3

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

Vicencio Oostra[1,4], Ana Rita A. Mateus[1, 2], Karin R. L. van der Burg[1], Thomas Piessens[1], Marleen van Eijk[1], Paul M. Brakefield[1,3], Patrícia Beldade[1,2], and Bas J. Zwaan[1,4]

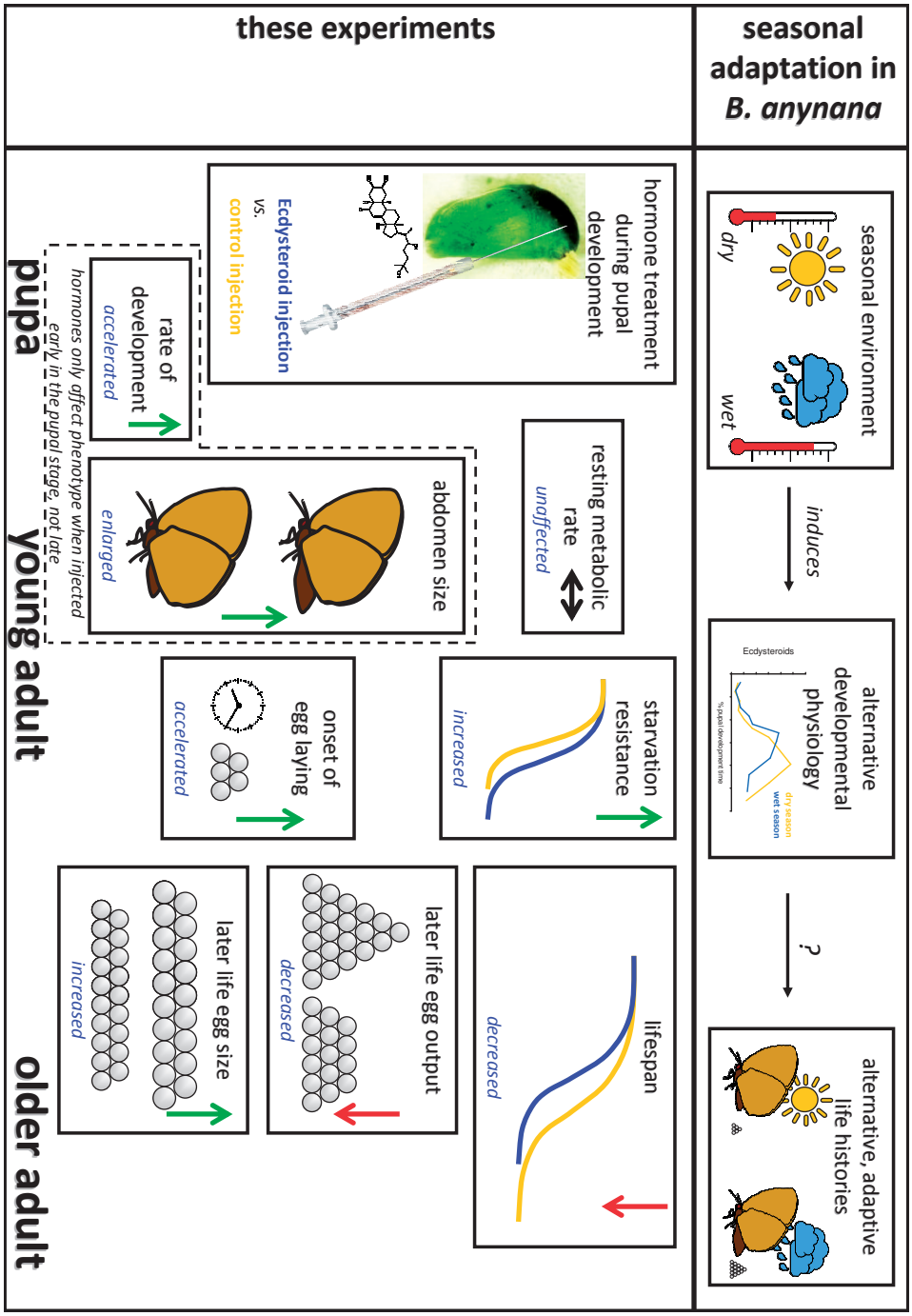
[1]Institute of Biology, Leiden University, PO Box 9505, 2300 RA, Leiden, The Netherlands [2]Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, P-2780-156 Oeiras, Portugal [3]Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK [4]Laboratory of Genetics, Wageningen University and Research Centre, P.O. Box 309, 6700 AH Wageningen, The Netherlands

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 4^{th} and 5^{th} 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

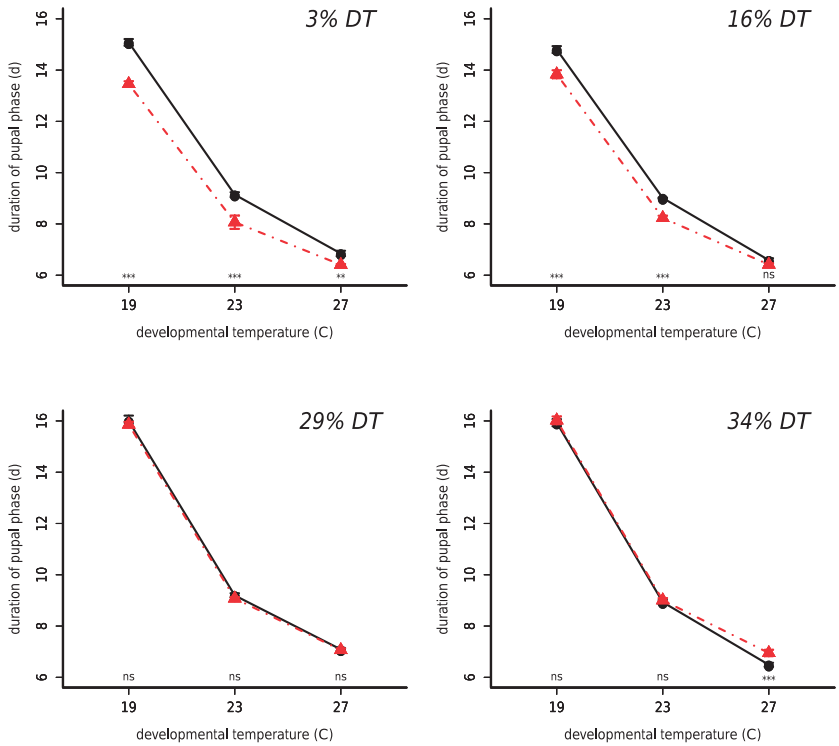


Figure 1. Early but not late 20-hydroxyecdysone (20E) treatment accelerates pupal development. Duration of pupal stage (days, ±SEM) is strongly affected by developmental temperature, as indicated by the shape of reaction norms and large differences between extreme temperatures (two-way ANOVA $p < 0.00001$). 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.

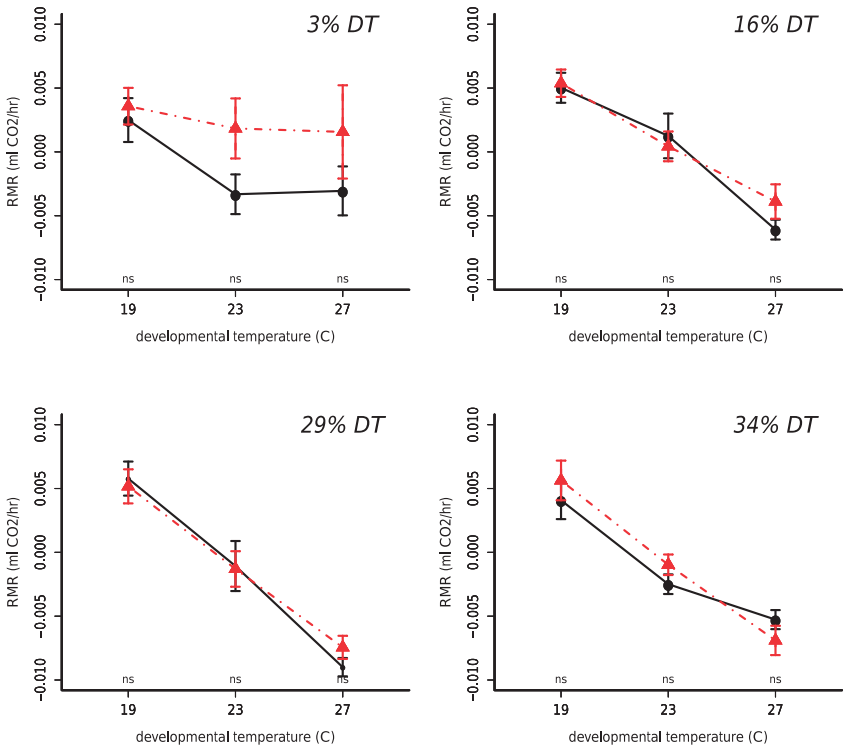


Figure 2. Developmental temperature imprint on adult resting metabolic rate (RMR) is not affected by pupal Ecdysteroids. Mass-corrected RMR (ml CO_2 hr^{-1}; see Table S1) is significantly affected by developmental temperature with individuals reared at lower temperature having higher RMR (two-way ANOVA $p < 0.05$). 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

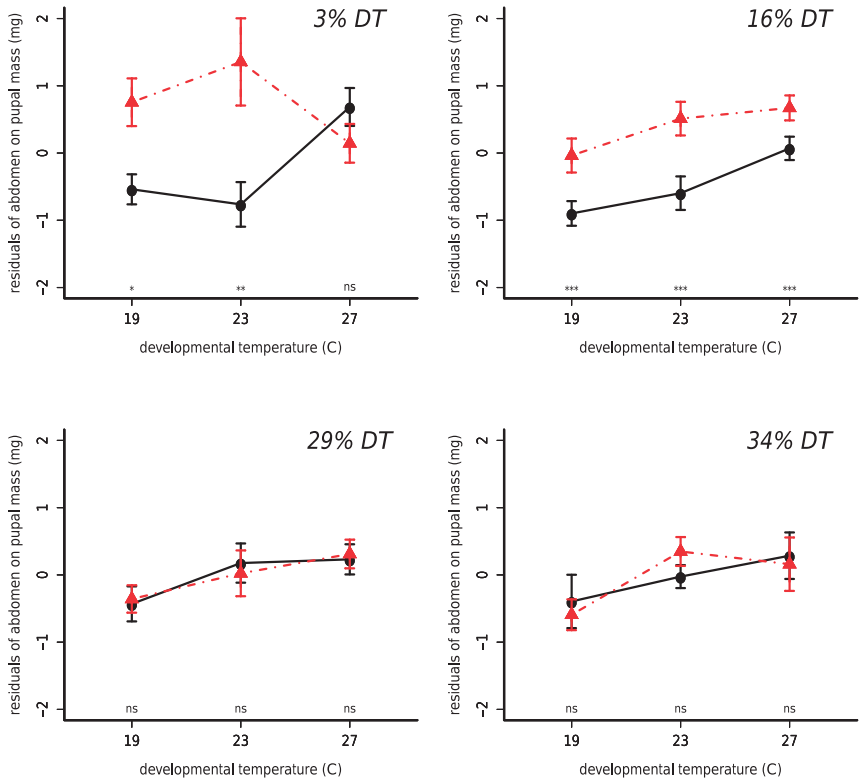


Figure 3. Pupal Ecdysteroids induce high, wet-season like allocation to abdomen mass. Mass-corrected abdomen dry weight (mg; see Table S1) is significantly affected by developmental temperature with females reared at high temperatures (wet season conditions) having a larger abdomen (two-way ANOVA $p < 0.05$). 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,

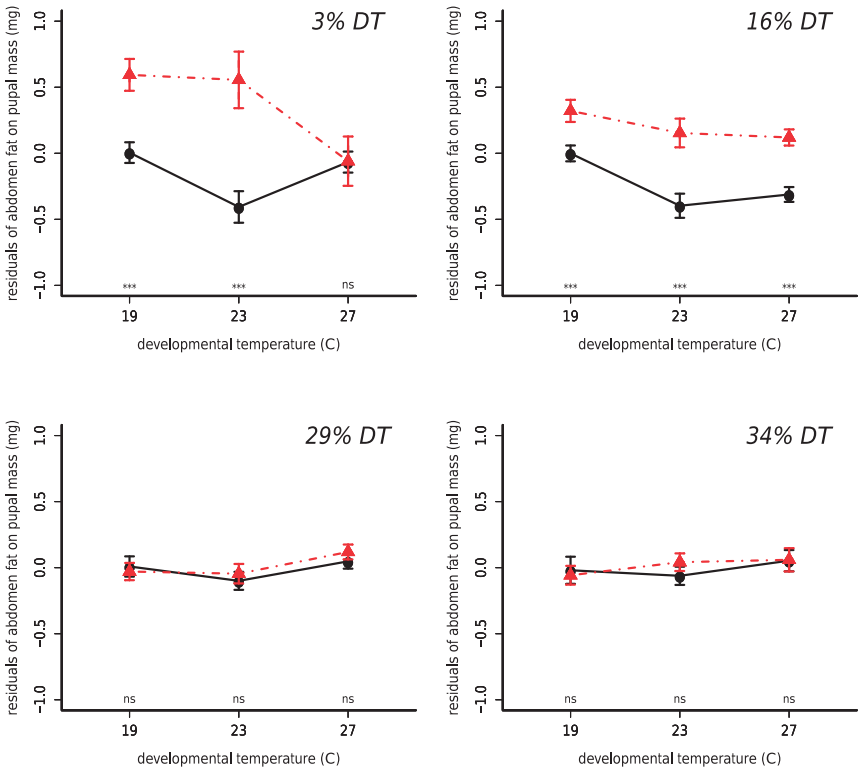


Figure 4. Pupal Ecdysteroids induce higher abdominal fat content in adult females. Mass-corrected abdomen fat content (mg; see Table S1) is significantly affected by temperature (two-way ANOVA $p < 0.05$), except in the cohorts injected at 34% DT. In addition, pupae injected with 20E (red) at 3 or 16, 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

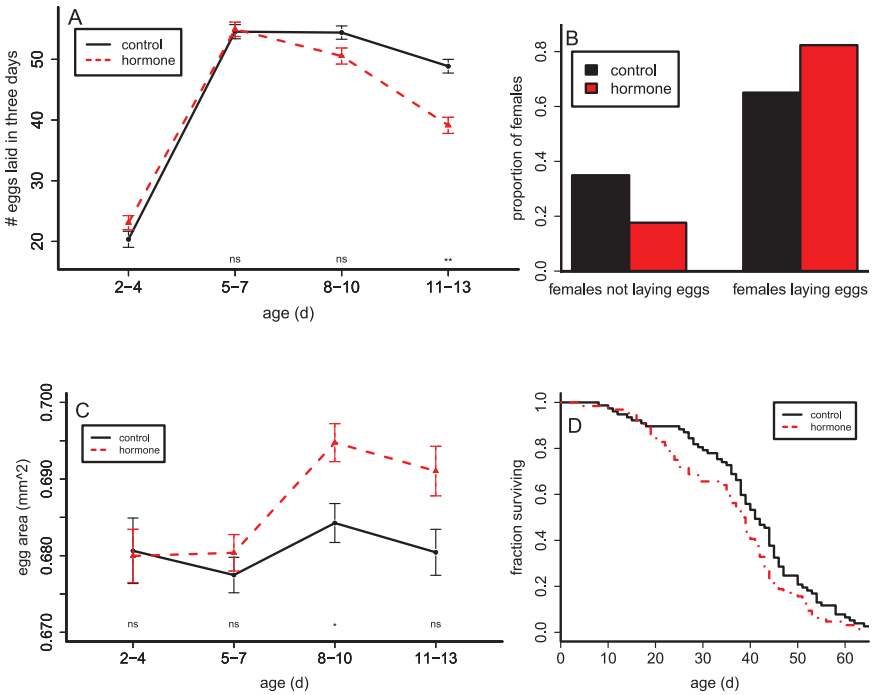


Figure 5. Pupal Ecdysteroids affect reproductive schedule and lifespan. A) Female fecundity (number of eggs laid) is highly affected by female age ($p < 0.00001$ for likelihood ratio test (LRT) between model with and without age). In addition, adult females injected as pupa with 20E (red) had lower fecundity 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.

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

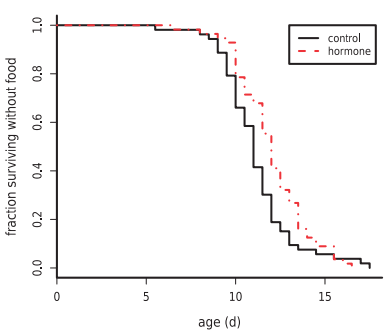


Figure 6. Pupal Ecdysteroids enhance adult starvation resistance in virgin females. Daily adult survival without food is increased in virgin females injected as pupa with 20E (red) compared to controls (black; Cox proportional hazard $p < 0.01$; hazard ratio = 0.68). See also Table S3.

(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.

Acknowledgments

The authors wish to thank M. Lavrijsen, D. Hallesleben, O. Brattström, E. van Bergen, N. Pul, K. Koops, J. Bot, and C. Jacobs for invaluable practical assistance during the experiments, M. Saastamoinen and M. de Jong for advice, C. Breuker for the ImageJ macro for egg size measurements, and J. van den Heuvel and H. van Mil for advice on the statistical analyses. This work was supported by the European Union's FP6 Programme (Network of Excellence LifeSpan FP6/036894 to VO and BJZ), the EU's FP7 Programme (IDEAL FP7/2007-2011/259679 to BJZ) and the Portuguese Foundation for Science and Technology (SFRH/BD/45486/2008 to ARAM and PTDC/BIA-BDE/100243/2008 to PB).

Supplementary information

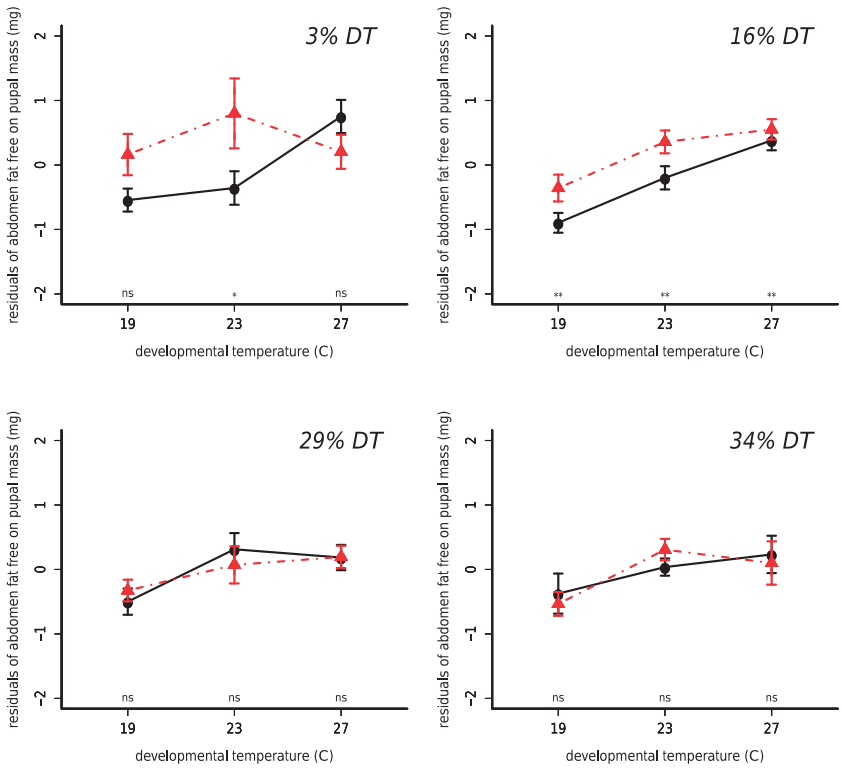


Figure S1. Pupal Ecdysteroids induce increase in fat-free abdomen mass. Mass-corrected abdomen fat fat-free dry weight (mg; see Table S1) is significantly affected by temperature (two-way ANOVA $p < 0.01$). In addition, pupae injected with 20E (red) at 16, but not at 29 or 34% DT, show a substantial 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.

References

Arpagaus M, Beydon P, Lafont R (1986) Effects of ecdysone injection on diapause termination in *Pieris brassicae* pupae – evidence for a stimulatory action on endogenous ecdysone production. *Reproduction Nutrition Development* **26**, 1305-1317.

Baayen RH (2011) *languageR: Data sets and functions with "Analyzing Linguistic Data: A practical introduction to statistics"* R package version 1.4. http://CRAN.R-project.org/package=languageR.

Bates DM, Maechler M, Bolker B (2011) *lme4: Linear mixed-effects models using S4 classes* R package version 0.999375-39. http://CRAN.R-project.org/package=lme4.

Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology* **20**, 1347-1363.

Berrigan D (1997) Acclimation of metabolic rate in response to developmental temperature in *Drosophila melanogaster*. *Journal of Thermal Biology* **22**, 213-218.

Braendle C, Heyland A, Flatt T (2011) Integrating mechanistic and evolutionary analysis of life history variation. In: *Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs* (eds. Flatt T, Heyland A), pp. 3-10. Oxford University Press, Oxford, UK.

Brakefield PM, Beldade P, Zwaan BJ (2009) The African butterfly *Bicyclus anynana*: a model for evolutionary genetics and evolutionary developmental biology. *Cold Spring Harbor Protocols* **2009**.

Brakefield PM, Kesbeke F, Koch PB (1998) The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana*. *American Naturalist* **152**, 853-860.

Brakefield PM, Reitsma N (1991) Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. *Ecological Entomology* **16**, 291-303.

Brakefield PM, Zwaan BJ (2011) Seasonal polyphenisms and environmentally-induced plasticity in the lepidoptera – the coordinated evolution of many traits on multiple levels. In: *Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs* (eds. Flatt T, Heyland A), pp. 243-252. Oxford University Press, Oxford, UK.

Brisson JA (2010) Aphid wing dimorphisms: linking environmental and genetic control of trait variation. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* **365**, 605-616.

Dawson A (2008) Control of the annual cycle in birds: endocrine constraints and plasticity in response to ecological variability. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* **363**, 1621-1633.

de Jong MA, Kesbeke FMNH, Brakefield PM, Zwaan BJ (2010) Geographic variation in thermal plasticity of life history and wing pattern in *Bicyclus anynana*. *Climate Research* **43**, 91-102.

Denlinger DL (2002) Regulation of diapause. *Annual Review of Entomology* **47**, 93-122.

Emlen DJ, Warren IA, Johns A, Dworkin I, Lavine LC (2012) A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science* **337**, 860-864.

Fischer K, Eenhoorn E, Bot AN, Brakefield PM, Zwaan BJ (2003) Cooler butterflies lay larger eggs: developmental plasticity versus acclimation. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **270**, 2051-2056.

Geister TL, Lorenz MW, Meyering-Vos M, Hoffmann KH, Fischer K (2008) Effects of temperature on reproductive output, egg provisioning, juvenile hormone and vitellogenin titres in the butterfly *Bicyclus anynana*. *Journal of Insect Physiology* **54**, 1253-1260.

Gilbert SF (2012) Ecological developmental biology: environmental signals for normal animal development. *Evolution and Development* **14**, 20-28.

Hofmann GE, Todgham AE (2010) Living in the now: physiological mechanisms to tolerate a rapidly changing environment. *Annual Review of Physiology* **72**, 127-145.

Klowden MJ (2007) *Physiological Systems in Insects* Elsevier Science / Academic Press, London, UK.

Koch PB (1996) Preadult changes of ecdysteroid and juvenile hormone titers in relation to diapause and pigmental variations in two lepidopteran species, *Cerura vinula* and *Araschnia levana* (Lepidoptera: Notodontidae Nymphalidae). *Entomologia Generalis* **20**, 143-155.

Koch PB, Brakefield PM, Kesbeke F (1996) Ecdysteroids control eyespot size and wing color pattern in the polyphenic butterfly *Bicyclus anynana* (Lepidoptera: Satyridae). *Journal of Insect Physiology* **42**, 223-230.

Le Lann C, Wardziak T, van Baaren J, van Alphen JJM (2011) Thermal plasticity of metabolic rates linked to life-history traits and foraging behaviour in a parasitic wasp. *Functional Ecology* **25**, 641-651.

Meylan S, Miles DB, Clobert J (2012) Hormonally mediated maternal effects, individual strategy and global change. *Philosophical Transactions of the Royal Society B-Biological Sciences* **367**, 1647-1664.

Munyiri FN, Ishikawa Y (2004) Endocrine changes associated with metamorphosis and diapause induction in the yellow-spotted longicorn beetle, *Psacothea hilaris*. *Journal of Insect Physiology* **50**, 1075-1081.

Nijhout HF (2003) Development and evolution of adaptive polyphenisms. *Evolution and Development* **5**, 9-18.

Oostra V, de Jong MA, Invergo BM*, et al.* (2011) Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **278**, 789-797.

Pijpe J, Brakefield PM, Zwaan BJ (2007) Phenotypic plasticity of starvation resistance in the butterfly *Bicyclus anynana*. *Evolutionary Ecology* **21**, 589-600.

R Development Core Team (2010) *R: A language and environment for statistical computing (v. 2.11.1)* R Foundation for Statistical Computing.

Ramaswamy SB, Shu S, Park YI, Zeng F (1997) Dynamics of juvenile hormone-mediated gonadotropism in the Lepidoptera. *Archives of Insect Biochemistry and Physiology* **35**, 539-558.

Richard DS, Jones JM, Barbarito MR*, et al.* (2001) Vitellogenesis in diapausing and mutant *Drosophila melanogaster*: further evidence for the relative roles of ecdysteroids and juvenile hormones. *Journal of Insect Physiology* **47**, 905-913.

Saastamoinen M, van der Sterren D, Vastenhout N, Zwaan BJ, Brakefield PM (2010) Predictive adaptive responses: condition dependent impact of adult nutrition and flight in the tropical butterfly *Bicyclus anynana*. *American Naturalist* **176**, 686-698.

Schmidt PS (2011) Evolution and mechanisms of insect reproductive diapause: A plastic and pleiotropic life history syndrome. In: *Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs* (eds. Flatt T, Heyland A), pp. 230-242. Oxford University Press, Oxford, UK.

Schwedes CC, Carney GE (2012) Ecdysone signaling in adult *Drosophila melanogaster*. *Journal of Insect Physiology* **58**, 293-302.

Simpson SJ, Sword GA (2009) Phase polyphenism in locusts: mechanisms, population, consequences, adaptive significance and evolution. In: *Phenotypic plasticity in insects. Mechanisms and consequences* (eds. Ananthakrishnan TN, Whitman DW), pp. 147-189. Science Publishers, Inc., Plymouth, UK.

Simpson SJ, Sword GA, Lo N (2011) Polyphenism in Insects. *Current Biology* **21**, R738-R749.

Singtripop T, Wanichacheewa S, Tsuzuki S, Sakurai S (1999) Larval growth and diapause in a tropical moth, *Omphisa fuscidentalis* Hampson. *Zoological Science* **16**, 725-733.

Smith CR, Toth AL, Suarez AV, Robinson GE (2008) Genetic and genomic analyses of the division of labour in insect societies. *Nature Reviews Genetics* **9**, 735-748.

Sommer RJ, Ogawa A (2011) Hormone signaling and phenotypic plasticity in nematode development and evolution. *Current Biology* **21**, R758-R766.

Steigenga MJ, Fischer K (2007) Within- and between-generation effects of temperature on life-history traits in a butterfly. *Journal of Thermal Biology* **32**, 396-405.

Therneau T (2012) *A package for survival analysis in S* R package version 2.36-14. http://CRAN.R-project.org/package=survival.

Tsuchida K, Nagata M, Suzuki A (1987) Hormonal control of ovarian development in the silkworm, *Bombyx mori*. *Archives of Insect Biochemistry and Physiology* **5**, 167-177.

van den Heuvel J, Saastamoinen M, Brakefield PM*, et al.* (2013) The predictive adaptive response: modeling the life-history evolution of the butterfly *Bicyclus anynana* in seasonal environments. *The American Naturalist* **181**, E28-E42.

Visser ME, Caro SP, van Oers K, Schaper SV, Helm B (2010) Phenology, seasonal timing and circannual rhythms: towards a unified framework. *Philosophical Transactions of the*

Royal Society of London, Series B: Biological Sciences **365**, 3113-3127.

Zera AJ (2007) Endocrine analysis in evolutionary-developmental studies of insect polymorphism: hormone manipulation versus direct measurement of hormonal regulators. *Evolution and Development* **9**, 499-513.

Zera AJ (2009) Wing polymorphism in crickets. In: *Phenotypic plasticity in insects. Mechanisms and consequences* (eds. Ananthakrishnan TN, Whitman DW). Science Publishers, Inc., Plymouth, UK.

Zera AJ, Harshman LG (2001) The physiology of life history trade-offs in animals. *Annual Review of Ecology and Systematics* **32**, 95-126.

Zera AJ, Harshman LG, Williams TD (2007) Evolutionary endocrinology: The developing synthesis between endocrinology and evolutionary genetics. *Annual Review of Ecology and Systematics* **38**, 793-817.

Zijlstra WG, Steigenga MJ, Koch PB, Zwaan BJ, Brakefield PM (2004) Butterfly selected lines explore the hormonal basis of interactions between life histories and morphology. *American Naturalist* **163**, E76-87.

Zwaan BJ, Bijlsma R, Hoekstra RF (1991) On the developmental theory of ageing. I. Starvation resistance and longevity in *Drosophila melanogaster* in relation to preadult breeding conditions. *Heredity* **68**, 123-130.