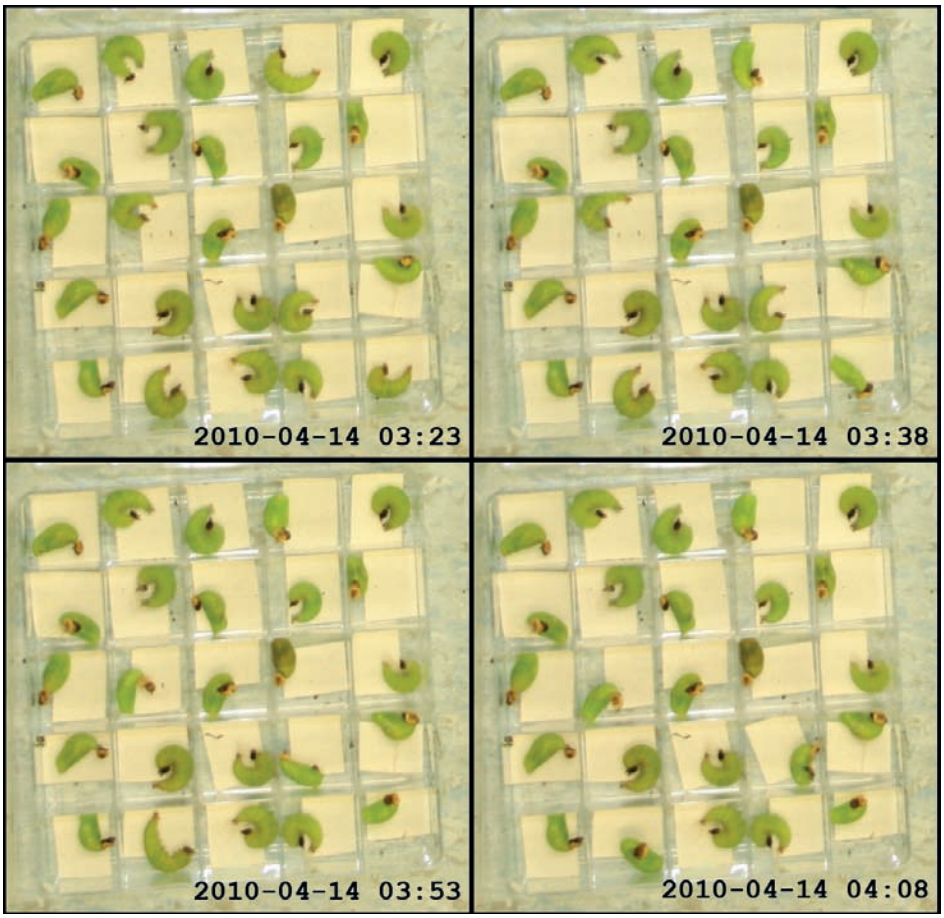


**Figure 2. Timing of pupations.** For the experiments described in Chapters 2 and 3, it was necessary to time the moment of pupation accurately in order to establish time series of hormone concentrations or to inject at the right moment during development. This was done by using time-lapse photography 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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