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Plastic larval development in a butterfly has complex environmental and genetic causes and consequences for population dynamics

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Summary

1. In insects, the length of larval development time typically influences adult body size and individual fitness, and hence development time can be expected to respond in an adaptive manner to variation in environmental conditions. In the wild, larval growth may be influenced by individual condition, which can be affected by population-level parameters such as population density and abundance and quality of resources.

2. We sampled larvae of the Glanville fritillary butterfly (*Melitaea cinxia*) from 514 local populations across a large metapopulation before the winter diapause and reared the larvae in common garden conditions after diapause. Here, we report that small post-diapause larvae prolonged their development via an extra larval instar, apparently to compensate for their 'bad start' after diapause. The number of instars was additionally a plastic response to environmental conditions, as the frequency of the extra instar increased under cooler thermal conditions.

3. The benefit of the extra instar is clear, as it allows individuals to develop into larger adults, but the cost is delayed adult eclosion, which is likely to select against the extra instar especially in males, in which early eclosion is critical for mating success. In support of this, the frequency of the extra instar was significantly lower in males (7%) than in females (42%).

4. Polymorphisms in three genes, *serpin-1, vitellin-degrading protease precursor* and *phosphoglucose isomerase*, which are known to influence development in insects, were associated with the occurrence of the extra instar.

5. At the level of local populations, the frequency of the extra instar was higher in newly established populations than that in old local ones, possibly reflecting maternal effects, as new populations are often established by females with heavy investment in dispersal. The frequency of the extra instar in turn correlated with the change in population size over 1 year and the risk of local extinction in the natural metapopulation of the Glanville fritillary.

6. Our results highlight the importance of the physiological condition of individuals in shaping subsequent life-history events and even population dynamics.

Key-words: body size, butterfly life-history, larval development, phenotype-genotype association, *phosphoglucose isomerase*, *serpin-1*, variation in instar number, *vitellin-degrading protease precursor*

Introduction

Individual development typically exhibits plasticity in response to the prevailing environmental conditions (Pigliucci 2001). Phenotypic plasticity often involves a strong genetic component (e.g. Bergland *et al.* 2008), but plasticity is also influenced by individual condition and individual state (Hiyama, Taira & Otaki 2012), and even by transgenerational effects (Greer *et al.* 2011). Multifactorial inheritance (Bergland *et al.* 2008), including polygenic, epistatic and gene-environment interactions as well as epigenetic modifications further contribute to phenotypic plasticity (Maleszka 2008).

Adult body size has significant consequences for fitness (Blanckenhorn 2000). In insects, size at pupation is proximately determined by the larval growth trajectory, and a

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trade-off between development time and final body size is commonly reported (Flatt & Heyland 2011). An important constraint is set by the critical minimal body mass at which further growth is not necessary for pupation to occur (Davidowitz, Amico & Nijhout 2003). Both environmental and genetic factors strongly influence development time and adult size, and hence growth trajectories are likely to vary within and between populations (e.g. Berven & Gill 1983). Dissimilar growth trajectories may reflect compromises between the costs and benefits of alternative trajectories (Arendt 1997). In insects, growth trajectories and the initiation of metamorphosis are furthermore influenced by hormonal cascades (e.g. Nijhout & Wheeler 1982), and therefore genetic variation in the respective pathways is expected and known to influence growth trajectories in a number of species (Flatt & Heyland 2011 and references therein). As growth rate is influenced by metabolic rate, genes affecting metabolism are expected to influence larval development, often in interaction with thermal conditions (e.g. Kallioniemi & Hanski 2011; Kvist *et al*. 2012).

A bad start in development may be compensated for via increased growth rate (compensatory growth) or increased development time (catch-up growth) (e.g. Metcalfe & Monaghan 2001; Auer *et al*. 2010). In insects, prolonged development time can be realized either via prolonged development time within a particular instar or by increasing the number of instars (Esperk, Tammaru & Nylin 2007a; Esperk & Tammaru 2010). Intraspecific variation in the number of larval instars (developmental polymorphism; Schmidt & Lauer 1977) is widespread across insect taxa. Environmental factors, such as temperature, food quality and quantity are known to influence the number of instars (Esperk, Tammaru & Nylin 2007a). The most likely reason for plasticity in the number of instars is compensation for a bad start (Esperk, Tammaru & Nylin 2007a), as instar number generally increases when larvae fail to reach a species-specific threshold size for metamorphosis. However, compensatory growth may come with a cost, such as reduced lifespan and fecundity (e.g. Metcalfe & Monaghan 2001; Auer *et al*. 2010). Some genes involved in compensatory growth have been identified and characterized in *Drosophila* (Gerhold *et al*. 2011), but generally, little is known about the proximate mechanisms influencing compensatory growth.

Here, we have investigated the occurrence of an extra eighth larval instar in the Glanville fritillary butterfly (*Melitaea cinxia*), sampled as pre-diapause larvae from 514 small local populations in a large metapopulation. We tested the prediction that larvae with a poor initial condition (small body size) following winter diapause are more likely to develop through the extra instar than individuals with a better condition. Whether this is the case or not, other factors may further influence the occurrence of the extra instar and the post-diapause larval growth trajectory. We studied the effect of post-diapause thermal conditions and larval host plant species on larval development and assessed associations of growth trajectories with several candidate genes. Furthermore, we examined the influence of population-level parameters such as population density and the pre-diapause environmental conditions in the natural populations. We investigated to what extent the larval growth trajectory studied under common garden conditions correlated with the survival, in the field, of the larval family groups from which the larvae reared in the laboratory had been collected. Finally, we examined possible correlation between the extra larval instar and population dynamics. Given that growth trajectories may influence individual survival and fecundity, such effects on population dynamics could be expected to occur, but they have remained little studied in natural populations (but see Werner & Gilliam 1984 and references therein).

Materials and methods

STUDY SYSTEM

In the Åland Islands in Finland, the Glanville fritillary inhabits a large network of dry meadows with one or both of the two larval host plant species, *Plantago lanceolata* and *Veronica spicata* (Nieminen, Siljander & Hanski 2004). The larvae live and overwinter in large groups of full sibs (Kuussaari *et al*. 2004). At the end of the summer, the larvae moult into the fifth instar in a 'winter nest', which the larvae spin at the base of the host plant and inside which they diapause. At this stage the colour of the larvae changes from pale brown to black, with bright orange head capsule, giving the larvae the aposematic appearance that they have in the spring (Kuussaari *et al*. 2004). Following the diapause, the larvae continue to live gregariously until the final instar during which they disperse to pupate.

The habitat in the Åland Islands is highly fragmented and the butterfly has a classic metapopulation structure with a high rate of population turnover (extinctions and re-colonizations; Hanski 1999, 2011). The dry meadows have been surveyed for the presence and numbers of the larval groups since 1993 (Hanski 1999, 2011). In the spring, the meadows that were occupied in the previous fall are revisited to assess the survival of the larval groups and entire local populations over the winter. All local populations are more or less ephemeral, as they are often very small and commonly have just a single or a few larval groups in a given year (Hanski 1999, 2011). Weather variables such as ambient temperature and precipitation are the primary factors influencing large-scale dynamics (Hanski & Meyke 2005), whereas biotic factors influencing larval mortality, for instance quality and quantity of host plants and parasitism, influence local population dynamics (Hanski 1999, 2011).

EXPERIMENTAL SET-UP

During the fall survey of the metapopulation in 2009, 9 930 fifth instar larvae (three larvae per larval group) from 645 local populations across the entire metapopulation were sampled from their winter nests and kept in diapause in the laboratory (+5 °C) until the following spring. For this experiment, 4 851 larvae from 514 larval groups were reared under common garden conditions in the laboratory (28 : 15 °C; 12 : 12, L/D). The average number of larval families per population was three (range from 1 to 54), but

many populations (48%) had just one larval group. The larvae were fed with the leaves of either *P. lanceolata* or *V. spicata*, with individuals from the same family having the same diet. Not all larvae survived to adulthood due to parasitism and other causes of mortality.

Post-diapause larvae were reared individually. They were weighed in the fifth instar immediately after diapause (accuracy 0·01 mg) and subsequently in the beginning of each remaining instar and in the pupal stage. The typical number of instars is seven, but a fraction of larvae developed through an extra eighth instar. Variation in the number of instars is more likely to occur in the post-diapause than in the pre-diapause stage, as the post-diapause treatment (e.g. temperature) influences the occurrence of the additional post-diapause instar (see Results). Occasionally, some larvae fail to moult to the morphologically distinct diapause (fifth) instar, while other larvae in the same larval family group do so, but these exceptional larvae invariably die before the end of diapause (S. Ikonen, pers. obs.). Unfortunately, individual data on pre-diapause larval development is practically impossible to obtain as small pre-diapause larvae cannot be successfully reared individually due to their gregarious behaviour. Therefore, it is difficult to conclusively disprove the possibility that some larvae would moult to the diapause phenotype in the fourth instar.

With the individual-level post-diapause material, we constructed growth trajectories for larvae with seven versus eight instars. In the beginning of the sixth instar, most of the larvae were reared in the standard conditions with 28 : 15 °C 12 : 12 L/D, but a smaller random sample of larvae (approximately 1/3) were reared in high temperature treatment with 32 : 15 °C 12 : 12 L/D. After eclosion, adult butterflies were sexed and marked individually. Adult butterflies were kept at 26 : 18 °C (9 : 15 L/D) and fed daily with honey : water solution (1 : 3) until they died.

SURVIVAL OF THE LARVAL GROUPS AND POPULATION DYNAMICS IN THE WILD

All populations in which larvae were detected in the fall 2009 were revisited in the spring and fall 2010. The spring survey allowed us to record the survival of each individually marked larval family group in the wild and to relate its survival to the traits of the three individuals sampled from that group in the fall 2009 and studied under common garden conditions. Using data from the fall surveys in 2009 and 2010, we calculated the proportional change in the size of each population as N_{2010}/N_{2009}. Additionally, we recorded the survival of populations that existed in the fall 2009 by observing whether there were one or more larval groups in the respective habitat patches in the fall 2010. We then analysed the effects of the explanatory variables on the change in population size and their survival over 1 year.

GENOTYPING

To investigate associations between candidate genes and the occurrence of the extra eighth larval instar, we selected 383 apparently unrelated individuals, maximally one individual/sex/family, across the entire metapopulation, including 109 and 111 females and 50 and 113 males with and without the extra instar respectively. Individuals used in the genotype-phenotype association analyses were all from the standard temperature treatment group. The candidate genes were chosen based on a comparison of pooled RNA sequencing data (Vera *et al.* 2008, cinxiabase http://cinxiabase.vmhost.psu.edu/ and unpublished data) from the Åland Islands and a Chinese population, and on prior knowledge from the literature of the role of the genes in larval development in insects (Appendix S1). We thus selected genes belonging to serine proteases, serine protease inhibitors and cuticular protein GO groups for genotyping. In addition, three SNPs from the gene *phosphoglucose isomerase* (*Pgi*), known to be associated with life-history variation in the Glanville fritillary (e.g. Orsini *et al.* 2009; Saastamoinen, Ikonen & Hanski 2009; Hanski 2011), were included. The set of markers includes 31 SNPs in 11 genes, of which four were subsequently excluded based on quality tests (Appendix S1).

DNA extraction was done in the Institute of Biotechnology, University of Helsinki, using Nucleo Spin ® 96 Tissue kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. SNP genotyping was performed at the Institute for Molecular Medicine Finland (FIMM; Helsinki, Finland) using Sequenom iPLEX Gold chemistry according to the manufacturer's instructions. The Sequenom assay design was validated in seven independent samples with direct genomic sequencing with AB 3730 (Applied Biosystems Europe, Bleiswijk, the Netherlands) according to the manufacturer's protocol. The primers (available by request) for Sequenom were designed using the Sequenom assay designing software, MASSARRAY Assay Design 3·1, and the validation primers covering the Sequenom PCR and extension probe regions were designed using Primer3 (Rozen & Skaletsky 2000).

We assessed the quality of the SNPs using an in-house quality control pipe-line (Wong 2011). We tested the high-quality SNPs for genetic associations with the occurrence of the eighth instar using logistic regression in the PLINK genome association analysis toolset (Purcell *et al.* 2007). The final genetic association analyses were conducted separately for males and females, as the association signal appeared to vary between the sexes. False discovery rate calculation (FDR_BH; Benjamini & Hochberg 1995 step-up FDR control) implemented in PLINK was used to correct for multiple testing.

STATISTICAL ANALYSES

We measured development time, body mass, eclosion date and lifespan for individual larvae and adult butterflies. As individual development time and body mass were measured for all post-diapause instars (fifth, sixth, seventh and eighth instars), analyses were performed with repeated measures and using the Kenward-Roger's adjustment to derive denominator degrees of freedom (Kenward & Roger 1997). Explanatory variables included instar, sex, host plant in the laboratory, host plant in the field (from which the larvae were sampled in the first place) and temperature treatment. Additional analyses were performed for the fifth instar body mass, pupal mass and adult lifespan. Survival to adulthood was analysed with binary error distribution with the host plant in the field and the fifth instar body mass as explanatory variables. Factors explaining whether individuals had the extra eighth instar were similarly analysed with binary error distribution and logit link function. These factors included sex, host plant in the laboratory, host plant in the field, fifth instar body mass and the temperature treatment. Finally, we analysed whether the survival of larval groups in the field was affected by the occurrence of the extra instar among the larvae from that group that were reared in the laboratory, as well as by additional explanatory

variables. In all individual-level analyses, population and family nested within population were included as random factors.

Population-level analyses were performed with phenotypic population averages. When necessary, these averages were corrected for sex differences. We used GLM (generalized linear mixed models) procedures to assess whether or not the frequency of individuals with the extra instar was affected by host plant in the wild, population age (Hanski, Saastamoinen & Ovaskainen 2006) and size, spatial connectivity and larval condition (body mass in the fifth instar). Spatial connectivity was measured as a function of the sizes (number of larval groups) in the neighboring populations and their distances to the focal population (for more details see Hanski, Saastamoinen & Ovaskainen 2006; Ovaskainen 2004). Except for the body mass in the fifth instar (the only phenotypic trait measured prior to the temperature treatment), the population-level analyses were performed on individuals reared in standard temperature conditions, as very few larvae per population (two on average) were reared in the high temperature treatment. Models were simplified by removing non-significant interaction terms to yield the final minimal adequate model. GLM procedures were used to assess whether or not proportional change in population size between the falls 2009 and 2010 (N_{2010}/N_{2009}) was affected by population size in 2009 (density dependence), spatial connectivity, regional trend in population sizes (see Hanski 1999), average body mass in the fifth instar, the frequency of the extra instar, host plant species and the percentage of dried-out host plants in the habitat patch. Additionally, we analysed with binary error distribution and logit link function the factors that influenced the survival of local populations from the fall 2009 until the fall 2010. Statistical analyses were carried out with SAS 9·2.

To characterize spatial autocorrelation among populations in the body mass of post-diapause larvae, frequency of the extra instar and over-wintering survival of larval groups we computed envelopes of Besag's *L*-function for 9,999 randomly reshuffled labelling (Illian *et al.* 2008).

Results

POST-DIAPAUSE LARVAL DEVELOPMENT

The average lengths of the post-diapause larval instars were 8, 8 and 11 days in the fifth, sixth and seventh instars respectively (the seventh was the longest; $F_{2,\ 5274} = 1802{\cdot}4$, $P < 0\ .0001$). In general, the length of each instar was shorter in males than in females (Table 1; $F_{1,6146} = 194{\cdot}0$, $P < 0{\cdot}0001$), and development times were shorter in the higher rearing temperature (Table 1; $F_{1,4622} = 384{\cdot}7$, $P < 0{\cdot}0001$). Host plant species used by the larvae in the field and in the laboratory did not influence the lengths of the larval instars ($P = 0{\cdot}59$ and $P = 0{\cdot}11$ respectively).

Larval body mass increased with increasing instar (Fig. 1; $F_{4,7949} = 3{,}7949$, $P < 0{\cdot}0001$) and females were heavier than males ($F_{1,5624} = 345{\cdot}3$, $P < 0{\cdot}0001$). The difference in body mass between the sexes increased with increasing instar (sex × instar; $F_{4,7962} = 516{\cdot}4$, $P < 0{\cdot}0001$), but the sex difference was not yet evident in the fifth instar ($F_{1,1102} = 3{\cdot}08$, $P = 0{\cdot}08$; the trend was towards males being heavier). Individuals reared in the high temperature treatment became heavier in the final instars ($F_{1,3848} = 41{\cdot}8$, $P < 0{\cdot}0001$), which increase was furthermore greater in females (temperature treatment × sex; $F_{1,1641} = 5{\cdot}9$, $P = 0{\cdot}015$). Host plant in the field and host plant in the laboratory did not affect body mass ($P > 0{\cdot}8$ for both). However, considering the body mass at the end of the diapause (fifth instar), larvae sampled in the field from *V. spicata* were heavier than larvae sampled from *P. lanceolata* ($F_{1,1102} = 18{\cdot}8$, $P < 0{\cdot}0001$).

Heavier individuals at the end of the diapause (fifth instar) were more likely than lighter individuals to survive until adulthood ($F_{1,2729} = 289{\cdot}4$, $P < 0{\cdot}0001$), but host plant in the field did not affect larval survival ($P = 0{\cdot}9$). The former result from the laboratory is consistent with the over-winter survival of larval groups in the field: larval groups with heavier larvae, based on the sample of three larvae reared in the laboratory, survived better (Table 2). Additionally, in the field larval groups living on *V. spicata* survived better than larval groups on *P. lanceolata* (Table 2). Male butterflies had a longer lifespan than females ($F_{1,982} = 20{\cdot}07$, $P < 0{\cdot}0001$), and individuals reared in the standard temperature treatment had a longer lifespan than those reared in the high temperature treatment ($F_{1,982} = 6{\cdot}15$, $P = 0{\cdot}013$). Host plant in the field and host plant in the laboratory did not affect adult lifespan ($P = 0{\cdot}53$ and $P = 0{\cdot}26$, respectively).

THE EXTRA INSTAR AT THE INDIVIDUAL LEVEL

In general, 42% and 30% of females and 7% and 3% of males in the standard and high temperature treatments, respectively, developed through eight larval instars. Thus, females were more likely than males to develop through the extra eighth instar ($F_{1,1027} = 171{\cdot}4$, $P < 0{\cdot}0001$), and a smaller frequency of individuals had the extra instar in the high temperature treatment ($F_{1,1027} = 8{\cdot}1$, $P = 0{\cdot}005$). In both sexes smaller larvae in the beginning of the post-diapause development were more likely to have the extra instar ($F_{1,1027} = 86{\cdot}6$, $P < 0{\cdot}0001$). The difference in body mass between the individuals with and without the extra instar increased from the fifth to the seventh instar (Fig. 1). The plant species on which the larvae developed after diapause did not have a significant effect on the occurrence of the extra instar ($P = 0{\cdot}11$). Table 1 summarizes the results for the growth trajectories in males and females in the two temperature treatments.

Having the additional instar paid off in terms of increased pupal mass ($F_{1,1100} = 174{\cdot}3$, $P < 0{\cdot}0001$; Fig. 1). For instance, in the standard temperature treatment, the relative difference in body mass between individuals with and without the additional instar was 9 and 10% in females and males, respectively (Fig. 1; Table 1). The cost of the extra instar was delayed eclosion ($F_{1,1115} = 1017{\cdot}5$ $P < 0{\cdot}0001$), 6 (6) days in females and 7 (10) days in males in the standard (high) temperature treatment. In general, males eclose earlier than females ($F_{1,1115} = 212{\cdot}1$, $P < 0{\cdot}0001$), but the difference in the eclosion time

Table 1. Average values (± SE) of the life history traits separately for the two temperature treatments (standard and high temperature), the two sexes, and individuals with 7 and 8 larval instars.

	Females			Males		
	seven instars	eight instars	*P*	seven instars	eight instars	*P*
Standard temperature						
Body mass						
fifth instar	5·2 (±0·08)	4·5 (±0·1)	***	5·1 (±0·04)	4·2 (±0·1)	***
sixth instar	19·0 (±0·2)	15·5 (±0·3)	***	19·0 (±0·2)	15·4 (±0·4)	***
seventh instar	65·5 (±0·8)	41·6 (±0·8)	***	60·2 (±0·5)	39·4 (±1·2)	***
eighth instar	–	114·2 (±3·2)	–	–	99·9 (±5·8)	-
pupae	178·0 (±1·0)	193·8 (±1·9)	***	146·2 (±0·6)	160·7 (±2·6)	***
Development time						
fifth instar	8·1 (±0·05)	8·3 (±0·08)	*	7·4 (±0·04)	7·7 (±0·1)	NS
sixth instar	8·3 (±0·06)	7·6 (±0·1)	***	7·6 (±0·04)	7·9 (±0·2)	NS
seventh instar	14·3 (±0·1)	8·6 (±0·2)	***	11·8 (±0·06)	8·1 (±0·3)	***
eighth instar	–	12·4 (±0·2)	–	–	10·6 (±0·4)	-
pupae	8·1 (±0·2)	8·2 (±0·4)	*	7·9 (±0·02)	8·2 (±0·07)	*
Age at eclosion	38·7 (±0·1)	45·1 (±0·3)	***	34·7 (±0·1)	42·4 (±0·5)	***
Adult lifespan	20·0 (±0·5)	21·2 (±0·6)	NS	22·7 (±0·3)	22·0 (±1·1)	NS
High temperature						
Body mass						
fifth instar	5·2 (±0·08)	4·2 (±0·1)	***	5·1 (±0·06)	4·7 (±0·3)	**
sixth instar	19·5 (±0·3)	15·3 (±0·5)	***	19·1 (±0·2)	16·8 (±1·1)	***
seventh instar	76·2 (±1·2)	44·8 (±1·5)	***	68·2 (±0·9)	44·3 (±2·8)	***
eighth instar	–	117·5 (±5·6)	–	–	82·5 (±6·7)	-
pupae	188·7 (±1·6)	207·4 (±4·8)	***	154·6 (±1·0)	158·2 (±7·1)	*
Development time						
fifth instar	7·6 (±0·9)	7·7 (±0·2)	NS	6·9 (±0·05)	7·6 (±0·3)	*
sixth instar	7·5 (±0·08)	6·8 (±0·1)	**	6·6 (±0·06)	7·4 (±0·3)	*
seventh instar	11·7 (±0·2)	7·1 (±0·2)	***	9·6 (±0·09)	6·5 (±0·6)	***
eighth instar	–	10·7 (±0·3)	–	–	10·5 (±0·6)	-
pupae	7·5 (±0·05)	8·0 (±0·06)	***	7·1 (±0·04)	8·2 (±0·2)	***
Age at eclosion	34·1 (±0·3)	40·1 (±0·5)	***	30·2 (±0·2)	40·3 (±0·1)	***
Adult lifespan	19·6 (±0·6)	20·7 (±1·1)	NS	21·2 (±0·5)	20·4 (±2·1)	NS

*$P \leq 0{\cdot}01$.
**$P \leq 0{\cdot}001$.
***$P \leq 0{\cdot}0001$.
NS, non-significant.

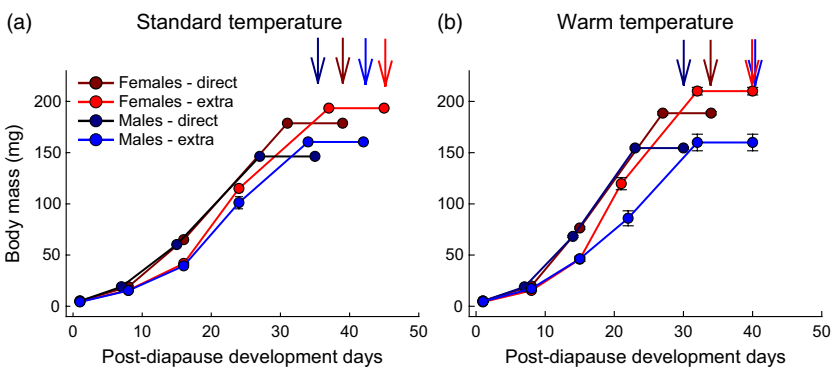

Fig. 1. Growth trajectories for females (red) and males (blue) with and without the extra instar under (a) standard and (b) high temperature treatments. The trajectories are based on the weights in the beginning of each instar and the durations of the instars. Darker colours represent individuals with seven instars, lighter colours individuals with eight instars. Each circle represents the average time of change of the instar. Arrows indicate the times of eclosion of the different groups.

between the sexes under both thermal conditions is smaller when individuals have eight instars (sex × extra instar interaction; $F_{1,1115} = 16{\cdot}1$, $P < 0{\cdot}0001$). Furthermore males with the extra instar under both temperature treatments eclosed later than the corresponding females with seven instars ($P < 0{\cdot}0001$ in both treatments; Fig. 1, Table 1). The number of instars did not affect adult lifespan ($P = 0{\cdot}21$; Table 1).

GENOTYPIC ASSOCIATIONS WITH THE EXTRA INSTAR

In males, significant allelic associations (additive model) were found for two closely located SNPs in the gene *serpin-1* (c172_est:512G>A: $t_{1,162} = 2{\cdot}5$, $P = 0{\cdot}014$ and c172_est:652A>G: $t_{1,160} = 2{\cdot}6$, $P = 0{\cdot}010$; Fig. 2a and b) and for one SNP in the gene *vitellin-degrading protease precursor* (c177_est:199G>A: $t_{1,160} = 2{\cdot}0$, $P = 0{\cdot}041$;

Table 2. Factors influencing over-winter survival of individual larval families and annual survival of local population.

	d.f.	*F*	*P*	Coefficient (SE)
Survival of larval groups				
Population size in N_{2009}	1,909	3·2	0·074	0·07 (0·04)
Average fifth instar body mass in the group	1,909	20·2	<0·0001	0·33 (0·07)
Frequency of extra instar in the group	1,909	1·6	0·202	0·38 (0·30)
Host plant species*	1,909	7·0	0·008	−0·46 (0·18)
Population survival				
Population size in N_{2009}	1,362	22·6	<0·0001	0·86 (0·18)
Average fifth instar body mass in the population	1,362	5·6	0·018	0·33 (0·14)
Frequency of extra instar in the population	1,362	4·4	0·037	0·78 (0·37)
Host plant species*	1,362	3·5	0·061	−0·61 (0·33)

*estimate for *V.spicata* in comparison to *P.lanceolata*.

Fig. 2c). In females, a significant association was found for a different SNP in *vitellin-degrading protease precursor* (c177_est:181A>G: $t_{1,219} = 2{\cdot}2$, $P = 0{\cdot}030$; Fig 2d). In addition, in females a significant association was detected for one SNP in the *Pgi* gene (EU888473·1(Pgi):c.331A>C: $t_{1,219} = 2{\cdot}0$, $P = 0{\cdot}043$; Fig. 2e). In all cases, an increase in the minor allele frequency increased the likelihood of the extra instar (Fig. 2; see also Table S2 in Online Supporting Information). The genetic associations were not significant after correcting for multiple testing (Table S3). Power calculations performed using PGA (Menashe, Rosenberg & Bingshu 2008) show that the power to detect a significant association with the current material varies between 27% and 76% (Fig. 3). The power calculation was based on the observed minor allele frequency (MAF) and calculated odds ratios (OR) of two of the top candidate SNPs using two different r^2 linkage disequilibrium values. These power simulations represent four likely scenarios assuming that our genotyped SNPs are strongly linked to the potential functional alleles, which is possible because we genotyped SNPs that could be expected to be functionally significant. The experimental setting including 50 individuals with the eighth instar as powerful in the best scenario (MAF 0·25, OR 1·88 and complete linkage between the marker and a functional variant), but is clearly underpowered in most of the simulated scenarios. The model assuming single major locus inheritance may furthermore overestimate the statistical power.

THE EXTRA INSTAR AT THE POPULATION LEVEL

At the population level, the frequency of individuals with the extra instar (corrected for sex) was higher in populations in which individuals were lighter at the end of diapause ($F_{1,190} = 10{\cdot}6$, $P = 0{\cdot}0013$). Small mass of post-diapause larvae may reflect unfavourable environmental conditions in the previous summer, during pre-diapause larval development, and indeed those populations that had a higher frequency of desiccated host plants in summer 2009 had lighter post-diapause larvae ($P = 0{\cdot}024$). Considering populations with different ages (time since the establishment of the population) and population connectivities, the frequency of the extra instar was higher in

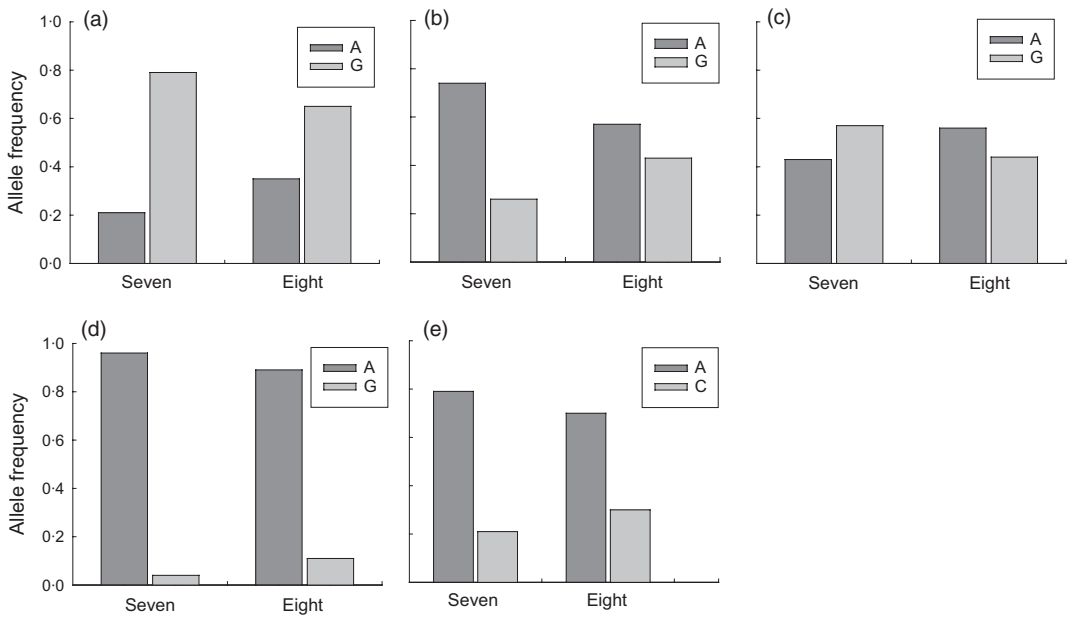

Fig. 2. A–C show the average allele frequencies in males in two SNPs in *serpin-1* (A and B; c172_est:512 and c172_est:652, respectively) and one SNP in *vitellin-degrading protease precursor* (C; c177_est:199) with seven and eight instars during larval development. D–E show the average allele frequencies in females in one SNP in *vitellin-degrading protease precursor* (D; c177_est:181) and one SNP in *Pgi* (E; EU888473·1(Pgi):c.331A) with seven and eight instars during larval development. See Table S2 for details.

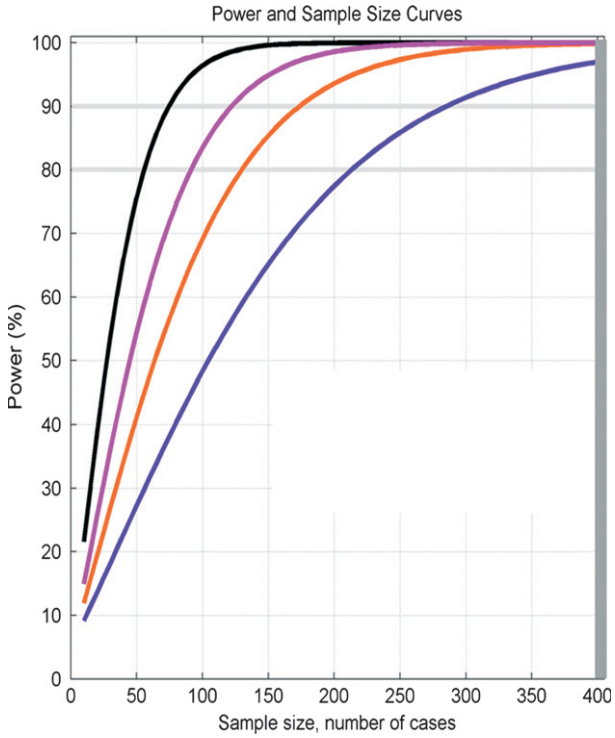

Fig. 3. Power calculation using PGA (Menashe, Rosenberg & Bingshu 2008). The parameter values were selected to match the data for males: frequency of the eighth instar 7%, case-control ratio 2·26, and the number of cases 50. The other parameters are MAF (minor allele frequency), FF (frequency of the functional allele), OR (odd ratio for the eighth instar, with two copies of an allele increasing the probability of the eighth instar) and r^2 (linkage disequilibrium value between the functional locus and a SNP). Black line: MAF 0·25, FF 0·25, OR 1·88, LD 1; red line: MAF 0·25, FF 0·17, OR 1·88, LD > 0·8; pink line: MAF 0·47, FF 0·47, OR 1·6, LD 1; blue line: MAF 0·25, FF 0·32, OR 1·88, LD 0·8. SNP-wise OR is calculated with PLINK (Purcell *et al.* 2007).

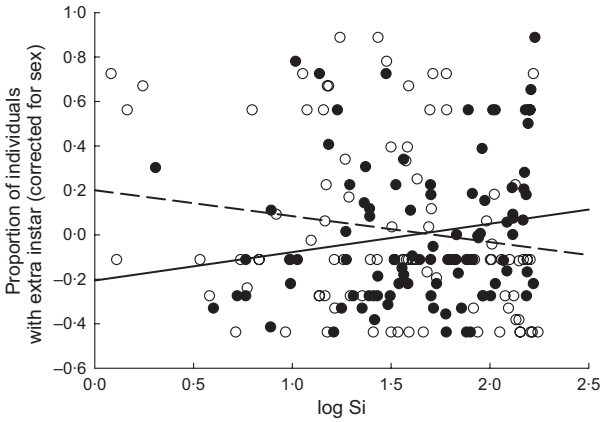

Fig. 4. The frequency of individuals with the extra instar in newly established (open symbol and dashed line) and old local populations (≥ 5 years old; closed symbol and solid line) in relation to population connectivity (S_i, defined in Materials and methods). Each symbol refers to one local population.

newly established populations than that in old local ones ($F_{1,190} = 6{\cdot}0$, $P = 0{\cdot}016$), and especially in isolated newly established populations ($F_{1,190} = 6{\cdot}0$, $P = 0{\cdot}014$; the interaction term in Fig. 4). The size of the population in 2009 (number of larval groups) and the host plant species used by the larvae in the field did not affect the occurrence of the extra instar ($P = 0{\cdot}85$ and $P = 0{\cdot}91$ respectively). At the population level, heavier larvae at the end of diapause were found in populations in which *V. spicata* was used more than *P. lanceolata* ($F_{1,386} = 7{\cdot}0$, $P = 0{\cdot}009$), in parallel with the result for individual larvae.

We did not find any spatial autocorrelation in the population average values of post-diapause larval mass, pupal mass, frequency of the extra instar and the survival of larval groups.

CONSEQUENCES FOR POPULATION DYNAMICS

Larger populations and those with heavier post-diapause larvae (Fig. 5a) were less likely to go extinct over 1 year than small populations and those with lighter post-diapause larvae (Table 2). Independent of post-diapause body mass, population survival was positively correlated with high frequency of extra instar (Table 2; Fig. 5b). In a model with the above factors as well as the abundance of the host plants and the percentage of host plants that dried out in summer 2010, population size had a highly significant effect ($P < 0{\cdot}0001$) on extinction and the P values for all the other factors were between 0·03 and 0·09; extinction was more likely in populations with a small amount of host plants and high percentage of dried-out host plant individuals, both factors reducing food availability to larvae.

Proportional change in population size from fall 2009 to fall 2010 was negatively related to (the logarithm of) population size in 2009 (indicating density dependence), positively related to regional trend (which reflects spatially correlated population dynamics; Hanski 1999; Hanski & Meyke 2005), and negatively related to the percentage of host plants in the habitat patch that had dried out in summer 2010 (Table 3). The last two effects were not significant at 5% level when we excluded populations that went extinct by late summer 2010 (Table 3). Additionally, the change in population size was negatively affected by the frequency of the extra instar, and there was a significant interaction with average post-diapause (fifth instar) larval body mass (Table 3).

Discussion

COSTS AND BENEFITS OF THE EXTRA INSTAR

The present results demonstrate that Glanville fritillary larvae can fully compensate for small initial body mass following winter diapause by developing through an extra larval instar, which allows individuals to reach greater adult body mass than individuals developing without the extra instar. Small post-diapause body mass reflects an individual's condition, as the likelihood of surviving to

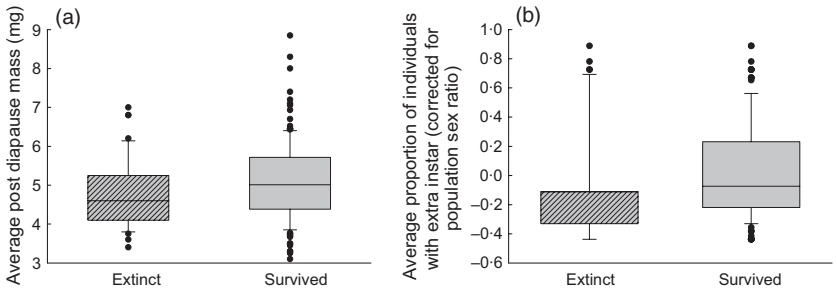

Fig. 5. The effects of (a) the average post-diapause body mass and (b) the frequency of individuals with the extra larval instar (corrected for sex) on the survival of local populations in the wild. For statistics see Table 2.

Table 3. Factors influencing proportional change in population size from 2009 to 2010 (N_{2010}/N_{2009}). The first two columns include populations that went extinct by 2010 (d.f. = 1, 338), the last two columns exclude those populations (d.f. = 1,150).

	F	*P*	Coefficient (SE)	*F*	*P*	Coefficient (SE)	Direction
Population size in N_{2009}	73·1	<0·0001	−0·12 (0·•01)	38·5	<0·0001	−0·13 (0·02)	–
Percentage of dry hosts in N_{2010}	11·4	0·001	−0·003 (0·001)	2·7	0·103	−0·003 (0·002)	–
Regional trend in pop· change	6·4	0·012	0·10 (0·04)	3·5	0·064	0·13 (0·07)	+
Average fifth instar body mass	0·03	0·874	−0·003 (0·02)	0·3	0·591	−0·02 (0·04)	
Frequency of extra instar	4·8	0·030	−0·54 (0·25)	6·6	0·012	−1·43 (0·56)	–
Body mass × Freq of extra instar	4·7	0·030	–	6·4	0·012	–	

adulthood depended on the initial body mass. The primary cost of the extra instar is increased time of development, which is an especially severe cost in males, as will be discussed below. Consistent with the sex-biased cost, the frequency of the extra larval instar was 6–10 times higher in females than males, depending on the temperature treatment.

Compensatory responses in larval development are common in insects, but they typically involve either faster growth rate or a prolonged period of development within a particular instar (Esperk & Tammaru 2010) rather than development through an extra instar. Only a few examples of the latter are known from butterflies (Esperk, Tammaru & Nylin 2007a). Compensation in the Glanville fritillary occurred mainly via increased development time (catch-up growth) rather than increased growth rate (compensatory growth), even though there appeared to be some differences in the growth rates of individuals with and without the extra instar (Fig. 1). However, more detailed data on daily growth rates would be required to test differences in the slopes of the growth trajectories. In general, increasing growth rate is energetically very costly, and it may increase the risk of starvation and predation (Arendt 1997).

Size compensation is typically only partial in insects (e.g. Esperk & Tammaru 2010), which has been interpreted to reflect constraints on within-instar growth and costs of prolonged development. Full compensation is more likely when compensation is due to variation in the number of instars (Esperk *et al.* 2007b; Esperk & Tammaru 2010) as in the present case. In view of the small number of reported cases of polymorphism in the number of instars in butterflies, we note that researchers may not become aware of such variation unless larvae are reared individually and observed very closely.

We found a strong negative relationship between the fifth instar body mass and the occurrence of the extra instar. There may exist a genetic correlation between the two traits, but in any case our results demonstrate that plasticity in post-diapause larval development via an extra instar is strongly condition-dependent and induced by environmental conditions. Apart from individuals with low fifth instar body mass being more likely to have the extra instar, lower temperature during post-diapause development increased the likelihood of the extra instar, consistent with studies on other insects (Esperk, Tammaru & Nylin 2007a). Furthermore, and as also reviewed by Esperk *et al.* (2007b), females were more likely to develop through an extra instar than males.

Whether an individual larva develops through an extra instar or not is likely to represent a compromise between the relative costs and benefits (Dmitriew 2011). For most organisms body size rather than age has the greatest influence on fecundity, while growth rate and development time define the relationship between body size and age (Arendt 1997). In this study, the extra instar paid off in terms of adult body mass, as both males and females with eight instars were heavier than individuals with only seven instars. The benefits of greater adult body mass in the Glanville fritillary are however complex and depend on the environmental conditions and individual state. For instance, previous studies have shown that heavier females have higher fitness (clutch size and lifetime egg production) than lighter females, but only in years with suboptimal environmental conditions (Saastamoinen 2007a,b). Similarly, even though in the present case body size did not affect female lifespan, such an effect has been reported for reproducing females (Saastamoinen, Ikonen & Hanski 2009). In males, in contrast, larger body size does not appear to be related to higher fitness (Saastamoinen,

Ikonen & Hanski 2009). For males, early eclosion (protandry) even at the expense of smaller body size is highly advantageous (e.g. Stillwell *et al*. 2010), as Glanville fritillary females mostly mate only once and do so within a few days from eclosion (Saastamoinen 2007a,b). In general, males eclose a few days before females but males with the extra instar will miss the eclosion of the directly developing females, and hence pay a high cost (Esperk *et al*. 2007b). Other costs of having the extra instar and hence prolonged development time may include increased risk of stage-specific predation and parasitism, and trade-offs between resource allocation for growth and maintenance and/or immunity (Arendt 1997).

Like many other Lepidoptera, the Glanville fritillary shows sexual size dimorphism with adult females being larger and heavier than males. We observed no difference in larval body mass in the fifth instar, but females started to become heavier in the subsequent instars. The proximate mechanism that gives rise to the sexual size difference in adults is a combination of prolonged development time within single instars and the additional eighth instar (see review by Esperk *et al*. 2007b). This result adds to the growing literature indicating that much of intraspecific variation in sexual size dimorphism seems to be due to differences in phenotypic plasticity between males and females (reviewed in Stillwell *et al*. 2010).

GENOTYPE–PHENOTYPE ASSOCIATIONS

We found significant associations between the extra instar and three candidate genes, *serpin-1* (two SNPs), *vitellin-degrading protease precursors* (two SNPs) and *Pgi* (one SNP). These genetic associations were somewhat different in the two sexes, which is a common phenomenon in for example human studies and may be due to different pathways or epistatic interactions in males and females, or other confounding factors (Liu *et al*. 2012). The associations were not statistically significant after correcting for false discovery in multiple testing, but based on the power analysis, we should not expect highly significant association signals in this material. The present results are suggestive of association, especially in view of the specific genes that are involved (below), but they require validation in a family setting or with a larger sample size.

Several studies on insects, including Lepidoptera (e.g. *Bombyx mori*; Sasaki & Kobayashi 1984; *Mamestra configurata*; Hegedus *et al*. 2008; *Manduca sexta*; Jiang, Wang & Kanost 1994), have highlighted the functional roles of *serpin-1* and *vitellin-degrading protease* in larval development. Serpins in general are important regulators of insect defence mechanisms as well as developmental processes (Hegedus *et al*. 2008), and *serpin-1* in particular is considered to play a role in developmental pathways (Chamankhah *et al*. 2003). The fact that *serpin-1* is alternatively spliced in many Lepidoptera (Jiang & Kanost 1997; Hegedus *et al*. 2008) implies its involvement in the regulation of physiological processes such as larval moulting (Chamankhah *et al*. 2003). Proteases are known to play a prominent role in a wide range of processes, including food digestion, embryogenesis, tissue reorganization (e.g. regeneration, moulting and metamorphosis), defence mechanisms and immune responses (Clynen, Schoofs & Salzet 2005). Vitellin itself is a yolk reserve, and hence crucial for successful embryonic development. Vitellin is degraded by a protease during larval development and the resulting free amino acids (generated by hydrolysis of vitellin protein), are thought to be important in the embryonic and larval development (Li *et al*. 1998). Finally, molecular variation in *Pgi* is known to be related to a number of life-history traits in the Glanville fritillary (reviewed in Bonte & Saastamoinen 2012; Hanski 2012). We are unaware of any previous studies showing genetic associations with instar polymorphism, but inheritance of the instar number has been demonstrated for several insect species (see references in Esperk, Tammaru & Nylin 2007a) including the Glanville fritillary (Kvist *et al*. 2012).

INDIVIDUAL GROWTH TRAJECTORIES AND POPULATION DYNAMICS

The frequency of the extra larval instar was higher than average in newly established and especially in newly established isolated populations, and the occurrence of the extra instar was associated with the *Pgi* genotype in females. These two findings are related to dispersal, and possibly to transgenerational (maternal) cost of dispersal. Previous studies have shown that AC heterozygous females in the SNP EU888473·1(Pgi):c.331A>C are more dispersive than AA homozygotes (Haag *et al*. 2005; Niitepõld *et al*. 2009) and that, consequently, the frequency of the C allele is especially high in newly established populations (Hanski 2011; Hanski & Mononen 2011). The butterflies in new populations are the offspring of females that established the population in the previous generation, and there is substantial heritability of the dispersal behaviour (Saastamoinen 2008). Therefore, given that the extra instar is a means of compensating for poor physiological condition of larvae at the end of diapause, and taking into account that dispersal is costly (Roff & Fairbarn 2007), females establishing new populations may have invested resources to dispersal at the expense of offspring condition, which could explain the high frequency of the extra larval instar in new populations. Some old populations may have inhabited habitat patches with higher quality of resources than in the patches occupied by many newly established populations, because by definition the former have persisted for at least 5 years in the same habitat patch. Variation in resource levels between population types might also explain differences in the prevalence of the extra instar.

The number of larval instars in the Glanville fritillary may have significant population-level consequences. In general, larger populations and populations with heavier post-diapause larvae were more likely to survive until the

following generation, highlighting the significance of larval condition for over-winter survival. On top of these effects, population persistence was positively correlated with a higher frequency of the extra larval instar. The effect of the frequency of the extra instar on the proportional change in population size was more complex. The direct effect of the frequency of the extra instar was negative, but a significant interaction with the average post-diapause body mass implies that change in population size is further influenced by individual condition. Given that the individual condition and the growth trajectory are influenced by the environmental conditions, we suggest that they represent mechanisms by which the environment influences population dynamics.

Conclusions

Post-diapause body mass was positively correlated with both individual and population-level survival in the Glanville fritillary, but the larvae are able to compensate for initial bad start after diapause by developing through an extra larval instar. The costs and benefits of compensatory growth are likely to be sex-specific, and indeed we found a lower frequency of the compensatory extra instar in males in which the cost of delayed adult eclosion is expected to be high due to protandry. Variation in individual condition is critical for life-history ecology and evolution, as it can explain, for example, apparent lack of the predicted trade-offs between energy demanding life-history traits. On the other hand, if individuals can compensate for their initial poor condition by accumulating more resources once the environment improves, we may not observe trade-offs that would have been apparent under continuously limited resource availability. The present results indicate that the individual physiological condition and the environmental conditions during subsequent development may affect individual life history, sexual dimorphism and even population dynamics, thereby coupling individual growth trajectories and population dynamics.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1: Selection of candidate genes and quality testing and call rates of the SNPs.

Table S1. Genotype counts and call rates for 27 SNPs used in the genotype-phenotype association analyses.

Appendix S2: Allele frequencies for the significant SNPs separately for males and females.

Table S2. Allele frequecies and sample sizes (in brackets) for two closely located SNPs in the gene *serpin-1* (172_est:512 & 172_est:652), for two closely located SNPs in the gene *vitellin-degrading protease precursor* (177_est:199 & 177_est:181), and for one SNP in the gene *Pgi* (EU888473.1(Pgi):c.331) separately for males and females.

Appendix S3: Results on allelic associations for the candidate SNPs and the presence of the eight instar.

Table S3. Allelic associations for the 27 SNPs (additive model) and the presence of the eight instar separately for males and females (FDR based P-values are in brackets). Significant associations are marked bold.