JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12051

Seasonal selection and resource dynamics in a seasonally polyphenic butterfly

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Keywords:

Araschnia levana;
diapause;
nutritional ecology;
polyphenism;
seasonal selection;
sexual dimorphism.

Abstract

Seasonal polyphenisms are widespread in nature, yet the selective pressures responsible for their evolution remain poorly understood. Previous work has largely focussed either on the developmental regulation of seasonal polyphenisms or putative 'top-down' selective pressures such as predation that may have acted to drive phenotypic divergence. Much less is known about the influence of seasonal variation in resource availability or seasonal selection on optimal resource allocation. We studied seasonal variation in resource availability, uptake and allocation in *Araschnia levana* L., a butterfly species that exhibits a striking seasonal colour polyphenism consisting of predominantly orange 'spring form' adults and black-and-white 'summer form' adults. 'Spring form' individuals develop as larvae in the late summer, enter a pupal diapause in the fall and emerge in the spring, whereas 'summer form' individuals develop directly during the summer months. We find evidence for seasonal declines in host plant quality, and we identify similar reductions in resource uptake in late summer, 'spring form' larvae. Further, we report shifts in the body composition of diapausing 'spring form' pupae consistent with a physiological cost to overwintering. However, these differences do not translate into detectable differences in adult body composition. Instead, we find minor seasonal differences in adult body composition consistent with augmented flight capacity in 'summer form' adults. In comparison, we find much stronger signatures of sex-specific selection on patterns of resource uptake and allocation. Our results indicate that resource dynamics in *A. levana* are shaped by seasonal fluctuations in host plant nutrition, climatic conditions and intraspecific interactions.

Introduction

Polyphenisms, instances where individuals within a population exhibit different phenotypes despite sharing genotypes, are widespread in living systems (Moran, 1992), and have been the subject of much inquiry dating back to 19th century work on the induction of butterfly colour polyphenisms (Weismann, 1875; Poulton, 1890). Thanks in large part to these studies, and more recent ones involving physiological manipulations via synthetic hormones, we know a considerable amount about the developmental regulation of polyphenisms, particularly in insect groups such as ants, butterflies and beetles (Koch, 1992; Emlen & Nijhout, 2000; Hartfelder & Emlen, 2005; Simpson *et al.*, 2011). Modern genomic techniques are now being applied to understand how evolution has shaped developmental programming in polyphenic organisms (Kijimoto *et al.*, 2009; Snell-Rood *et al.*, 2011). However, despite the increasing sophistication of our knowledge in this area, much is not known about the ecological and evolutionary pressures that drive and maintain such polyphenisms. What factors, biotic or abiotic, interact to favour distinct phenotypes

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in different contexts? Are these selective pressures top-down (e.g. driven by predation) or bottom-up (e.g. responses to variation in available nutrients)? To what extent are some polyphenic traits the pleiotropic effect of selection acting in other contexts?

Seasonal polyphenisms are compelling systems for exploring these issues, in part because seasonal environments offer a variety of tractable candidate variables which may select for seasonal phenotypic matching. In general, seasonal polyphenisms may result from responses to three different types of selective pressures. First, seasonal differences in resource availability may drive selection on the phenotypes individuals are able to realize during different seasons. In herbivorous insects, such seasonal resource differences may arise if developing individuals feed on different host plants during different times of the year or if host plant chemistry and tissue characteristics vary consistently with season. As selection should often act to increase developmental rate or efficiency by reducing seasonal mismatches between resource supply and demand, we might expect that seasonal shifts in host plant biochemistry should translate into related biases in the body composition of herbivorous insects. This selective pressure should be highest for individuals under strong developmental time constraints, such as those who must achieve a particular developmental state ahead of seasons of predictably inclement weather (e.g. Blanckenhorn, 1998; Pöykkö & Hyvärinen, 2012). Evaluation of this selective pressure can be accomplished in the first instance by comparing seasonal shifts in diet to seasonal shifts in body composition.

Second, seasonal environments often differ dramatically in their climatic conditions, imposing selection on traits related to the regulation of body temperature, hydration state and metabolism. This is particularly true in temperate climates, where individuals must cope with cold or freezing temperatures during winter months. Many animals, especially ectotherms, enter a period of developmental arrest or diapause during the winter months, which requires physiological changes to body composition that impart freeze tolerance and/or fund baseline maintenance metabolism. For insects, many of these biochemical changes are well understood, particularly in relation to freeze tolerance (Sinclair *et al.*, 2003). This allows for informed predictions about changes in body composition related to diapause. However, links between diapause-related and nondiapause-related traits are not well understood. For example, the idea that diapause-related traits may have pleiotropic effects on seasonally polyphenic characters such as adult body shape, colouration or life history strategies remains underexplored (but see Gotthard & Berger, 2010).

Lastly, seasonal polyphenisms may be driven by seasonal differences in the social environment of organisms, which may lead to divergence in the life history strategies favoured during different times of year. These strategies may relate to a variety of interactions, both interspecific (e.g. predator avoidance) and intraspecific (e.g. territoriality or mate acquisition). Seasonal changes in population size and/or metapopulation structure may also influence traits related to dispersal and reproductive tactics (Hanski, 1998; Fric & Konvicka, 2000). Selective pressures arising from differential predation are frequently invoked in the case of seasonal colour polyphenisms, although evidence in support of this intuitive hypothesis is relatively scant and largely equivocal (e.g. even for the extensively studied polyphenic butterfly *Bicyclus anynana*, Lyytinen *et al.*, 2003, 2004; Vlieger & Brakefield, 2007).

The selective pressures identified above are by no means mutually exclusive, but rather may act in concert to shape the characteristics of seasonal phenotypes. For instance, resource availability may strongly influence the ability of individuals to successfully prepare for diapause and survive overwintering (Trudeau *et al.*, 2010). Seasonal shifts in climatic conditions may affect selection on alternative mate acquisition strategies and associated phenotypic traits (Wickman & Wiklund, 1983; Van Dyck & Wiklund, 2002). However, relatively little research has been conducted to quantify the contributions of these selective pressures to the body phenotypes produced by seasonally polyphenic organisms. Here, we present work focussed on identifying signatures of resource availability and resource use consistent with a role for biotic, abiotic and social selective pressures in the evolutionary maintenance of seasonal polyphenism in the European Map Butterfly, *Araschnia levana* (Linnaeus 1758).

Araschnia levana is a nymphalid butterfly widely distributed throughout Eurasia that exhibits a distinct seasonal colour polyphenism associated with non-overlapping generations that have different developmental pathways (Fig. 1, Reinhardt, 1984). Generations that develop as larvae during the spring and summer months emerge after a short pupal phase as *prorsa* or 'summer form' adults with dark, melanic, dorsal wing colouration crossed by distinctive white bands (Fig. 1a). Generations that accomplish larval development during the late summer and early fall months enter a pupal diapause and emerge the following spring as *levana* or 'spring form' adults. This overwintering, spring generation exhibits dorsal wing colouration dominated by large areas of ommochrome-based orange pigmentation (Fig. 1b). These differences in wing colouration draw on different amino acid precursors, with melanin synthesis requiring large quantities of tyrosine and ommochrome synthesis relying on supply of tryptophan (Koch, 1991; True, 2003). More subtle polyphenisms in body size, wing morphology and flight muscle investment have also been identified in this species (Fric & Konvicka, 2002; Fric *et al.*, 2006), traits which may be related to seasonal shifts in population structure (Fric & Konvicka, 2000). Whether other aspects of *A. levana* body pheno-

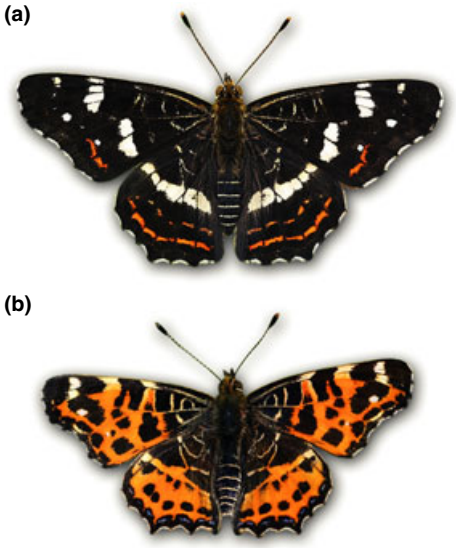

Fig. 1 Seasonal polyphenism of *Araschnia levana* wing colouration, illustrating typical 'summer form' (a) and 'spring form' (b) adult wing colouration.

type differ with season has not been explored, although we expect that traits related to diapause and overwintering should be present in 'spring form' pupae, but absent in 'summer form' pupae.

A considerable amount is known about the physiological basis for both the wing colour polyphenism and seasonal differences in developmental pathway in *A. levana*. Both wing colouration and pupal diapause are regulated largely by responses to photoperiod, although temperature does modulate these responses to some degree (Reinhardt, 1984). Larvae reared on long photoperiods characteristic of summer day lengths develop directly and emerge as 'summer form' adults. Larvae reared on short photoperiods similar to those experienced during fall months enter pupal diapause and emerge after a period of cold exposure as 'spring form' adults. Thanks to the pioneering work of Koch (1992) and colleagues, we know that these differences in photoperiod are translated physiologically via shifts in the timing of release of the hormone ecdysone during pupal development. 'Summer form' pupae release ecdysone early in pupal development, with concentrations in the haemolymph peaking around day 3 of pupal development (Koch & Bückmann, 1987). 'Spring form' pupae, on the other hand, release no ecdysone during early development (Koch & Bückmann, 1987), and may instead release a peak of ecdysone after diapause as spring temperatures rise to a level that supports the resumption of development. Differences in wing colour development between the two forms also appear to be regulated via this ecdysteroid pathway (Koch & Bückmann, 1987; Koch, 1991). While the actual developmental switch point is controlled via pupal ecdysone, recent work indicates that commitment to a diapausing phenotype may occur as early as the penultimate larval instar, after which the majority of individuals are no longer responsive to shifts in photoperiod cues (Friberg *et al.*, 2011). Thus, by pupation, any adaptive resource biases associated with a particular developmental pathway are likely to be evident.

Current understanding of the seasonal polyphenism in *A. levana* mirrors the state of research on many other polyphenisms. Our knowledge of the developmental mechanisms responsible for the seasonal colour polyphenism is rather sophisticated (see above). However, despite the fact that *A. levana* has been under scientific scrutiny since at least 1830 (Weismann, 1875), we still understand very little about the selective pressures or constraints that maintain polyphenism in this species. The influence of seasonal fluctuations in larval resource availability has not been explored, nor has the importance of pupal diapause to resource acquisition and allocation. Polyphenic differences in body plan related to flight capacity have been reported, but only for field-caught individuals (Fric & Konvicka, 2002; Fric *et al.*, 2006). Recent work to test the hypothesis that seasonal differences in predation pressure are responsible for the evolution of two different colour forms has revealed weak or no seasonal benefits for either form in the context of avian predation (Joiris *et al.*, 2010; Ihalainen & Lindstedt, 2012; K. Fischer, pers. comm.). Thus, although we understand how seasonal polyphenism is produced in *A. levana*, we know almost nothing about why.

We sought evidence for the role of different selective pressures by evaluating seasonal patterns of resource availability in the host plant *Urtica dioica* as well as patterns of resource acquisition and allocation to adult body tissues in the different seasonal forms of *A. levana*. In particular, we probed resource relationships for evidence of (i) 'bottom-up' selective pressures driven by seasonal variation in the nutritional content of host plant tissues, (ii) abiotic selective pressures resulting in differences in pupal phenotype related to overwintering and freeze tolerance and (iii) patterns of adult resource investment consistent with selection arising from inter- or intraspecific interactions.

Materials and methods

Plant nutrient composition

We collected leaf tissue samples of the host plant *U. dioica* from riparian areas along the Choisille River (47° 24′17″ N 0°38′38″ E) and Loire River (47°23′26″ N 0°38′19″ E) near Saint-Cyr-sur-Loire, France. Leaf tissue samples were collected from plants found within areas actively defended by male *A. levana*, ensuring that the plants sampled corresponded to those likely to be

used by ovipositing females and developing larvae. We sampled areas with large, persistent male populations ('favoured' sites). We also sampled sites where males were only found when population densities forced some males to defend new areas ('marginal' sites). The latter sites were generally smaller patches of *U. dioica* that received greater sun exposure (N. I. Morehouse, pers. obs.). Preliminary analyses indicated that host plant nutrient composition did not qualitatively differ between these two site types. As a result, the analyses reported here were performed with both site types pooled, and thus seasonal changes in plant composition correspond to the range of possible habitats used for larval development in these populations.

Leaf samples were collected at time points corresponding to larval development for both direct-developing 'summer form' individuals (two generations per year, plant sampled in early May 2011 and early July 2010) and diapausing 'spring form' individuals (plant sampled in late August 2010). Leaf samples were pooled from multiple plants within each site, and a total of eight sites were sampled twice at each time point. Samples were returned to the lab fresh, weighed, frozen at −80 °C, lyophilized for 24 h (Alpha 2-4 LDplus; Martin Christ GmbH, Osterode am Harz, Germany) and re-weighed. Plant water content was calculated as the difference between wet and dry weights. Plant samples were then hand-ground to a homogenous powder and sampled for lipid, sugar, protein and free amino acid content.

We measured plant lipid and sugar concentrations following colorimetric methods modified from Giron *et al.* (2002). Briefly, approximately 10 mg of powdered plant material was weighed, placed in a 1.5 mL Eppendorf tube and manually ground with a pestle in 800 μL of a chloroform–methanol–water mixture (1 : 2 : 0.4, v : v : v). Samples were left refrigerated overnight to complete extraction. We then centrifuged each sample and transferred four aliquots to borosilicate tubes. As our extraction procedure resulted in significant quantities of chlorophyll and flavonoids in solution, which absorb in the wavelength regions used during colorimetric quantification, we used two of these four aliquots as reference samples to quantify background absorbance of samples in the absence of vanillin (lipid quantification) and anthrone (sugar quantification).

For lipid quantification, 35 μL samples were heated in a water bath to 90 °C to evaporate the solvent. We then added 40 μL of 95% sulphuric acid, re-heated the samples to 90 °C for 2 min and removed them from the bath to cool. We added 960 μL of vanillin reagent (1.2 mg mL^{-1} of purified vanillin dissolved in 68% phosphoric acid) to each cooled tube and allowed the samples to sit at room temperature for 15 min. In reference samples, vanillin reagent was replaced with an equivalent volume of 68% phosphoric acid. Samples were then transferred to a microcuvette and their absorbance at 525 nm quantified using a spectrophotometer (DU®-64; Beckman, Villepinte, France). Lipid content for each plant sample was determined as the difference in absorbance between vanillin-containing and vanillin-free extracts. Standard curves used to translate absorbances to concentrations were created using a dilution series of purified vegetable oil (Van Handel, 1985b).

To quantify sample sugar content, we heated 15 μL of extract in a water bath to 90 °C until nearly all solvent was evaporated. We then added 1 mL of anthrone reagent (1.4 mg mL^{-1} of purified anthrone dissolved in 68% sulphuric acid), re-heated the solution to 90 °C for 15 min and then removed the tubes to cool. Samples were transferred to microcuvettes and their absorbance quantified at 625 nm. In reference samples, anthrone reagent was replaced with an equivalent volume of 68% sulphuric acid. Sugar content for each plant sample was determined as the difference in absorbance between anthrone-containing and anthrone-free extracts. Standard curves were created using purified glucose (Van Handel, 1985a).

We quantified plant protein content using a modified Bradford protein microassay (Bradford, 1976). As with lipid and sugar quantification, we weighed approximately 10 mg samples of powdered plant tissues and transferred them to 1.5 mL Eppendorf tubes. Samples were then manually ground with a pestle in 800 μL of physiological water (0.15 M NaCl) containing 0.001% Triton X-100 (Sigma-Aldrich, St Quentin Fallavier, France). We refrigerated the samples for 5 days to allow for full digestion and protein extraction. Samples were then brought back to room temperature, centrifuged and 10 μL of supernatant transferred to tubes containing 790 μL of physiological saline and 200 μL of Bradford reagent (Bio-Rad Laboratories, Munich, Germany). Sample absorbance was measured at 595 nm. In reference samples, Bradford reagent was replaced by an equivalent volume of the Bradford reagent mixture without Coomassie blue G-250 (85% phosphoric acid, de-ionized water and methanol, 29 : 17 : 4, v : v : v). Standard curves were produced using a dilution series of suspended bovine serum albumin (Sigma-Aldrich).

Tissue concentrations of free amino acids were analysed by derivatization using an EZ:faast amino acid analysis kit (Phenomenex Ltd, Aschaffenburg, Germany) and subsequent quantification via GC-MS system composed of an AutoSystem XL gas chromatograph coupled to a TurboMass mass spectrometer (Perkin-Elmer, Courtaboeuf, France). As in prior analyses, we weighed approximately 10 mg samples of powdered plant tissues and manually ground them in 1.5 mL Eppendorf tubes in 200 μL of a mixture of 25% acetonitrile and 0.01 M HCl (1 : 3, v : v). Samples were allowed to undergo extraction for one hour, followed by centrifugation. Supernatant samples (120 μL) were then subjected to derivatization, concentrated under a stream of nitrogen

gas and injected immediately into the GC-MS system. Samples were separated on a ZB-AAA column (10 m × 0.25 mm, Phenomenex Ltd) using helium as the carrier gas at a constant flow of 1.1 mL min^{-1}. The GC oven temperature ramped from 110 to 320 °C at a rate of 30 °C min^{-1}, with the temperature at the injection port maintained at 250 °C. The MS was maintained with a temperature of 200 °C at the electron impact source and 310 °C at the inlet line. The scan range was 3.5 scans per second. Under these conditions, we injected 2 μL samples in the splitless mode (30 s).

We used norvaline as an internal standard. Calibration curves for each of 17 amino acids were produced using a mix of amino acids at an original concentration of 200 nmol mL^{-1}. Purified amino acids were sourced from Phenomenex and Sigma-Aldrich. Detectable quantities were found for all 17 amino acids. Preliminary analyses of individual amino acid titres revealed no compelling seasonal trends. We therefore only discuss variation in total amino acid titres, and seasonal concentrations of tryptophan and tyrosine, due to their relevance to the seasonal colour polyphenism in this species (see above).

Experimental design

All experimental animals analysed for body composition were lab-reared offspring of females captured in July 2010 at the same sites used for plant sampling. Larvae were fed fresh *U. dioica* collected during July and early August 2010. Larvae were reared in plastic containers in groups of 8–10 individuals within a climate chamber that maintained a 14 h : 10 h, 22 °C : 18 °C temperature cycle and approximately 55% relative humidity. Offspring were split between two photoperiod treatments within this chamber, long days (LD, 16 h : 8 h, light : dark) simulating summer conditions of direct-developing generations and short days (SD, 12 h : 12 h, light : dark) corresponding to fall conditions experienced by larvae of the diapausing generation. Individuals reared on the LD treatment developed directly to adulthood, whereas individuals experiencing the SD treatment entered diapause. We placed this latter group in a cold room at 4 °C for 4 months before returning them to the chamber to complete development into adulthood.

On the third day of pupation, we weighed all individuals, sexed them using external genitalic features on the 8th abdominal segment, and transferred them to individual containers for pupal development. A subset of individuals from each photoperiod treatment were culled at this stage for measurement of pupal resource pools (10 per sex per photoperiod treatment per resource analysis, $N = 80$). Remaining individuals were allowed to complete development, eclose as adults, harden their wings and excrete their meconium (5 per sex per photoperiod treatment per resource analysis, $N = 40$). Adults were then measured for their fresh weight. Both adults and culled pupae were freeze-killed at −80 °C, lyophilized for 24 h and weighed again for dry weight. Pupal and adult water content was calculated as the difference between wet and dry weights.

Pupae were analysed whole for their body composition, whereas adult individuals were separated into three body compartments which were weighed and analysed independently: wings, head–thorax and abdomen. For all sample types, lyophilized tissues were manually ground to a homogenous powder prior to extraction and analysis. We measured the lipid, sugar and protein content using the same colorimetric methods described above, with the exception that vanillin- and anthrone-free reference samples were not necessary in the absence of interference from extractable pigments in our lipid and sugar analysis.

Statistics

All statistical procedures were run in SPSS 19.0 (SPSS, Inc., Chicago, Illinois). Data were found to be homoscedastic and normally distributed using visual evaluation of normal probability plots, spread-versus-level plots and Levene's test. Seasonal changes in plant nutritional composition were tested using a repeated measures analysis of variance (RMANOVA), with collection site as a random between-subjects factor and season and sample as within-subjects factors. Individual RMANOVAS were run for each nutrient variable (i.e. water content, lipids, sugars, protein and free amino acids). We verified the assumption of sphericity using Mauchly's test. *Post hoc* multiple comparisons of the effect of season were corrected using the Tukey–Kramer method to maintain an experiment-wide α of 0.05.

Pupal and adult body composition results were analysed using two-way ANOVAS with Photoperiod and Sex as fixed factors. Allocation of resources into the three body compartments were likewise analysed as a series of two-way ANOVAS with Photoperiod and Sex as fixed factors, and the response variables representing the percentage of body resources invested in the target body compartment. Allocation patterns were analysed separately for each body compartment to avoid issues of nonindependence between body sections.

Results

Plant nutrient composition

Leaf tissues of *U. dioica* changed in composition over the course of the growing season. Late spring plants had higher water content than late summer plants ($F_{2,8} = 18.926$, $P < 0.001$, Fig. 2a). Late spring plants were also higher in sugars than either mid- or late

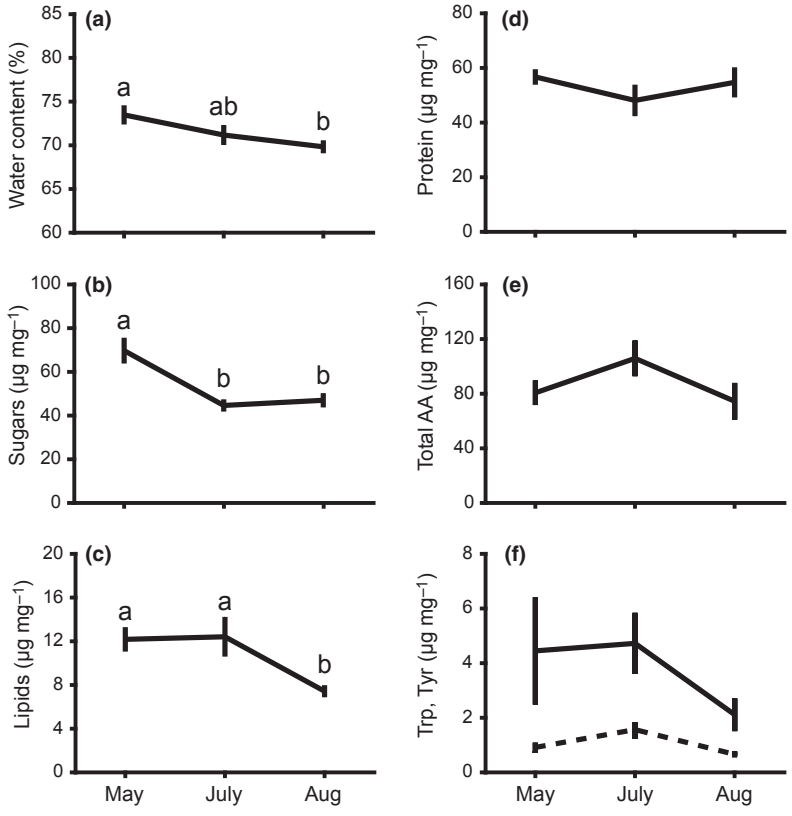

Fig. 2 Seasonal dynamics of *Urtica dioica* leaf tissue composition, including water content (a) and concentrations of sugars (b), lipids (c), proteins (d), total amino acids (e), tryptophan (f, solid line) and tyrosine (f, dashed line). Data are plotted as means ± standard errors. Statistically significant differences between sample months are labelled with different lowercase letters.

summer plants ($F_{2,8}$ = 18.259, P = 0.001, Fig. 2b). Likewise, late spring and mid-summer plant tissues were higher in lipids than late summer plants ($F_{2,8}$ = 5.859, P = 0.027, Fig. 2c). However, we detected no seasonal changes in either bulk protein ($F_{2,8}$ = 0.639, P = 0.553, Fig. 2d) or total free amino acids ($F_{2,8}$ = 2.374, P = 0.155, Fig. 2e). Similarly, we found no significant shifts in tryptophan ($F_{2,8}$ = 3.070, P = 0.102, Fig. 2f) or tyrosine concentrations ($F_{2,8}$ = 3.885, P = 0.066, Fig. 2f).

Pupal body composition

The body composition of pupae differed between sexes and photoperiod treatments, although these effects were subtle. Female pupae exhibited higher dry masses than male pupae, but this difference was smaller in SD pupae compared with LD pupae (Sex × Photoperiod, $F_{1,76}$ = 8.079, P = 0.006, Fig. 3a). Pupal water content was higher in males than females (Sex, $F_{1,76}$ = 38.968, P < 0.001, Fig. 3e), and LD pupae had consistently higher water content than SD pupae (Photoperiod, $F_{1,76}$ = 114.202, P < 0.001, Fig. 3e). We found no significant effect of sex or photoperiod treatment on pupal sugar concentrations (Fig. 3b) or protein concentrations (Fig. 3d), but male LD pupae were marginally higher in lipid concentration than male SD pupae and both LD and SD female pupae (Sex × Photoperiod, $F_{1,36}$ = 7.679, P = 0.009, Fig. 3c).

Adult body composition

We detected effects of both sex and photoperiod treatment on adult body composition. As in pupae, adult females had higher body dry weights (Sex, $F_{1,113}$ = 249.515, P < 0.001, Fig. 3f). LD adults were also heavier than SD adults irrespective of sex (Photoperiod, $F_{1,113}$ = 35.716, P < 0.001, Fig. 3f). Adult water content exhibited an interaction between sex and photoperiod treatment, with SD individuals of both sexes having generally higher water contents than LD adults (Sex × Photoperiod, $F_{1,113}$ = 8.966, P = 0.003, Fig. 3j). Consistent with pupal results, we found no effect of sex or photoperiod on adult sugar concentrations (Fig. 3g). However, lipid concentrations were higher in adult males irrespective of photoperiod treatment (Sex, $F_{1,16}$ = 15.317, P = 0.001, Fig. 3h). Adult protein concentrations were higher in females than males (Sex, $F_{1,16}$ = 53.635, P < 0.001, Fig. 3i) and higher for LD individuals than SD individuals (Photoperiod, $F_{1,16}$ = 29.355, P < 0.001, Fig. 3i).

Qualitative comparison between pupal (Fig. 3a–e) and adult (Fig. 3f–j) body compositions revealed reductions in body mass, water content and protein concentration from pupal to adult stages. For protein, absolute

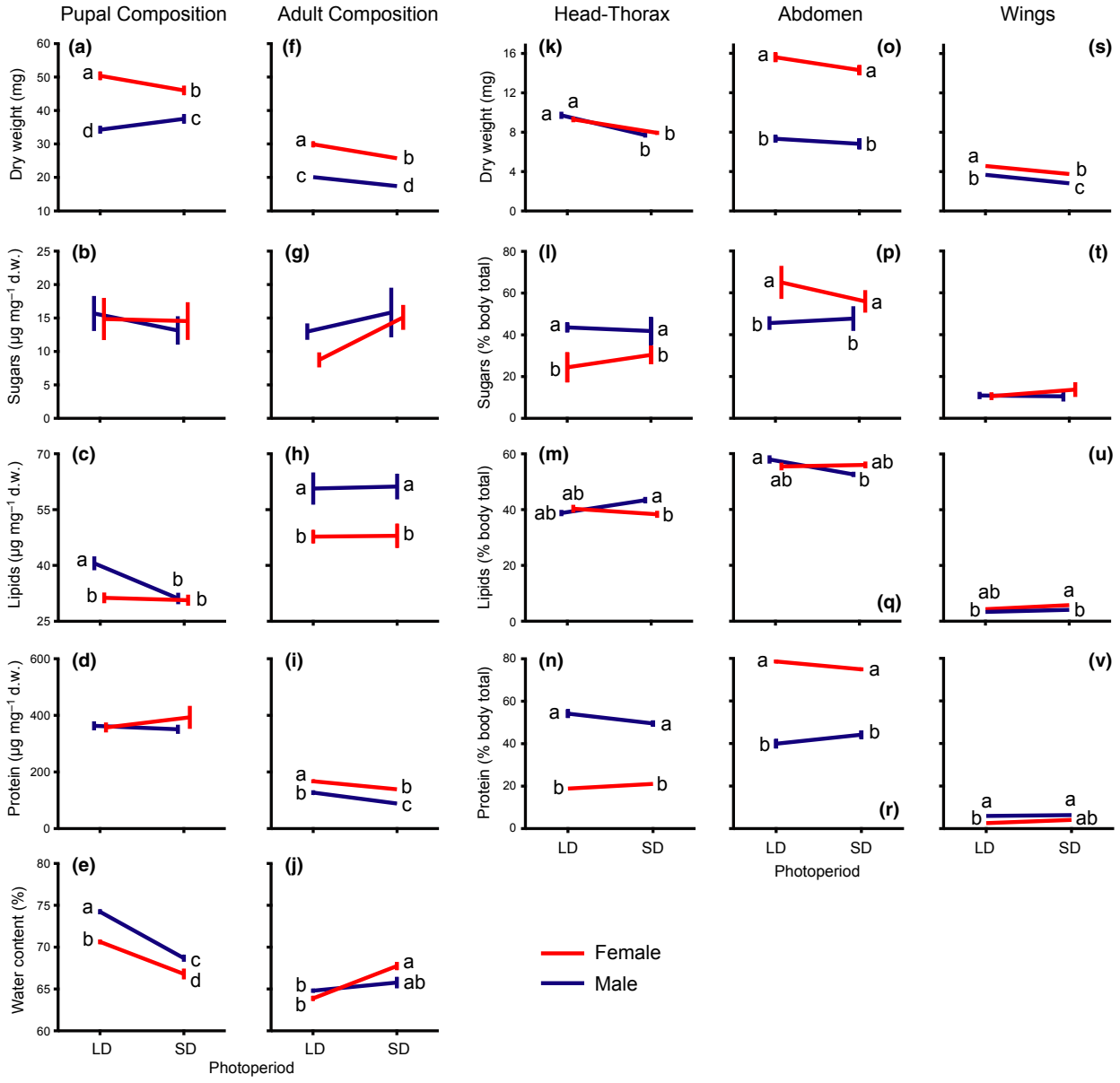

Fig. 3 Patterns of resource acquisition (a–j) and allocation (k–v) in female (red lines online; solid lines in print) and male (blue lines online; dashed lines in print) *Araschnia levana* reared either on long photoperiods ['long days' (LD)] leading to direct-developing 'summer form' individuals or short photoperiods ['short days' (SD)] leading to diapausing 'spring form' individuals. Pupae and adults were evaluated for dry weight (a, f) body concentrations of sugars (b, g), lipids (c, h), protein (d, i) and water content (e, j). Investment of these resources into adult body compartments are also represented, including the head and thorax (k–n), abdomen (o–r) and wings (s–v). Resource allocation of sugars, lipids and proteins is represented in percentages of the total quantities reported to their left (g–i). Data are plotted as means ± standard errors. Statistically significant differences between groups are labelled with different lowercase letters.

levels of protein dropped between the pupal stage and adult stage ($F_{1,52}$ = 191.962, $P < 0.001$). Lipid concentrations, on the other hand, increased in adult tissues compared with pupal levels. However, absolute quantities of lipids did not change from the pupal to adult stage in either sex ($F_{1,52}$ = 2.879, P = 0.096).

Adult resource investment

Patterns of resource investment differed between the sexes, consistent with their divergent life history strategies. However, effects of photoperiod treatment were restricted to mass investment in the head–thorax

(Photoperiod, $F_{1,36} = 35.019$, $P < 0.001$, Fig. 3k) and wings (Photoperiod, $F_{1,36} = 41.691$, $P < 0.001$, Fig. 3s). In both instances, LD individuals exhibited higher mass allocations than SD individuals, which follow from our finding that LD individuals were on average higher in mass (see above, Fig. 3f). We found significant effects of sex on mass investment in the abdomen and wings, but not the head and thorax. Females consistently invested more dry mass in their abdomens (Sex, $F_{1,36} = 265.583$, $P < 0.001$, Fig. 3o) and wings (Sex, $F_{1,36} = 50.572$, $P < 0.001$, Fig. 3s) than males. Again, these follow patterns detected at the whole animal level.

For adult sugars, we found that males invested a higher proportion in their head and thorax (Sex, $F_{1,16} = 7.645$, $P = 0.014$, Fig. 3l), whereas females invested more sugars in their abdomens (Sex, $F_{1,16} = 5.588$, $P = 0.031$, Fig. 3p). Likewise, males invested a great proportion of their protein in their head and thorax, although this sex difference was smaller for SD individuals (Sex × Photoperiod, $F_{1,16} = 6.530$, $P = 0.021$, Fig. 3n). Females allocated a greater proportion of their protein to their abdomen; this sex difference was also smaller in SD individuals (Sex × Photoperiod, $F_{1,16} = 5.934$, $P = 0.027$, Fig. 3r). We found complex, statistically significant interactions between sex and photoperiod treatment for lipid allocation to head–thorax (Sex × Photoperiod, $F_{1,16} = 6.995$, $P = 0.018$, Fig. 3m) and abdomen (Sex × Photoperiod, $F_{1,16} = 5.348$, $P = 0.034$, Fig. 3q). The general trend was for invariance in male lipid allocation with photoperiod treatment, contrasted with increases in SD female head–thorax lipid investment and commensurate reductions in abdomen lipid allocation.

We detected minor but significant effects of sex and photoperiod treatment on lipid allocation to wings, with higher levels of allocation in females (Sex, $F_{1,16} = 12.572$, $P = 0.003$, Fig. 3u) and SD individuals (Photoperiod, $F_{1,16} = 8.433$, $P = 0.010$, Fig. 3u). We also found higher allocation of protein to wings in males (Sex, $F_{1,16} = 16.867$, $P < 0.001$, Fig. 3v). However, in all cases, the absolute value of these differences was small in comparison to the differences identified from abdomen and head–thorax allocation patterns.

Discussion

We sought to identify seasonal patterns of resource availability, acquisition or allocation that would provide evidence for seasonal selection acting on body morphology in *A. levana*. In particular, we were interested in whether patterns of resource availability and use indicated a role for (i) 'bottom-up' selection acting via seasonal differences in nutritional composition of host plant tissues, (ii) selection arising from the abiotic challenges associated with overwintering and diapause and/or (iii) selection generated by inter- and intraspecific interactions during adulthood. To this end, we quantified seasonal resource dynamics in the tissues of the host plant *U. dioica*. We then evaluated resource acquisition during the larval phase by quantifying the resource composition of pupae of *A. levana* reared on a common diet but split between photoperiod treatments associated with 'spring form' (SD) and 'summer form' (LD) developmental trajectories. Patterns of resource allocation were evaluated by characterizing the resource composition of body tissues from lab-reared adult *A. levana* of each seasonal form.

Variation in resource availability

We identified modest evidence for a role of seasonal variation in resource availability. First, we find seasonal variation in the nutritional composition of *U. dioica* leaves. Leaf tissues collected during the late summer months had lower concentrations of water, sugars and lipids (Fig. 2a–c). Interestingly, we find no evidence for a decrease in protein or amino acids (Fig. 2d–f), contrary to the seasonal decline previously reported for German populations of *U. dioica* (Rosnitschek-Schimmel, 1985). Importantly, we find no evidence for seasonal shifts in host plant tryptophan or tyrosine which might select for differential synthesis of ommochromes versus melanins, the pigment synthesis pathways which underlay the conspicuous colour differences between seasonal forms in *A. levana*. Nevertheless, our results suggest that the average quality of *U. dioica* as a food source declines over the course of *A. levana*'s growing season, with the plant tissues available to developing 'spring form' larvae during the late summer being the least nutritious and potentially most difficult to digest (Scriber & Slansky, 1981).

Although relationships between resource availability and resource acquisition are complex due the variety of strategies herbivores employ to maintain nutritional homeostasis, we might expect in the simplest case that herbivores encountering consistent seasonal declines in host plant quality would alter their resource demands to reduce nutritional mismatches between their food and the requirements of their body construction. Thus, we predicted that seasonal declines in host plant resources might be accompanied by correlated reductions in relevant resource pools in *A. levana* larvae and pupae adopting the 'spring form' developmental trajectory (i.e. those reared in the SD photoperiod treatment). Importantly, we predicted that such biases should persist even when individuals of each developmental trajectory were reared on a common diet. Consistent with this prediction, we find that 'spring form' SD pupae of both sexes are lower in water content (Fig. 3e) and male 'spring form' pupae exhibit lower lipid concentrations (Fig. 3c) even when reared on a common diet. We find no evidence for differences in dry weight, sugar or protein content between pupae of each seasonal form. Interestingly, these pupal patterns do not translate to adult tissues. Rather, 'spring

form' (SD) adults are higher in water content at eclosion than their 'summer form' (LD) counterparts (Fig 3j) and exhibit no detectable seasonal differences in lipid stores (Fig 3h). Thus, while we see some evidence that pupal characteristics may have evolved to accommodate seasonal declines in host plant quality, we find no support for a role of larval nutrition in driving differences in adult morphology or adult resource pools. We should note that while our experimental animals fed on a 'common diet', this diet was host plant collected in mid-summer, which corresponds with the timing of larval feeding for the second 'summer form' generation. Because host plants during this time exhibit intermediate nutritional values within the range of tissue compositions we recorded, we think this is a reasonable empirical compromise, but it is plausible that 'summer form' larvae are better adapted to feeding on this seasonal plant phenotype.

Overwintering, diapause and adult selection

If diapause and overwintering are the predominant selective pressures acting on pupal body composition, we would expect that differences between pupae should be largely explained by phenotypic changes in 'spring form' pupae associated with rapid completion of development in advance of cold winter months (i.e. reduced pupal size, Abrams *et al.*, 1996), desiccation and freeze tolerance (i.e. reduced water content and elevated concentrations of cryoprotectant sugars, Sinclair *et al.*, 2003) and preparation for funding maintenance metabolism during an extended period of developmental arrest (i.e. elevated lipid stores, Ellers & Van Alphen, 2002; Wang *et al.*, 2007).

In evaluating pupal resource characteristics and translation of pupal resources to adult tissues, we do find limited evidence for traits associated with pupal diapause and overwintering. 'Spring form' pupae entered diapause with lower body water content, a strategy that may reduce freezing of body tissues (Zachariassen, 1985). Interestingly, we also find that 'spring form' individuals experience only negligible reductions in body water content over the course of pupal development, emerging with equivalent water contents in adult tissues (Fig. 3e,j). This stands in contrast to direct-developing 'summer form' individuals, which begin pupation with much higher water contents, but arrive at adulthood with substantially reduced water contents (Fig. 3e,j). In comparison, these results suggest that diapausing 'spring form' pupae may have specific traits that prevent further dehydration during the pupal phase, allowing them to enter pupation with reduced water contents to aid in freeze tolerance.

Contrary to predictions arising from a role of overwintering on pupal composition, we find no evidence for increases in body sugars. However, our colorimetric analyses of sugar concentrations are insensitive to differences between typical metabolic sugar substrates such as glucose and sugars that act as cryoprotectants such as trehalose. Our methods are also less sensitive overall to polyols (Graham, 1963), which are often used by insects as cryoprotectants. Thus, while we see no net increase in body sugar concentrations, we may be missing more subtle shifts in sugars related to freeze tolerance in 'spring form' pupae. Similarly, we find no evidence for increased lipid resources in diapausing pupae, suggesting that there are little or no additional resources devoted to funding basal metabolism during diapause. However, interestingly, we find that while both seasonal forms enter pupation with equivalent protein resources, adult 'spring form' individuals have lower overall protein contents. This suggests that 'spring form' pupae may utilize body protein sources as an energetic substrate during pupal development.

We found broad differences between seasonal adult phenotypes consistent with a role for both overwintering and seasonal selection on adult flight capacity. 'Spring form' adults weighed less than their 'summer form' counterparts (Fig. 3f), despite the fact that no body size differences were identified at the beginning of the pupal phase. This reduction in body mass during pupation may reflect the metabolic cost of diapause and/or overwintering experienced by 'spring form' individuals. The resultant reduced body mass appears to translate predominantly into reductions in adult head and thorax mass (Fig. 3k) and wing mass (Fig. 3s). Similarly, 'spring form' adults emerged with smaller concentrations of protein in their bodies (Fig. 3i). Combined with the reduced head, thorax and wing mass, these reductions in adult protein are likely to affect the size of flight musculature and associated flight capacity. This latter result is thus consistent with the findings of Fric & Konvicka (2002) and Fric *et al.* (2006) for field-caught individuals of each generation. Critically, our results indicate that the reduced flight capacity of 'spring form' individuals found in the field persists even when individuals are reared on a common diet and climatic regime, suggesting that this is not simply a plastic response to seasonal differences in nutrition or climate.

Sex differences

Beyond differences in seasonal phenotypes, we also identified clear differences in resource acquisition and allocation between the sexes. Females were consistently heavier than males, both as pupae (Fig. 3a) and adults (Fig. 3f). Females invested this additional mass into their abdomens (Fig. 3o) and wings (Fig. 3s), a pattern consistent with evolutionary responses to fecundity selection (Honěk, 1993; Bauerfeind & Fischer, 2008). Although no sex differences in sugar content were identified for whole pupae (Fig. 3b) or adults (Fig. 3g), we find that males invest more sugars into their heads and thoraxes (Fig. 3l), whereas females allocate more sugars

to their abdomens (Fig. 3p). These investment patterns are consistent with a role for these sugars as metabolic substrates for male territory defence and female egg production (Kemp & Wiklund, 2001; Boggs, 2003, 2009). Likewise, we find that males invest more of their body protein resources into their head and thorax (Fig. 3n), whereas females allocate much of their protein to their abdomens (Fig. 3r). Females are also more protein rich at adult eclosion (Fig. 3I). From these patterns, it remains clear that female body structure has evolved under selection to prioritize fecundity (Boggs, 2003, 2009), whereas males appear to prioritize flight capacity and thus territory defence (Kemp & Wiklund, 2001). Lastly, males emerge as adult butterflies with higher lipid concentrations than do females (Fig. 3h), although patterns of lipid allocation do not appear different between the sexes (Fig. 3m,q,u). Again, this may reflect a need for males to fund territory defence from capital rather than income sources (Kemp & Alcock, 2003).

In summary, our results indicate that the seasonal phenotypes of *A. levana* are likely influenced by a complex interplay between multiple sources of selection. At the beginning of the pupal stage, we identify shifts in pupal body composition consistent with adaptive tuning of resource requirements to seasonal declines in host plant quality. During pupation, we see that diapausing 'spring form' pupae retain more body water but eclose with less dry mass and protein than their direct-developing 'summer form' conspecifics. These patterns are consistent with both preparations for and costs of pupal diapause, which are not imposed on individuals in the direct-developing generation. During adulthood, we find that 'spring form' pupae are smaller and less protein rich. This is likely to translate into reduced flight capacity, particularly in males, and may reflect reduced selection on mobility in the 'spring form' generation. Consistent throughout our data is a strong signature of sexual differentiation in both resource acquisition and allocation, suggesting that inter- and intrasexual selection may be one of the more potent forces driving phenotypic evolution in this species.

The nutrient pools and fluxes we quantified in this study are likely to interact directly and/or indirectly with the seasonal colour polyphenism for which this species remains known. However, our results indicate no broad seasonal shifts in adult body composition that would explain seasonal differences in colouration. In addition, we find no seasonal patterns in the availability of tryptophan or tyrosine, the amino acids required for synthesis of the pigments involved in each seasonal colour phenotype. More specific explorations of resource dynamics involved in the melanin and ommochrome synthesis pathways are thus needed to better understand how seasonal differences in wing colouration interact with the resource dynamics identified above.

While our study provides no direct links between nutritional dynamics and the colour polyphenism for which *A. levana* is famous, it does reveal signatures of selection acting on resource acquisition and allocation that have to date been mostly characterized in species exhibiting much more obvious polyphenisms in metric traits (e.g. wing polymorphic crickets, Zera & Harshman, 2001; and horn polyphenic beetles, Moczek & Nijhout, 2004). This suggests that even rather subtle morphological responses to seasonal selection may correspond to nontrivial alterations of underlying nutritional dynamics. More work is needed to understand both pattern and process in the evolution of the metabolic pathways associated with these seasonally selected phenotypes (Zera, 2011).

Acknowledgments

This research was supported by a Marie Curie International Incoming Fellowship from the European Union to NIM (PIIF-GA-2008-221466). We thank Bernard Lemesle for assistance identifying field populations of *A. levana*, and two anonymous reviewers for comments that helped to improve the manuscript. The authors have no conflicts of interest to declare.

References

Abrams, P.A., Olof, L., Nylin, S. & Wiklund, C. 1996. The effect of flexible growth rates on optimal sizes and development times in a seasonal environment. *Am. Nat.* **147**: 381–395.

Bauerfeind, S.S. & Fischer, K. 2008. Maternal body size as a morphological constraint on egg size and fecundity in butterflies. *Basic Appl. Ecol.* **9**: 443–451.

Blanckenhorn, W.U. 1998. Adaptive phenotypic plasticity in growth, development, and body size in the yellow dung fly. *Evolution* **52**: 1394–1407.

Boggs, C.L. 2003 Environmental variation, life histories and allocation. In: *Butterflies: Ecology and Evolution Taking Flight* (C.L. Boggs, W.B. Watt, P.R. Ehrlich, eds), pp. 185–206. University of Chicago Press, Chicago, IL.

Boggs, C.L. 2009. Understanding insect life histories and senescence through a resource allocation lens. *Funct. Ecol.* **23**: 27–37.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.

Ellers, J. & Van Alphen, J.J.M. 2002. A trade-off between diapause duration and fitness in female parasitoids. *Ecol. Entomol.* **27**: 279–284.

Emlen, D.J. & Nijhout, H.F. 2000. The development and evolution of exaggerated morphologies in insects. *Annu. Rev. Entomol.* **45**: 661–708.

Friberg, M., Aalberg Haugen, I.M., Dahlerus, J., Gotthard, K. & Wiklund, C. 2011. Asymmetric life-history decision-making in butterfly larvae. *Oecologia* **165**: 301–310.

Fric, Z. & Konvicka, M. 2000. Adult population structure and behaviour of two seasonal generations of the European Map Butterfly, *Araschnia levana*, species with seasonal polyphenism (Nymphalidae). *Nota Lepidopterol.* **23**: 2–25.

Fric, Z. & Konvicka, M. 2002. Generations of the polyphenic butterfly *Araschnia levana* differ in body design. *Evol. Ecol. Res.* **4**: 1017–1032.

Fric, Z., Klimova, M. & Konvicka, M. 2006. Mechanical design indicates differences in mobility among butterfly generations. *Evol. Ecol. Res.* **8**: 1511–1522.

Giron, D., Rivero, A., Mandon, N., Darrouzet, E. & Casas, J. 2002. The physiology of host-feeding in parasitic wasps: implications for survival. *Funct. Ecol.* **16**: 750–757.

Gotthard, K. & Berger, D. 2010. The diapause decision as a cascade switch for adaptive developmental plasticity in body mass in a butterfly. *J. Evol. Biol.* **23**: 1129–1137.

Graham, H.D. 1963. Reaction of sugar alcohols with the anthrone reagent. *J. Food Sci.* **28**: 440–445.

Hanski, I. 1998. Metapopulation dynamics. *Nature* **396**: 41–49.

Hartfelder, K. & Emlen, D.J. 2005. Endocrine control of insect polyphenism. *Compr. Mol. Insect Sci.* **3**: 651–703.

Honěk, A. 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. *Oikos* **66**: 483–492.

Ihalainen, E. & Lindstedt, C. 2012. Do avian predators select for seasonal polyphenism in the European map butterfly *Araschnia levana* (Lepidoptera: Nymphalidae)? *Biol. J. Linn. Soc.* **106**: 737–748.

Joiris, A., Korati, S. & Van Dyck, H. 2010. Differential bird predator attack rate on seasonal forms of the map butterfly (*Araschnia levana* L.): does the substrate matter? *Ethology* **116**: 1020–1026.

Kemp, D.J. & Alcock, J. 2003. Lifetime resource utilization, flight physiology, and the evolution of contest competition in territorial insects. *Am. Nat.* **162**: 290–301.

Kemp, D.J. & Wiklund, C. 2001. Fighting without weaponry: a review of male-male contest competition in butterflies. *Behav. Ecol. Sociobiol.* **49**: 429–442.

Kijimoto, T., Costello, J., Tang, Z., Moczek, A. & Andrews, J. 2009. EST and microarray analysis of horn development in *Onthophagus* beetles. *BMC Genomics* **10**: 504.

Koch, P.B. 1991. Precursors of pattern specific ommatin in red wing scales of the polyphenic butterfly *Araschnia levana* L.: haemolymph tryptophan and 3-hydroxykynurenine. *Insect Biochem.* **21**: 785–794.

Koch, P.B. 1992. Seasonal polyphenism in butterflies: a hormonally controlled phenomenon of pattern formation. *Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere* **96**: 227–240.

Koch, P.B. & Bückmann, D. 1987. Hormonal control of seasonal morphs by the timing of ecdysteroid release in *Araschnia levana* L. (Nymphalidae: Lepidoptera). *J. Insect Physiol.* **33**: 823–829.

Lyytinen, A., Brakefield, P.M. & Mappes, J. 2003. Significance of butterfly eyespots as an anti-predator device in ground-based and aerial attacks. *Oikos* **100**: 373–379.

Lyytinen, A., Brakefield, P.M., Lindström, L. & Mappes, J. 2004. Does predation maintain eyespot plasticity in *Bicyclus anynana*? *Proc. R. Soc. Lond. B Biol. Sci.* **271**: 279–283.

Moczek, A.P. & Nijhout, H.F. 2004. Trade-offs during the development of primary and secondary sexual traits in a horned beetle. *Am. Nat.* **163**: 184–191.

Moran, N.A. 1992. The evolutionary maintenance of alternative phenotypes. *Am. Nat.* **139**: 971–989.

Poulton, E.B. 1890 *The Colours of Animals, Their Meaning and Use, Especially Considered in the Case of Insects*. D. Appleton & Co., New York, NK.

Pöykkö, H. & Hyvärinen, M. 2012. To grow fast or to grow big? Time-limited larvae of *Eilema depressum* speed up their growth and reduce number of instars. *Entomol. Exp. Appl.* **142**: 145–152.

Reinhardt, R. 1984 *Der Landkärtchenfalter*, 2nd edn. A. Ziemsen Verlag, Wittenberg Lutherstadt.

Rosnitschek-Schimmel, I. 1985. Seasonal dynamics of nitrogenous compounds in a nitrophilic weed I. Changes in inorganic and organic nitrogen fractions of the different plant parts of *Urtica dioica*. *Plant Cell Physiol.* **26**: 169–176.

Scriber, J.M. & Slansky, F. 1981. The nutritional ecology of immature insects. *Annu. Rev. Entomol.* **26**: 183–211.

Simpson, S.J., Sword, G.A. & Lo, N. 2011. Polyphenism in insects. *Curr. Biol.* **21**: R738–R749.

Sinclair, B.J., Vernon, P., Jaco Klok, C. & Chown, S.L. 2003. Insects at low temperatures: an ecological perspective. *Trends Ecol. Evol.* **18**: 257–262.

Snell-Rood, E.C., Cash, A., Han, M.V., Kijimoto, T., Andrews, J. & Moczek, A.P. 2011. Developmental decoupling of alternative phenotypes: insights from the transcriptomes of horn-polyphenic beetles. *Evolution* **65**: 231–245.

Trudeau, M., Mauffette, Y., Rochefort, S., Han, E. & Bauce, E. 2010. Impact of host tree on forest tent caterpillar performance and offspring overwintering mortality. *Environ. Entomol.* **39**: 498–504.

True, J.R. 2003. Insect melanism: the molecules matter. *Trends Ecol. Evol.* **18**: 640–647.

Van Dyck, H. & Wiklund, C. 2002. Seasonal butterfly design: morphological plasticity among three developmental pathways relative to sex, flight and thermoregulation. *J. Evol. Biol.* **15**: 216–225.

Van Handel, E. 1985a. Rapid determination of glycogen and sugars in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**: 299–301.

Van Handel, E. 1985b. Rapid determination of total lipids in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**: 302–304.

Vlieger, L. & Brakefield, P.M. 2007. The deflection hypothesis: eyespots on the margins of butterfly wings do not influence predation by lizards. *Biol. J. Linn. Soc.* **92**: 661–667.

Wang, X., Yang, Q., Zhou, X., Zhao, F. & Lei, C. 2007. Effect of photoperiod associated with diapause induction on the accumulation of metabolites in *Sericinus montelus* (Lepidoptera: Papilionidae). *Appl. Entomol. Zool.* **42**: 419–424.

Weismann, A. 1875 *Studien zur Descendez-Theorie. I. Ueber den Saison-Dimorphismus der Schmetterlinge*. Verlag von Wilhelm Engelmann, Leipzig.

Wickman, P.-O. & Wiklund, C. 1983. Territorial defence and its seasonal decline in the speckled wood butterfly (*Pararge aegeria*). *Anim. Behav.* **31**: 1206–1216.

Zachariassen, K.E. 1985. Physiology of cold tolerance in insects. *Physiol. Rev.* **65**: 799–832.

Zera, A.J. 2011. Microevolution of intermediary metabolism: evolutionary genetics meets metabolic biochemistry. *J. Exp. Biol.* **214**: 179–190.

Zera, A.J. & Harshman, L.G. 2001. The physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.* **32**: 95–126.

Received 4 June 2012; revised 17 September 2012; accepted 8 October 2012