Thermal adaptation in butterflies: patterns, significance and mechanisms

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7. The genetic background of altitudinal variation in life-history and temperature stress resistance traits*

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1. Introduction

1.1. Temperature – a key environmental factor

Temperature exerts important influences on all aspects of an individual's ecology and evolution (e.g. Hoffmann et al. 2003, Sinclair et al. 2003) and affects biological organization directly and indirectly on nearly all spatial and temporal scales. For instance, temperature may impact on immune function (Mondal and Rai 2001), sensory input (Stevenson et al. 1985), foraging ability (Carrière and Boivin 1997) and locomotion (Berwaerts and Van Dyck 2004), courtship behaviour (Geister and Fischer 2007), reproduction (Fischer et al. 2003) and rates of feeding and growth (Kingsolver and Woods 1997). Temperature is also a significant source of mortality in nature (Willmer et al. 2000) and therefore an important selective agent (Clarke 2003, Hoffmann et al. 2003). Consequently, temperature is considered to be one of the most important ecological factors for ectothermic organisms (Johnston and Bennett 1996, Angilletta and Dunham 2003, Clarke 2003, 2006, Sinclair et al. 2003).

In nature, most organisms face variable thermal environments, posing substantial challenges for key elements of fitness (Dahlhoff and Rank 2007). Hence, given the typically wide range of temperatures in space (along geographical ranges) and time (i.e. daily and seasonal cycles), organisms will have to adapt to such conditions or, if not, will risk extinction (Angilletta et al. 2002, Helmuth 2002, Dahlhoff and Rank 2007). Facing rapidly changing climatic conditions at the global scale (e.g. Parmesan et al. 1999, Hitch and Leberg 2007), the evolution of the thermal sensitivity of performance in ectotherms has become a major focus of research programs in evolutionary study (Angilletta et al. 2002). Such questions, i.e. how organisms adapt to complex and changing environments, lie at the very heart of ecology and evolutionary biology.

1.2. Adaptation to temperature – plastically and/or genetically?

To cope with environmental change, organisms need to adjust phenotypic values to environmental needs. Such an adjustment can be achieved on the one hand via phenotypic plasticity (i.e. direct environmental effects on the phenotype as an

adaptive strategy to cope with short-term environmental variation; Bradshaw 1965, Pigliucci 2001), or on the other hand via genetic differentiation (i.e. long-term genetic adaptation). Phenotypic plasticity refers to the phenomenon of a genotype producing different phenotypes in response to different environmental conditions. It is a ubiquitous aspect of organisms (Travis 1994, West-Eberhard 2003) and is a property that may be adaptive, maladaptive or neutral with regard to an individual's fitness. Thus, phenotypic plasticity is not necessarily an adaptation to variable environments, but may alternatively be merely a biochemical or physiological interaction of an organism with its environment (Bradshaw 1965). Long-term exposure to a homogeneous environment, in contrast, may lead to a fixation of alleles being favourable in that environment, while the same alleles may be disadvantageous in novel environments (Via and Hawthorne 2002). Thus, the genetic variation necessary to adapt to novel environments may be exhausted after long periods of evolution in a constant environment (Barrett and Bell 2006). However, although both sources of variation typically contribute jointly to adaptation, the relative importance of genetic adaptation versus phenotypic plasticity in shaping adaptive evolution is still a matter of a controversial discussion (e.g. Ayrinhac et al. 2004, Samietz et al. 2005). To understand adaptive aspects of the evolution of developmental plasticity, the relationship between environmental change and morphological or physiological plasticity, its functional significance and hence fitness implications, are crucial (Atkinson et al. 2006).

In this context, one of the most widespread patterns of phenotypic plasticity is the relationship between adult (final) size and environmental temperature. In most ectotherms, a higher temperature during development increases growth and development rates, but decreases adult size at maturity. This pattern, known as the temperature–size rule (TSR), has been observed in more than 80% of ectothermic species studied, and occurs in diverse organisms including animals, plants, protozoa and bacteria (Atkinson 1994; but see also Walters and Hassall 2006, Kingsolver et al. 2007). Such plasticity may be driven by several mechanisms: behavioral (e.g. food uptake; Arendt 1997) and/or physiological mechanisms (e.g. through changes in the efficiency of converting ingested food into body mass; see Arendt 1997, Van Doorslaer and Stoks 2005), but also mechanisms at the cellular or intracellular level

might be responsible (Partridge et al. 1994, Van der Have and de Jong 1996, Pörtner 2002, Blanckenhorn and Llaurens 2005, Walters and Hassall 2006).

However, despite much effort over recent years (e.g. Blanckenhorn 1997, Gotthard et al. 2000, Frazier et al. 2001, Atkinson et al. 2006, Cabanita and Atkinson 2006, Walters and Hassall 2006), the role of physiological constraints in causing the TSR is currently unclear. Although recent (theoretical) approaches seem to suggest that the TSR might be adaptive, it seems that body size per se is not the target of selection. As body size generally is one of the most significant features of organisms, which influences many ecological, physiological and life-history traits (Roff 1992, Stearns 1992, Blackburn and Gaston 2001, Chown and Klok 2003, Davidowitz et al. 2003, Teuschl et al. 2007), the TSR is called a puzzle for life historians (Berrigan and Charnov 1994).

In addition to disentangling the potential adaptive nature of phenotypic plasticity, estimating genetic variation in such plasticity, measured as genotype by environment interactions, and analysing geographical variation in fitness-relevant traits is crucial for understanding the mechanisms of adaptive evolution in relation to temperature. Because of a strong covariance between temperature and geographic clines, clinal variation along climatic gradients may indicate a possible contribution of directional selection to differences among populations (Bubliy and Loeschcke 2005). Thus, given the typically wide range of temperatures in space and time, organisms are expected to show both, plastic and genetic adaptations (e.g. along geographic clines) to different temperatures (Arnett and Gotelli 1999, Robinson and Partridge 2001, Chown and Klok 2003, Van Doorslaer and Stoks 2005).

Indeed, many species show genetically determined geographical variation in traits being under thermal selection (ranging from life-history, stress resistance and morphology through to behaviour), and such population-specific differences are thought to be the result of adaptive evolution (Hoffmann et al. 2002, Castañeda et al. 2005, Collinge et al. 2006, Sambucetti et al. 2006). However, although high altitudes and latitudes share similarly extreme environmental conditions, recent studies mainly investigated latitudinal patterns that are arguably related to changes in temperature or related factors (Addo-Bediako et al. 2000, Loeschcke et al. 2000, Schmidt et al.

2005, Van Doorslaer and Stoks 2005), while altitudinal patterns have been studied much less frequently (Bubliy and Loeschcke 2005, Sørensen et al. 2005).

Either way, if variation in physiological responses is found over short geographical distances such as altitudes, patterns strongly suggest adaptive evolution via directional thermal selection (Dahlgaard et al. 2001). However, while a contribution of directional selection to the differentiation among populations is often supposed (Chown and Klok 2003, Van Doorslaer and Stoks 2005), in the majority of cases the selective forces underpinning such variation and its associated consequences at the genetic level were not explicitly investigated. Only relatively few studies, based on allozymes or DNA sequences, have revealed associations between gene frequencies and clinal variation in environmental factors, such as temperature or salinity (reviewed in Eanes 1999, Watt 2000). As for several ectotherms correlations between allozyme variation and an array of fitness-related traits including morphological and physiological ones could be shown (e.g. Watt 1992, Neargarder et. al. 2003, McMillan et al. 2005, Dahlhoff and Rank 2007, Saastamoinen 2007), such studies are important for understanding the genetic basis of thermal selection.

1.3. Study organism – the butterfly *Lycaena tityrus*

Lycaena tityrus (Poda, 1761) is a widespread temperate zone butterfly, ranging from Western Europe to central Asia (Ebert and Rennwald 1991). The species is bivoltine with two discrete generations per year in most parts of its range, although populations with one or three generations per year occur (Ebert and Rennwald 1991, Tolman and Lewington 1998). Butterflies used in this study belonged to several lowland populations from Germany and to the alpine subspecies *Lycaena tityrus subalpinus* (Speyer, 1851), caught between 1350 and 2020m in the Italian and Austrian Alps. In these high-altitude regions, *Lycaena tityrus* is generally monovoltine with adults being on the wing from mid-July through late August (Scheuringer 1972). However, as shown by several rearing experiments, even those alpine populations are potentially multivoltine (Fischer and Fiedler 2000). Central European low-altitude populations, in contrast, are typically bivoltine.

Lycaena tityrus colonizes different types of unimproved grassland and wetland as well as natural grassland such as swampy clearings or mountainous canyons and ridges. Adult butterflies predominantly suck on composite plants (*Compositae*). This species exhibits a distinct sexual dichroism, with female wings showing more orange colouration compared to males. However, in the *Lycaena tityrus subalpinus* subspecies, this sexual dichroism is only weak, being much more pronounced in low-altitude populations. Larvae of the last brood enter diapause, overwintering half-grown in the third instar (Descimon 1980). Pupation occurs after completion of four larval instars. The principal larval host-plant is *Rumex acetosa* L., but some congeneric plant species such as *R. acetosella* L. and *R. scutatus* L. are utilised as well (SBN 1987, Ebert and Rennwald 1991, Tolman and Lewington 1998). Although this species is still relatively widespread in most parts of Europe, the intensification of agriculture caused a clear decline in population numbers associated with local and regional extinctions in most parts of its range. Thus, in many parts of Europe, this species is considered to be vulnerable (Van Swaay and Warren 1999).

1.4. Objective and key elements of this thesis

Using *Lycaena tityrus* as a model organism, this study focuses on the patterns, significance and mechanisms of thermal adaptation in ectotherms. As outlined above, understanding how organisms adapt to complex environments through plastic and/or genetic adjustment is crucial in the face of rapidly changing climatic conditions at the global scale (e.g. Parmesan et al. 1999, Hitch and Leberg 2007), and thus of special concern. Regarding the TSR both, adaptive and mechanistic models have been proposed to explain its prevalence, but a single general explanation remained elusive (Angilletta and Dunham 2003). Thus, there is a pressing need to better understand the effects of temperature on body size in ectotherms (Atkinson and Sibly 1997). Accordingly, chapter 5 of this thesis investigates some mechanisms potentially underlying the TSR, namely the effects temperature on life-history traits (e.g. larval time and body mass), behaviour (e.g. food intake), and physiology (e.g. body composition, growth rate, conversion efficiency) in the Copper butterfly *Lycaena tityrus*, in order to disentangle the mechanistic basis of plastic responses of body size to temperature:

1) What is the relative impact of behavioural and physiological mechanisms on the plastic increase in body size at cooler developmental temperatures?

Chapter 5

Next, clinal variation in fitness-related traits, which has become a key element in investigating adaptive evolution (Sambucetti et al. 2006), was examined. While most frequently only variation in life-history traits was analyzed, thermal performance (i.e. temperature stress resistance) and the expression of stress-inducible heat-shock proteins (HSPs) probably play a much more important ecological and evolutionary role in thermal adaptation (Sørensen et al. 2003), and may ultimately limit the distribution and abundance of organisms along steep ecological (e.g. thermal) gradients in nature (Dahlhoff et al. 2001). Therefore, chapters 6.1 and 6.2 focus on altitudinal patterns in traits potentially related to thermal performance, addressing the following questions:

2) Is there altitudinal variation in life-history traits and thermal stress resistance traits in *Lycaena tityrus,* and are these traits influenced by genotype x environment interactions?

Chapter 6.1

3) Does the expression of stress-inducible heat-shock proteins vary across populations from different altitudes and do they depend on ambient temperature?

Chapter 6.2

Finally, as the ability to adapt to different environments throughout a given species' range depends on the existence of variation in ecologically relevant genes (Veliz et al. 2004), and because identifying the precise molecular changes that contribute to adaptation remains a principal challenge (Hoekstra and Coyne 2007), this thesis also investigates the genetic differentiation across altitudes, potentially underlying variation in life-history and temperature stress resistance traits, thus trying to detect specific enzymes that are likely under thermal selection (chapters 7.1 and 7.2):

4) Is there a genetic differentiation between alpine and lowland populations of *Lycaena tityrus* **and if yes, is the pattern caused by specific enzymes?**

Chapter 7.1

5) Is there a direct link between specific (allo-)enzymes and variation in life history traits and temperature stress resistance in *Lycaena tityrus***?**

Chapter 7.2

2. Synopsis

2.1. The mechanistic basis of the temperature-size-rule

The temperature-size rule (Bergmann's rule extended to ectotherms: TSR), which states that body size increases at lower developmental temperatures, appears to be a near universal law for ectotherms (e.g. Atkinson 1994, French et al. 1998, Stelzer 2002, Angilletta and Dunham 2003, Cabanita and Atkinson 2006). As expected and as was previously found in *Lycaena tityrus* (Fischer and Fiedler 2000), this species conforms to this widespread pattern of phenotypic plasticity. However, although recent studies seem to suggest that the temperature-size rule might be adaptive, the underlying developmental mechanisms are still largely unknown (see above). Thus, by analyzing a broad spectrum of temperature effects on several life-history traits (e.g. larval time and body mass), behaviour (e.g. food intake), and physiology (e.g. body composition, growth rate, conversion efficiency), I tried to disentangle the mechanistic basis of the temperature-size rule.

Owing to higher growth rates, development time was much shorter at the higher compared to the lower rearing temperature (e.g. Atkinson 1994, Berrigan and Charnov 1994, Fischer and Fiedler 2000, 2001, 2002, Gibert and de Jong 2001, Bochdanovits and de Jong 2003, Fischer et al. 2003, Clarke and Fraser 2004). Rearing at different temperatures additionally affected body composition of adult butterflies (see also Hoffmann 1973, 1974, Woods et al. 2003). While water content was not influenced by temperature regime, fat, the most efficient and most commonly used energy source in insects, and protein, which serves multiple functions including a prominent role in reproduction and which is not readily available from the butterflies' adult diet (Fischer et al. 2004, Bauerfeind and Fischer 2005), increased in butterflies reared at the higher temperature (see also Fischer et al. 2003). These findings suggest an advantage of developing at higher temperatures, and caution against using measures of body mass as the sole indicator of condition or energy content (cf. Angilletta and Dunham 2003).

Caused by protandry selection (Fagerström and Wiklund 1982), males showed generally shorter development times than females with concomitantly higher growth rates. Females were larger than males in the adult stage (see also Fischer and Fiedler 2000, 2001, 2002), but not in preadult stages. This difference is caused by

males showing a higher mass loss during metamorphosis than females, which has been interpreted as a potential cost of the males' accelerated development (Fischer et al. 2004). Moreover, a higher protein content was found in females compared to males, which may reflect the females' higher need of protein for egg production. Increased fat reserves in males, in contrast, may serve as flight fuel during mate location (e.g. Zera et al. 1998).

In *Lycaena tityrus* a higher body mass at the lower temperature was proximately due to a higher mass increment, which was in turn caused by both behavioural and physiological mechanisms: a much-increased food intake and, despite a lower assimilation (AD, see Fig. 1a), a higher efficiency in converting ingested food into body matter at the lower temperature (ECD, see Fig. 1b). Similar patterns could be shown in *Drosophila melanogaster*, were individuals reared at a lower temperature used limited food more efficiently than those reared at a higher temperature (Neat et al. 1995, Robinson and Partridge 2001). The seeming discrepancy between assimilation and conversion efficiency is most likely related to higher metabolic losses at the higher temperature (Kingsolver and Woods 1997, Renault et al. 2002).
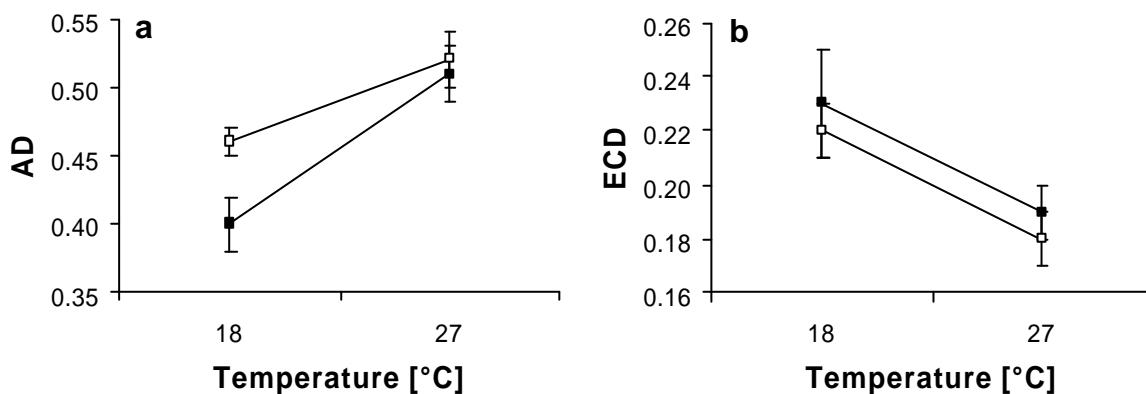
Fig. 1. Assimilation (AD; a) and efficiency of converting food into biomass (ECD; b) of *Lycaena tityrus* males (black symbols) and females (white symbols) reared at two different temperatures. Group means ± 1 SE are shown.

In contrast, sexual differences in body mass were caused by another mechanism. The males' higher growth rates are evidently facilitated by a higher daily food consumption, while total food consumption (due to the females' longer developmental period) and assimilation was higher in females. In contrast to temperature-induced

variation in body size, sexes did not differ in the efficiency of converting ingested food into body matter (for further details see also chapter 5).

The results obtained support the theoretical model developed by von Bertalanffy (1960) and Perrin (1995), assuming that the TSR arises as a consequence of differential effects of temperature on anabolism and catabolism. Growth efficiency (including anabolic and catabolic processes) should be negatively, but growth rate positively related to temperature (Angilletta and Dunham 2003). In other words, the temperature that maximizes growth efficiency is predicted to be lower than the temperature maximizing growth rate. Given the fact that a large body size is generally advantageous but that the costs of achieving large size increase with increasing temperature (due to a reduction in growth efficiency at higher temperatures), a negative relation based on diminishing returns should be optimal and thus adaptive.

2.2. Altitudinal variation in traits potentially related to thermal performance

2.2.1. Altitudinal variation in life-history traits and thermal stress resistance

Clinal variation in traits related to fitness suggests a contribution of directional selection, and analyzing such variation has consequently become a key element in investigating adaptive evolution. Presumably due to genetic differentiation, most life-history traits investigated in replicated populations of *Lycaena tityrus* from low-, (mid-) and high-altitudes, each reared at two different temperatures, showed variation across altitudes. High- compared to low-altitude populations showed a longer development time accompanied by reduced larval growth rates, increased cold- but decreased heat-stress resistance (see Fig. 2), and increased flight duration across a range of ambient temperatures. The increased development time for high-altitude butterflies contrasts with the general prediction of intrinsically higher growth rates and/or shorter development times at higher altitudes as an adaptation to the shorter season length (Atkinson 1994, Abrams et al. 1996). However, as high-altitude populations of this species are monovoltine and low-altitude ones are bivoltine

(Tolman and Lewington 1998), the pattern found here can be easily explained by this change in voltinism. It seems that the time stress imposed by fitting in an additional generation a year is more severe than the one imposed by the shorter growing season length in higher altitudes (Roff 1980).
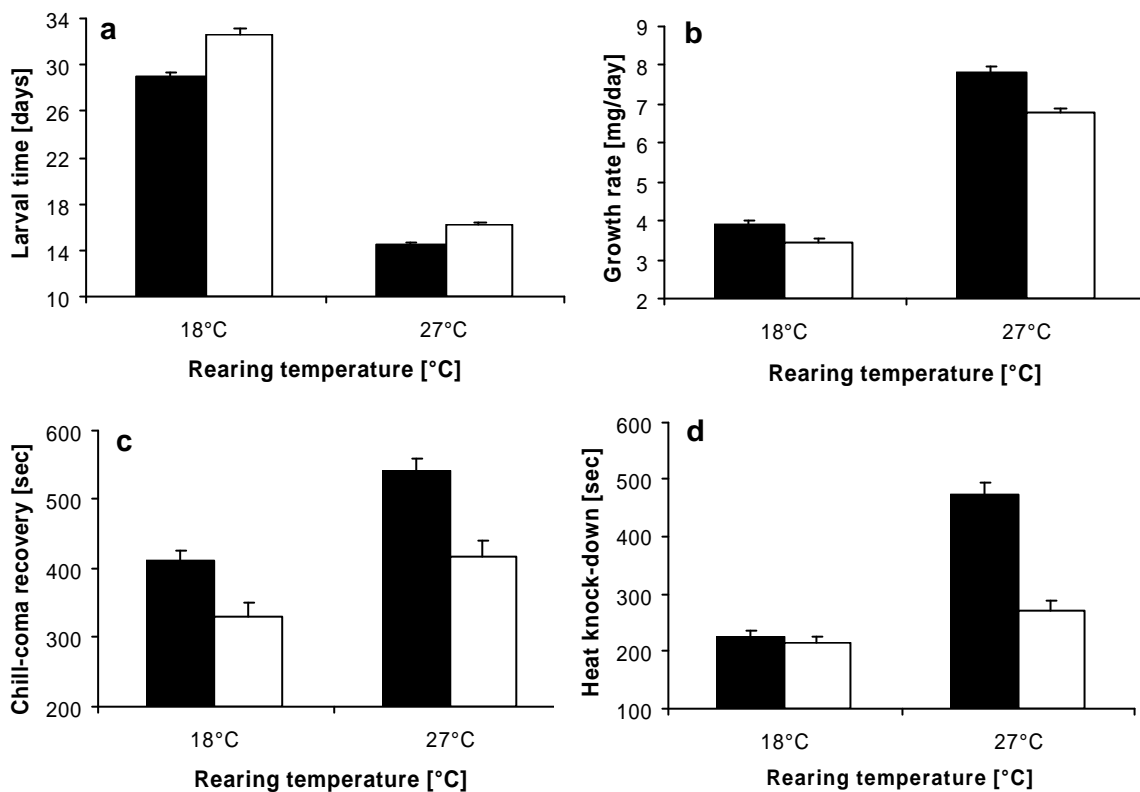
Fig. 2. Means (+ 1 SE) for larval time (a), larval growth rate (b), chill-coma recovery time (c) and heat knock-down time (d) for *Lycaena tityrus* from low- (black bars) and high-altitude populations (white bars) across two rearing temperatures (18°C and 27°C) Data were pooled across sexes and two replicates each.

Decreased chill-coma recovery times with increasing altitude refers to an enhanced cold stress resistance of higher-altitude animals which may facilitate earlier activity in the morning and later activity in the evening, allow for generally higher levels of activity under colder conditions due to a lower thermal threshold, and potentially enhance over-winter survival (Gibert et. al. 2001, Watt et al. 2003, Haag et al. 2005). Conversely, decreased heat knock-down resistance with increasing altitude in this species indicates a lower heat stress resistance of high-altitude animals, while butterflies from warmer habitats at low altitudes exhibited a much increased heat

tolerance. Similar patterns in knock-down resistance were for example also detected along a latitudinal cline for *Drosophila melanogaster* in Australia (Hoffmann et al. 2005). Thus, my results support the notion that chill-coma recovery and heat knock-down time are ecologically relevant traits reflecting adaptive variation (Sørensen et al. 2001; for review see Hoffmann et al. 2003). Adaptive variation across altitudes is also suggested by the results obtained from flight performance experiments, with high-altitude butterflies showing a better performance at lower temperatures as compared to low-altitude butterflies. The ability to fly even under sub-optimal conditions like low temperatures or strong winds is likely to be closely related to fitness in flying organisms such as butterflies (Barnes and Laurieahlberg 1986, Merckx et al. 2006), and should be particularly advantageous in mountainous areas (Norry et al. 2001, Hodkinson 2005).

In contrast, differences in morphological traits such as pupal mass, thorax mass, thorax/abdomen-ratio, wing length, wing area, wing loading or wing aspect ratio across altitudes across different altitudes were negligible. Recent studies show that the associations between temperature or environmental clines and body size are complex ranging from positive to negative (Chown and Klok 2003, Blanckenhorn and Demont 2004). The results shown here are likely attributable to differences in voltinism. They suggest that not only temperature regime but also its interactions with generation time, voltinism, and season length are likely to have strong impacts on insect body size (Roff 1980, Blanckenhorn 1997, Chown and Gaston 1999). Differences in flight performance across altitudes (see above) without morphological differentiation strongly suggest variation in physiological traits. Accordingly, a higher amount of fat stored in butterflies from low- as compared to higher-altitudes in *Lycaena tityrus* was found. However, as the reverse pattern with high-altitude butterflies showing an increased fat content as an adaptation to the harsher environmental conditions was expected, fat stores do not seem to play a decisive role for the differences in flight performance.

In addition to the population differences discussed above, plastic responses to different rearing temperatures resulted, as expected, in reduced larval and pupal development times at higher temperatures accompanied by higher growth rates. Interactions between temperature and altitude for larval and pupal time reflect some

marginal variation in the responses to temperature, with differences in larval time being less but differences in pupal time being more pronounced in low-altitude populations. However, based on their rather small size these effects are probably of marginal relevance only. Further, also as expected (cf. Fischer and Fiedler 2000), *Lycaena tityrus* conforms to the temperature-size rule: the lower developmental temperature caused a plastic increase in body size (e.g. Angilletta and Dunham 2003, Atkinson 1994; but see Kingsolver et al. 2007). Potential mechanisms resulting in this pattern may include an increase in food intake as well as an increase in the efficiency in converting ingested food into body matter, in combination with temperature-mediated changes in cell size and/or number (Partridge et al. 1994, Blanckenhorn and Llaurens 2005, Atkinson et al. 2006).

Thermal stress resistance was also influenced by prevailing environmental conditions. A lower developmental and early adult temperature caused shorter chill-coma recovery times (cf. Zeilstra and Fischer 2005), and a reduction in heat knock-down resistance. Similar results were obtained in studies on *Drosophila* (Chen and Walker 1994, Ayrinhac at al. 2004, Hoffmann et al. 2005). There was no evidence for an interaction between genotype (populations from different altitudes) and environment (rearing temperature) for chill-coma recovery, but for heat knock-down time. Butterflies from low-altitude populations showed a much more pronounced plastic response to temperature than high-altitude ones. The latter leaves substantial potential to quickly adjust heat stress resistance under heat spells for low-altitude butterflies, which is apparently not needed in populations from higher altitudes.

In summary, this study demonstrates local adaptations to regional climates, and that environmentally-induced plasticity can be as important as genetic factors in mediating adaptive responses. Consequently both sources of variation need to be considered when trying to predict responses to short- (such as particularly hot or cold days / nights) or long-term temperature variation (such as global warming). Exploring the limits within such mechanisms can help to buffer predictable changes in global temperatures remains an important task for future research (e.g. Van Doorslaer et al. 2007). Results are also given in more detail in chapter 6.1.

2.2.2. Altitudinal variation in the expression of heat-shock proteins

The expression of heat-shock proteins under thermal stress is an essential mechanism for ectotherms to cope with unfavourable conditions. Here, differences in HSP expression were investigated in Copper butterfly populations originating from different altitudes and / or being exposed to different rearing and induction temperatures. Although differentiation in HSP70 expression across altitudinal and latitudinal clines can be expected (Garbuz et al. 2003, Sørensen et al. 2005), there was no overall effect of altitude in the butterfly populations investigated. However, while high-altitude butterflies responded only marginally to differences in rearing temperature, HSP70 expression increased substantially at the higher compared to the lower rearing temperature in low-altitude butterflies, which might represent an adaptation to occasionally occurring heat spells (Fig. 3a). In this context it should be mentioned that the higher rearing temperature (27°C) used is relatively high for a temperate-zone butterfly, and that such conditions may amplify otherwise obscured phenotypic differences between genotypes (Hoffmann and Parsons 1991, Hoffmann and Merilä 1999, Blanckenhorn and Heyland 2004). As high-altitude butterflies showed only little plasticity in response to prevailing temperatures, they seem to rely more on genetically fixed stress resistance. Such reduced plasticity in high-altitude animals is likely related to a chronic exposure to thermal stress (cf. Sørensen et al. 1999, Lansing et al. 2000, Sørensen et al. 2001). Due to the lack of plasticity, high-altitude populations appear more vulnerable to rapid human-induced climatic change than low-altitude ones.
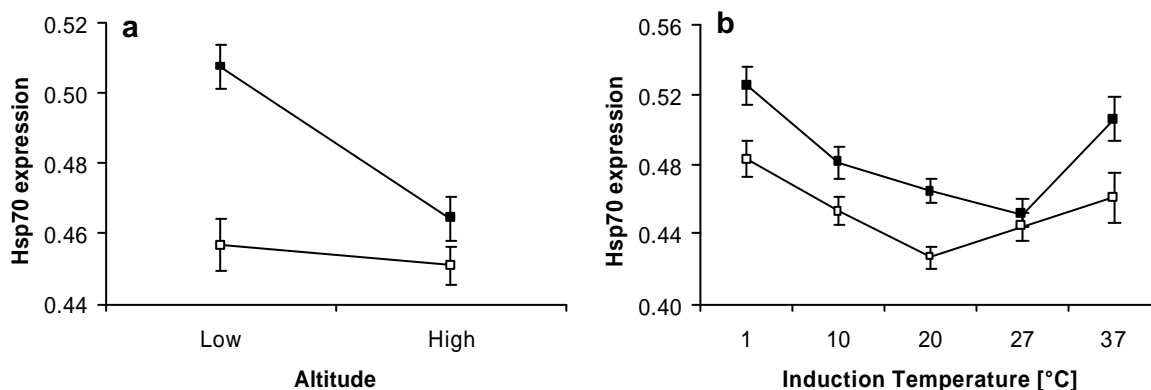

Fig. 3. Means (± 1 SE) of HSP70 expression for *Lycaena tityrus* across rearing temperatures (20°C: white symbols; 27°C: black symbols) and altitude (A) or induction temperature (B).

Generally, environmentally-induced plasticity (through different rearing or induction temperatures, see Fig. 3b) had a stronger effect on HSP70 expression than genetic factors (across populations). First, HSP70 expression in *Lycaena tityrus* was higher at the higher rearing temperature (indicating that a temperature of 27°C imposes mildly stressful conditions), similarly to an increase in HSP expression during warm seasons in other ectotherms (Fader et al. 1994, Roberts et al. 1997). Second, although often only high temperatures are used for HSP induction (e.g. Dahlgaard et al. 1998, Sørensen et al. 2001, 2005), HSP70 expression in *Lycaena tityrus* increased not only with warmer, but also with colder induction temperatures, like in other species were colder temperatures are also known to upregulate HSPs (Yocum 2001, Hoffmann et al. 2003, Michaud and Denlinger 2005). Finally, and most interestingly, in accordance to the beneficial acclimation hypothesis (Huey et al. 1999, Woods and Harrison 2002), lowest expression levels were found at the same temperature the respective individuals were reared at (i.e. for individuals reared at 27°C at an induction temperature of 27°C; and for individuals reared at 20°C at 20°C), thus indicating that a change in the thermal environment generally induces some stress (for results in more detail see also chapter 6.2).

Thus, as most of our knowledge on patterns of HSP expression stems from studies using *Drosophila* as a model organism, this is the first study on HSP70 expression in a Copper butterfly and laying the fundament within a comparative context, future investigations may deliver more insight in stress responses also in non-model organisms.

2.3. The genetic background of altitudinal variation in life-history and temperature stress resistance traits

2.3.1. Genetic differentiation between alpine and lowland populations

As for the Copper butterfly *Lycaena tityrus* altitudinal differences in life-history traits, flight performance, temperature stress resistance and the expression of stress-inducible heat-shock proteins could be demonstrated (see above and chapters 6.1

and 6.2), understanding the ecological process of population differentiation and identifying the molecular changes that contribute to adaptation is of special interest. By analyzing geographic variation in allozyme allele frequencies (based on 15 enzyme systems representing 18 loci) across 18 populations of the butterfly *Lycaena tityrus* from different altitudes, I tried to detect enzymes that are likely under natural selection.

In the analysed *Lycaena tityrus* populations, intrapopulational genetic diversity, namely the mean number of alleles per loci and the expected heterozygosity, was comparable to the values typically found in the Lepidoptera (Graur 1985). However, for lycaenids genetic diversity is known to be high (Marchi et al. 1996, Schmitt and Seitz 2001, Schmitt et al. 2002), and in particular the mean number of alleles per locus (1.74) is lower than in other common lycaenids (e.g. *Polyommatus icarus*: 2.98, Schmitt et al. 2003; *Polyommatus coridon*: 2.72, Schmitt et al. 2002). The populations of *Lycaena tityrus* investigated showed a remarkable genetic differentiation (F_{ST}: 0.065), being within the range of other strongly differentiated species like *Euphydryas gilettii* (Debinski 1994) or *Polyommatus coridon* (Schmitt and Seitz 2001). Populations were clearly separated into an alpine (high-altitude) and a non-alpine (low-altitude) cluster (Fig. 4).
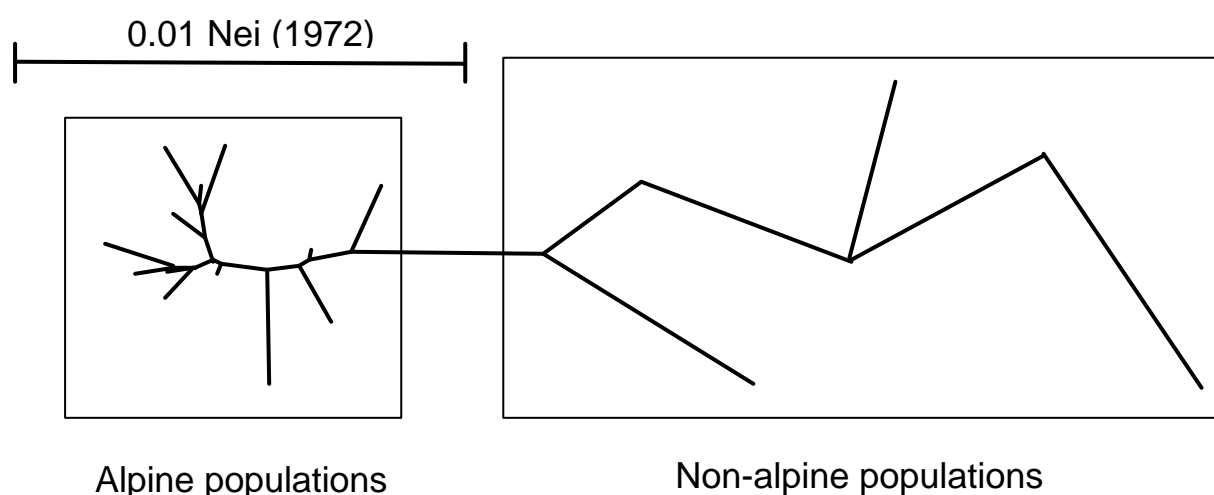

Fig. 4. Schematic neighbour joining dendrogram based on the genetic distances (Nei 1972) of 18 populations of *Lycaena tityrus*, revealing a clear distinction of populations into two main clusters, an alpine (high-altitude) and a non-alpine (low-altitude) one.

This geographic differentiation in the *Lycaena tityrus* populations analysed is primarily caused by variation at the PGI locus, an enzyme involved in important glycolytic pathways, being thus at the central point of all ATP-based energy supplies (Watt 1985). Based on the general assumption that low heterozygosity is associated with fitness costs (Ochando and Ayala 1999, Hotz and Semlitsch 2000), a positive relation between heterozygosity and altitude, with the harsher environmental conditions of higher altitudes warranting high individual fitness, was expected. However, results showed a negative rather than a positive correlation between heterozygosity and altitude, both, with regard to expected and observed heterozygosity. This pattern found in *Lycaena tityrus* seems to be related to variation at the PGI locus, with one homozygote genotype, PGI-2-2, dominating in all alpine populations, while low-altitude populations showed much more heterogeneous distributions with many heterozygotes (for further details see also chapter 7.1).

In summary, although the involvement of historical events cannot be ruled out, several lines of evidence strongly suggest that the specific pattern of allozyme (PGI) variation found in *Lycaena tityrus* is caused by thermal selection. First, effects of natural selection are generally locus-specific, whereas effects of migration, drift or inbreeding are expected to have relatively uniform effects across the entire genome (Storz and Nachmann 2003). The latter, however, is clearly not the case in *Lycaena tityrus*, as differentiation is mainly caused by variation only at the PGI locus. Second and more importantly, the PGI 2-2 genotype dominating in alpine (in contrast to lowland) populations is known to exhibit increased cold stress resistance and other features typical of alpine populations (see 2.3.2.). Thus, these findings suggest that PGI is an obvious target for thermal selection in *Lycaena tityrus* and probably a variety of other insects (e.g. Dahlhoff and Rank 2000).

2.3.2. Effects of genetic variation at the PGI locus on life history traits and temperature stress resistance

As evidence accumulated that the phosphoglucose isomerase (PGI) locus might be under thermal selection in the Copper butterfly *Lycaena tityrus* (see above), I investigated variation in life-history traits and temperature stress resistance across

PGI genotypes in *Lycaena tityrus* from different lowland populations reared at two temperatures (19°C and 24°C). As shown in chapter 2.3.1, PGI allele frequencies show altitudinal variation, with a single genotype occurring in ca. 90 % of all high-altitude animals. In low-altitude populations variation at this locus is much higher. Thus, if patterns caused by variation in PGI genotype are in broad agreement with those across high- and low-altitude populations, this strongly supports the notion that the PGI locus is involved in thermal adaptation (Neargarder et al. 2003, McMillan et al. 2005).

In *Lycaena tityrus*, seven different PGI genotypes could be detected. The two most common genotypes, PGI 1-2 and PGI 2-2 represent 79.1 % of all individuals. They were followed by PGI 2-3 (9 %), PGI 1-1 (6 %) and the very rare genotypes PGI 1-3 (4 %), PGI 1-4 (2 %), and PGI 3-3 (0.5 %). Because of the high variation in frequency, variation in life-history traits and stress resistance was investigated only across the four most common genotypes.

Concerning thermal stress resistance, most interestingly, the genotype dominating in high-altitude populations (PGI 2-2) exhibited the shortest chill-coma recovery times (see Fig. 5), consistent with an increased cold stress resistance in high-altitude *Lycaena tityrus* populations, suggesting that the PGI locus is indeed under thermal selection (see also Watt 1994, Dahlhoff and Rank 2000, McMillan et al. 2005).
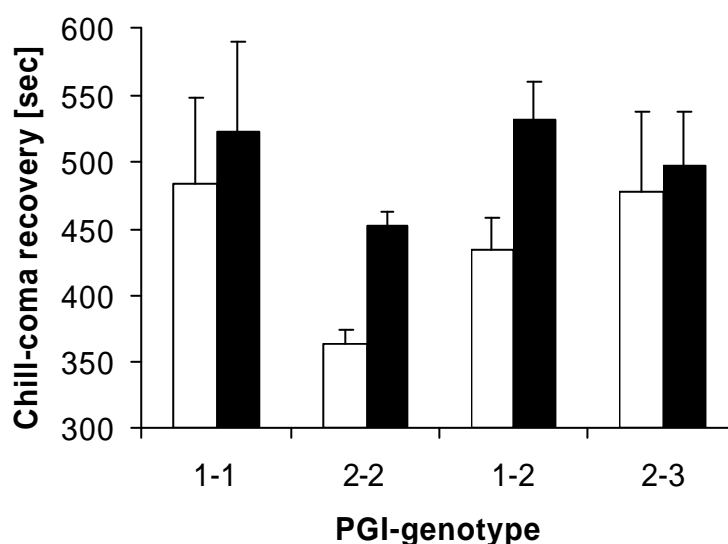

Fig. 5. Means (+1 SE) for chill-coma recovery time for *Lycaena tityrus* reared at 19°C (white symbols) and 24°C (black symbols) across four PGI genotypes. Data are pooled across sexes.

In contrast, despite variation in heat stress resistance across high- and low-altitude *Lycaena tityrus* populations, no significant variation across PGI genotypes in heat stress resistance was detected. This suggests on the one hand that heat and cold stress resistance are based on differential mechanisms, and on the other hand that PGI is not the only locus under thermal selection, but other yet unknown loci may also contribute to thermal adaptation, some of which may cause the reduced heat resistance in high-altitude populations (more details in chapter 7.2).

PGI genotype also affected larval and pupal development time, growth rate, and pupal mass in *Lycaena tityrus*. The 'high-altitude' genotype (PGI 2-2) showed intermediate to long development times in agreement with differences across populations. Growth rates in PGI 1-1 and PGI 2-3 butterflies, having comparatively low frequencies, were clearly higher than in PGI 1-2 and PGI 2-2 butterflies. It is a common belief that "faster is better" in ecology and evolutionary biology, and accumulating evidence suggests that growth rate in itself is a target of natural selection (Arendt 1997, Nylin and Gotthard 1998, Munch and Conover 2003). Thus, it is interesting to note that in *Lycaena tityrus* growth rates were highest for rare genotypes, which may suggest, that selection is not favoring genotypes promoting fastest development. In this context, another interesting pattern is that there was no trade-off between fast growth and body size across genotypes, i.e. slow-growing individuals did not become large, and fast-growing ones not small (cf. Blanckenhorn 1999, Davidowitz et al. 2004). For instance, PGI 2-3 individuals with the highest growth rates also showed the highest pupal masses, while PGI 1-2 butterflies with the lowest growth rates were smallest. It remains unclear though why the obviously highly efficient genotype PGI 2-3 is relatively rare (9 %) in nature. However, as the two most common genotypes were smaller, these findings suggest that the costs associated with achieving and / or maintaining large body size largely outweigh any potential benefit such as increased fecundity or mating success (Roff 1992, Blanckenhorn 2000).

Thus, because of the large variation in several life-history and stress resistance traits associated with variation at the PGI locus, PGI can be considered a pleiotropic gene of large effect (although genes linked to the PGI locus may also contribute to the variation found). Patterns caused by variation in PGI genotype are in broad

agreement with those across high- and low-altitude populations and support the notion that the PGI locus is heavily involved in thermal adaptation in arthropods (Neargarder et al. 2003, McMillan et al. 2005).

3. Summary (English and German)

3.1. Summary

Temperature is one of the most important ecological factors affecting biological organization directly and indirectly on nearly all spatial and temporal scales. As in nature organisms are often faced with variation in mean temperatures as well as in temperature extremes, they have to adapt plastically and/or genetically to their respective environmental conditions or will otherwise risk extinction. Using the Copper butterfly *Lycaena tityrus* as model organism, this study focuses on the patterns, significance and mechanisms of thermal adaptation in ectotherms on three main issues: (1) the mechanistic basis of the temperature-size rule (TSR), (2) altitudinal patterns potentially related to thermal performance and (3) the genetic background of such variation.

Following the TSR (being bigger at colder rearing temperatures) in *L. tityrus* is mainly caused by two different components: a behavioural and a physiological one. During the prolonged development at colder temperatures, larvae showed an increased food intake, a lower assimilation, but a higher efficiency in converting the ingested food into body matter (chapter 5). Sexual differences in body mass, however, were caused by another mechanism. The males' higher growth rates are evidently combined by a higher daily food consumption, while total food consumption and assimilation was higher in females. And, in contrast to temperature-induced variation in body size, sexes did not differ in the efficiency of converting ingested food into body matter.

In addition to such phenotypic patterns, a contribution of directional selection on traits related to fitness is inferred from clinal variation in such traits, and analyzing such variation has consequently become a key element in investigating adaptive evolution. In *L. tityrus*, altitudinal variation in life-history traits, temperature-stress resistance and flight performance (chapter 6.1), but also in the expression of heat-shock proteins (chapter 6.2), is present. While longer developmental times in high-altitude populations can be explained by a change in voltinism, reduced heat resistance and plasticity in the expression of heat-shock proteins, but increased cold resistance and flight duration across a range of ambient temperatures demonstrate local adaptations to regional climates. Furthermore, by rearing butterflies in both studies at different temperatures, environmentally-induced plasticity is demonstrated to be as important as genetic factors in mediating adaptive responses. Consequently both sources of variation need to be considered when trying to predict responses to short- (such as

particularly hot or cold days / nights) or long-term temperature variation (such as global warming).

Finally, this thesis also deals with answering the genetic background of such altitudinal variation. Butterflies from *L. tityrus* populations varying in altitude are clearly separated into an alpine (high-altitude) and a non-alpine (low-altitude) cluster (chapter 7.1). This geographic differentiation is primarily caused by variation at one single locus, the PGI locus, with one homozygote genotype, PGI-2-2, dominating in all alpine populations, while low-altitude populations show much more heterogeneous distributions with many heterozygotes. Interestingly, the genotype dominating in high-altitude populations (PGI 2-2) exhibited the shortest chill-coma recovery times compared to all other genotypes, and also shows intermediate to long development times, thus showing characters typical of high-altitude populations (chapter 7.2). These findings support the notion that the PGI locus is involved in thermal adaptation in *L. tityrus* and possibly other arthropods.

3.2. Zusammenfassung

Temperatur ist einer der wichtigsten ökologischen Faktoren, der sowohl direkt als auch indirekt die biologische Organisation auf beinahe allen räumlichen und zeitlichen Ebenen beeinflusst. Da in der Natur Organismen häufig mit Variation sowohl von Durchschnittstemperaturen aber auch mit Temperaturextremen konfrontiert werden, müssen sie sich plastisch und/oder genetisch den entsprechenden Umweltbedingen anpassen oder andernfalls ihr Aussterben riskieren. Die vorliegende Studie, in welcher der Feuerfalter *Lycaena tityrus* als Modelorganismus verwendet wurde, richtet ihr Augenmerk auf die Muster, die Signifikanz und die Mechanismen thermaler Anpassung, wobei insbesondere auf folgende drei Schwerpunkte eingegangen wird: (1) die mechanistischen Grundlagen der Temperatur-Größen-Regel, (2) höhenabhängige Variation in Eigenschaften, die potenziell mit thermaler Anpassung in Zusammenhang stehen und (3) den genetischen Grundlagen solcher Variation.

Bei *Lycaena tityrus* ist ein größeres Endgewicht bei kühleren Zuchttemperaturen (entsprechend der Temperatur-Größen-Regel) auf zwei unterschiedliche Faktoren, einem verhaltensgesteuerten und einem physiologischen, zurückzuführen. Während der verlängerten Entwicklungsdauer bei niedrigeren Temperaturen nehmen die Larven mehr Futter zu sich, während gleichzeitig, trotz geringerer Assimilation, eine erhöhte Effizienz in der Umwandlung aufgenommenen Futters in Körpermasse gezeigt wird (Kapitel 5). Unterschiede in der Körpergröße zwischen den Geschlechtern dagegen wird durch andere Mechanismen verursacht. Die höheren Wachstumsraten bei den Männchen sind offensichtlich mit einer höheren täglichen Futtermenge kombiniert, während die Gesamtfuttermenge und Assimilation bei den Weibchen höher ist. Und im Gegensatz zu der temperaturinduzierten Variation in Körpergröße unterscheiden sich die Geschlechter nicht in ihrer Effizienz in der Umwandlung aufgenommenen Futters in Körpermasse.

Zusätzlich zu solchen phänotypischen Mustern wird als Ursache klinaler Variation in fitnessbezogenen Merkmalen ein Einfluss von direkter Selektion vermutet, und in Folge dessen wurde die Analyse solcher Variationen zu einem Schlüsselelement in der Erforschung adaptiver Evolution. Bei *L. tityrus* sind altitudinale Variationen in Elementen der Lebensgeschichte, Temperaturresistenz und Flugleistung (Kapitel 6.1), aber auch in der Expression von Hitzeschockproteinen (Kapitel 6.2)

gegenwärtig. Während längere Entwicklungszeiten in Hochlagenpopulationen durch Unterschiede in der Anzahl der Generationen erklärt werden können, stellen eine verringerte Resistenz gegenüber Hitze und eine geringere Plastizität in der Expression von Hitzeschockproteinen, bei gleichzeitig erhöhter Resistenz gegen Kälte und höheren Flugleistungen bei unterschiedlichen Umgebungstemperaturen lokale Anpassungen an regionales Klima dar. Zudem konnte in beiden Studien durch Zucht der Schmetterlinge bei je zwei unterschiedlichen Temperaturen gezeigt werden, dass umweltbedingte Plastizität ebenso wichtig wie genetische Faktoren in der Vermittlung adaptiver Antworten ist. Daher müssen beide Möglichkeiten berücksichtigt werden wenn versucht werden soll, Reaktionen auf kurzzeitige (wie einzelne heiße oder kalte Tage/Nächte) oder längerfristige Variation in der Temperaturen (wie globale Erwärmung) vorherzusagen.

Schließlich befasst sich diese Studie auch mit der Analyse der genetischen Architektur solcher höhenabhängigen Variation. Populationen verschiedener Höhelagen zeigten eine klare Unterteilung in zwei Cluster, einem alpinen (Hochlagen) und einem außeralpinen (Tieflagen; Kapitel 7.1). Diese geographische Differenzierung ist im Wesentlichen durch Variation an einem einzigen Locus, dem PGI Locus, verursacht, wobei ein homozygoter Genotyp, der PGI 2-2 Genotyp, in allen alpinen Populationen vorherrscht, während in Tieflagenpopulationen eine viel heterogenere Verteilung bei gleichzeitig höherer Heterozygosität vorliegt. Interessanterweise weisen Individuen des in den Hochlagenpopulationen vorherrschenden PGI 2-2 Genotyps die kürzesten Erholungszeiten nach Kältestarre im Vergleich zu den anderen Genotypen auf, wie auch mittlere bis lange Entwicklungszeiten, welche für Hochlagen-Populationen typisch sind (Kapitel 7.2). Diese Ergebnisse unterstützen die Hypothese, dass der PGI Locus stark in die thermale Anpassung von *L. tityrus* wie auch möglicherweise anderer Arthropoden involviert ist.

4. References

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5. The mechanistic basis of the temperature-size-rule

Why get big in the cold? Towards a solution of a life-history puzzle

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Abstract

The temperature-size rule (TSR), which states that in body size increases at lower developmental temperatures, appears to be a near universal law for ectotherms. Although recent studies seem to suggest that the temperature-size rule might be adaptive, the underlying developmental mechanisms are thus far largely unknown. Here, we investigate temperature effects on life-history traits, behaviour and physiology in the copper butterfly *Lycaena tityrus* in order to disentangle the mechanistic basis of the above rule. In *Lycaena tityrus*, a larger body size produced at a lower temperature was proximately due to a higher increase in mass, which was caused by both behavioural and physiological mechanisms: a much-increased food intake and a higher efficiency in converting ingested food into body matter. These mechanisms, combined with temperature-induced changes at the cellular level, may provide general explanations for the TSR. Body fat and protein content increased in butterflies reared at the higher temperature, indicating favourable growth conditions. As predicted from protandry theory, males showed reduced development times, caused by higher growth rates compared to females. The latter was itself related to a higher daily food consumption, while the total food consumption (due to the females' longer developmental period) and assimilation was higher in females and may underly the sexual size dimorphism in body size.

Introduction

Temperature affects biological organization directly and indirectly on all spatial and temporal scales and levels, and is considered one of the most important ecological factors (Johnston and Bennett 1996, Angilletta and Dunham 2003, Clarke 2003, 2006, Sinclair et al. 2003). Consequently, the study of temperature effects has received considerable attention in recent decades. While there has been remarkable progress in some aspects of thermal biology (e.g. the role of heat shock proteins; see Yahara 1999, Sørensen et al. 2003), others remain poorly understood. In particular, the negative relationship between developmental temperature and adult size in ectotherms, often referred to as temperature-size rule (TSR), have remained enigmatic despite much effort over recent years (e.g. Blanckenhorn 1997, Partridge and Coyne 1997, Arnett and Gotelli 1999, Gotthard et al. 2000, Frazier et al. 2001,

Gibert and de Jong 2001, Angilletta et al. 2004, Weetman and Atkinson 2004, Atkinson et al. 2006, Cabanita and Atkinson 2006, Santos et al. 2006, Walters and Hassall 2006). Importantly, there is hardly any direct support for an adaptive explanation involving the demonstration of fitness advantages of being larger or maturing later at lower temperatures, and the underlying developmental mechanisms are largely unknown. Therefore, the role of physiological constraints is currently unclear, and consequently the TSR has been called a puzzle for life historians (Berrigan and Charnov 1994).

However, there is a pressing need to better understand the effects of temperature on body size in ectotherms (Atkinson and Sibly 1997). First, the TSR seems to be near universal across a wide variety of taxa: Atkinson (1994) showed that more than 80% of 109 studies on temperature effects in ectotherms observed an increase in body size with decreasing temperature (but see Walters and Hassall 2006, Kingsolver et al. 2007). Second, body size is one of the most significant features of organisms, which impacts on many ecological, physiological and life-history traits (Roff 1992, Stearns 1992, Blackburn and Gaston 2001, Chown and Klok 2003, Davidowitz et al. 2003, Teuschl et al. 2007).

Along with body size, growth rate is also strongly affected by temperature. It has long been known that lower temperatures generally reduce metabolic rates and thereby the rate of growth in ectotherms, which may impact on patterns in body size (Berrigan 1997, Yamahira and Conover 2002, Clarke and Fraser 2004, Van Doorslaer and Stoks 2005). However, knowing rates of growth and differentiation is not sufficient to fully understand variation in body size. Slow-growing individuals may become very large given enough time while fast-growing ones may be small if growth period is truncated (Blanckenhorn 1999, Davidowitz et al. 2004). Therefore, understanding body size variation requires also an understanding of the (endocrine) mechanisms regulating the termination of the growth period (Davidowitz et al. 2004). Further, plasticity in growth trajectories may be driven by behavioral (e.g. food uptake; Arendt 1997) and/or physiological mechanisms (e.g. through changes in the efficiency of converting ingested food into body mass; see Arendt 1997, Van Doorslaer and Stoks 2005).

Much of our current knowledge on the interrelations between temperature, feeding and growth comes from studies on fish (e.g. Elliot and Hurley 1997, 1999, 2000, Koskela et al. 1997, Jonsson et al. 2001, Angilletta and Dunham 2003). In the Arctic Charr, for instance, a plastic response to temperature was found with growth efficiency decreasing linearly with increasing temperature (Larsson and Berglund 2005; see also Present and Conover 1992 for the Atlantic silverside). Comparatively little, in contrast, is known on the temperature dependence of growth efficiency in arthropods (Robinson and Partridge 2001, Angilletta and Dunham 2003).

Both, adaptive and mechanistic models have been proposed to explain the TSR, but a single general explanation for the rule and its exceptions remains elusive (Angilletta and Dunham 2003). Recent (theoretical) approaches seem to suggest that the TSR might be adaptive, but that body size per se is not the target of selection. Rather, mechanisms at the cellular or intracellular level might be responsible. The models seem to agree that growth should become less efficient at higher temperatures, either as the result of oxygen limitation of thermal tolerance (Pörtner 2002), of differential temperature coefficients of growth versus differentiation (Van der Have and de Jong 1996, Walters and Hassall 2006), or of variation in cell size and/or cell number (Partridge et al. 1994, Blanckenhorn and Llaurens 2005; but see Atkinson et al. 2006), with smaller cells being more beneficial at higher temperature, while larger ones being favored in the cold (Kozlowski et al. 2004). A recent study by Davidowitz et al. (2004) suggests that plasticity of body size in relation to temperature is regulated by variation in growth rate and the time interval between the attainment of the critical weight (the size at which juvenile hormone synthesis ceases) and entry into the prepupal wandering stage in *Manduca sexta*.

A particularly appealing attempt to explain the TSR is the von Bertalanffy-Perrin model, as it provides a relatively simple yet potentially general explanation for temperature-body size relationships (von Bertalanffy 1960, Berrigan and Charnov 1994, Perrin 1995, Atkinson and Sibly 1997, Angilletta and Dunham 2003). Based on differential effects of temperature on anabolism and catabolism, this model predicts that organisms should grow larger in colder environments whenever growth efficiency decreases, while growth rate increases with increasing temperature (Angilletta and Dunham 2003). However, a recent review by Angilletta and Dunham (2003) revealed

that the required condition of reduced growth efficiency at higher temperatures was met in 6 out of 20 species only. Potential reasons for the lack of support for the model may include: (i) the species included in the review comprise exceptions from the TSR; or (ii) some of the underlying assumptions regarding thermal physiology are invalid for most ectotherms.

Against this background, here we investigate temperature effects on life-history traits (e.g. larval time and body mass), behaviour (e.g. food intake), and physiology (e.g. body composition, growth rate, conversion efficiency) in the Copper butterfly *Lycaena tityrus.* This approach enables us to test for the contributions of such factors in mediating plastic responses in body size, and thus to disentangle potential mechanisms underlying the TSR. As sexes commonly differ in growth trajectories, we also investigate the mechanisms underlying sexual dimorphisms in development time and body size.

Material and methods

Study organism

Lycaena tityrus (Poda, 1761) is a widespread temperate zone butterfly, ranging from Western Europe to central Asia (Ebert and Rennwald 1991). The species is bivoltine with two discrete generations per year in most parts of its range, although populations with one or three generations per year also occur (Ebert and Rennwald 1991, Tolman and Lewington 1998). *Lycaena tityrus* hibernates as half-grown larva. The principal larval host-plant is *Rumex acetosa* L., but some congeneric plant species such as *R. acetosella* L. and *R. scutatus* L. are utilised as well (SBN 1987, Ebert and Rennwald 1991, Tolman and Lewington 1998). The butterflies used in this study belong to a Bavarian, bivoltine lowland population. Nine freshly eclosed, mated females were caught in June 2006 in the field and transferred to Bayreuth University.

Experimental design

Oviposition and butterfly rearing

Captured females were kept in a climate chamber at 27°C and L18:D6 (24h light cycle). For oviposition they were placed individually in translucent plastic pots (1 L)

covered with gauze, and were provided with *R. acetosa* (oviposition substrate), fresh flowers (*Crepis sp., Achillea millefolium*, *Polygonum bistorta*, *Leucanthemum vulgare*) and a highly concentrated sucrose solution (for adult feeding). Eggs were collected each day, pooled across females and transferred to small glass vials. After hatching, larvae were randomly divided among two climate chambers differing in rearing temperature (set at 18°C and 27°C, respectively). Both climate chambers used are located within the same building next to each other. They are identical in terms of construction, lightning and air conditioning. Throughout, the photoperiod was set at L18:D6, relative humidity at 70 %. Larvae were placed individually in translucent plastic boxes (125 ml), containing moistened filter paper and fresh cuttings of *R. acetosa* in ample supply. Boxes were checked daily and supplied with new food when necessary until the beginning of data acquisition.

Data acquisition

Data acquisition started when larvae had reached the last (fourth) instar. After ecdysis larvae were weighed to the nearest 0.01 mg (Sartorius microscale MC 210 P) and transferred to a new, clean plastic box. Fresh food sufficient for > 24 h was weighed to the nearest 0.01 mg and added to the boxes. 24 ± 2 h later, the remaining food and the frass produced were removed, the larvae were weighed and they were then transferred to new boxes supplied with fresh, preweighed food. This procedure was repeated daily for each larva until pupation. To control for variation in water loss, dry mass of the remaining food and the frass was measured after drying samples for 24 h at 70°C to constant weight. The resulting dry masses were converted into fresh masses using calibration equations (dry against fresh food: $N = 45$, $r^2 = 0.89$, $t = 18.6$, $P < 0.0001$; dry against fresh frass: $N = 50$, $r^2 = 0.98$, $t = 48.3$, $P < 0.0001$). Calibrations are based on additional samples weighed before and after drying. The daily amount of food ingested was calculated as the difference between the fresh mass of food prior to feeding and the estimated fresh mass of the remnant.

Following eclosion, the sex of each butterfly was determined, after which they were frozen at -20°C for 30 min. Thereafter, heads, legs and wings were removed and body fresh mass was determined, while body dry mass was taken after drying the butterflies for 24 h at 70°C. Thus, we obtained for all individuals data on larval mass and mass increment, frass production and food ingestion on a daily basis throughout

the last larval instar, in which the bulk of the total weight gain is achieved. Additionally, duration of the last larval instar, pupal development time, prepupal mass, pupal mass, adult fresh and dry body mass, adult water content and adult relative fat content were recorded. The latter was determined as the mass difference between the adult dry mass and the remaining dry mass after two fat extractions. In each extraction, fat was extracted for 48 hours using 2 ml of dichloromethane (CH_2Cl_2) / methanol (CH_3OH) (2:1) solution for each butterfly (cf. Fischer et al. 2003). The above body mass data were used to compute the efficiency of mass conversion prior to and upon metamorphosis, using the following ratios: maximal larval mass to prepupal mass (MP), prepupal mass to pupal mass (PP), and pupal mass to adult fresh (PF) and dry mass (PD).

Additionally, 46 individuals (18°C: N = 20, 27°C: N = 26) were reared at both temperatures for measuring the protein content of adult butterflies. After eclosion, adult fresh mass was determined and total protein content was measured (EL 808 Ultra Microplate Reader Bio-Tek Instruments, Inc. Winooski, VT, USA) using the RotiQuant Universal assay (Roth, Karlsruhe, Germany) and bovine serum albumin as a standard (cf. Lorenz 2003).

Nutritional indices

The following indices were calculated in order to characterize growth patterns (1-4 modified after Zera et al. 1998; 5-6 according to Lindgren and Laurila 2005):

1. Growth increment (hereafter GI; in (mg) = maximum larval mass (note that mass drops considerably prior to pupation) in mg) – initial larval mass, i.e. at the beginning of last instar; in mg)

2. Consumption (CON; in mg) = fresh mass of food ingested during the feeding trial (here summed across the total feeding period; in mg)

3. Approximate digestibility (assimilation, AD) = [CON (in mg) – fresh mass of frass produced (in mg)] / CON (mg)

4. Efficiency of converting digested food into body matter (ECD) = GI (mg) / [CON (mg) – fresh mass of frass produced (mg)]

5. Mean daily growth rate (DGR; in mg / d) = GI (mg) / larval time (day)

6. Mean daily food consumption (DFC; in mg / day) = CON (mg) / larval time (day)

7.   Relative growth rate (RGR) = slope of regression of larval mass against time during the active feeding period.

Statistical analyses

All statistical tests were performed by using JMP (4.0.0) or Statistica 6.1. Life-history data were analysed using analyses of variance (ANOVAs) with temperature and sex as fixed effects. As suggested by Raubenheimer and Simpson (1992), the nutritional indices AD and ECD (see above) were analyzed by ANCOVA, including the numerator of the respective ratio as the independent variable and the denominator as the covariate. Further, all nutritional indices were analyzed by ANCOVAs with initial mass added as a covariate to control for differences in larval mass at the beginning of the last instar. To test for effects of ECD and CON on mass, data were additionally analysed by ANCOVAs with ECD, CON or both added as covariate. Variation in mass conversion upon metamorphosis was controlled for differences in body mass by using ANCOVAs with maximal larval mass (in MP), prepupal mass (in PP) and pupal mass (in PF and PD) added as covariates. Throughout, minimum adequate models were constructed by removing non-significant interaction terms. Pair-wise comparisons were performed employing Tukey's HSD. Calibration equations and correlations were computed by using Pearson's product moment correlations. Throughout the text only significant interactions terms are presented; all means are given ± 1 SE.

Results

Variation in life-history traits

The duration of the last larval instar varied significantly across temperatures ($F_{1,168} = 1325.3$, $P < 0.0001$) and sexes ($F_{1,168} = 75.3$, $P < 0.0001$). Development time was much reduced at the higher temperature, and was shorter in males compared to females (Table 1). The latter difference was less pronounced at 27°C than at 18°C (temperature by sex interaction: $F_{1,168} = 7.2$, $P = 0.0082$).

At the beginning of the last larval instar, larvae reared at 27°C were significantly heavier than those reared at 18°C ($F_{1,168} = 82.7$, $P < 0.0001$), and males were

Table 1. Life-history data (means ± 1 SE) for male and female *Lycaena tityrus* at two rearing temperatures. The last four lines represent the efficiency of mass conversion upon metamorphosis in per cent (with PM = prepupal mass / max. larval mass, PP = pupal mass / prepupal mass, PF (adult fresh mass / pupal mass) PD (adult dry mass / pupal mass).

Trait	18°C				27°C			
	Males	*N*	**Females**	*N*	**Males**	*N*	**Females**	*N*
Larval time [days]	8.5 ± 0.2	34	10.1 ± 0.2	43	3.8 ± 0.1	49	4.7 ± 0.1	45
Initial larval mass [mg]	34.2 ± 1.2	34	32.4 ± 1.0	43	59.9 ± 3.2	49	51.6 ± 2.8	45
Maximal larval mass [mg]	139.3 ± 1.5	34	137.7 ± 2.1	43	126.6 ± 1.5	49	124.8 ± 1.3	45
Final larval mass [mg]	128.0 ± 1.9	34	127.5 ± 2.2	43	120.7 ± 2.0	49	114.9 ± 1.4	45
Prepupal mass [mg]	120.9 ± 1.3	34	121.6 ± 2.1	43	110.4 ± 1.4	49	106.4 ± 1.1	45
Pupal mass [mg]	112.8 ± 1.2	34	111.2 ± 2.0	43	105.2 ± 1.5	49	101.6 ± 1.0	45
Adult fresh mass [mg]	35.8 ± 0.8	33	40.5 ± 1.6	43	31.8 ± 0.6	48	34.3 ± 0.7	45
Adult dry mass [mg]	9.1 ± 0.1	33	11.8 ± 0.3	41	8.5 ± 0.2	45	10.2 ± 0.2	43
PM [%]	86.9 ± 0.6	34	88.3 ± 0.6	43	87.3 ± 0.6	49	85.3 ± 0.6	45
PP [%]	93.4 ± 0.7	34	91.5 ± 0.4	43	95.3 ± 0.4	49	95.5 ± 0.2	45
PF [%]	31.90 ± 0.90	33	36.06 ± 0.92	43	30.42 ± 0.40	48	33.76 ± 0.55	45
PD [%]	8.12 ± 0.09	33	10.54 ± 0.15	41	8.18 ± 0.09	45	10.03 ± 0.16	43

significantly heavier than females ($F_{1,168}$ = 4.8, P = 0.0294; Table 12). In contrast to these initial patterns, individuals reared at the lower temperature reached significantly higher maximal larval masses ($F_{1,168}$ = 61.7, P < 0.0001), final larval masses (i.e. on the last day before becoming a prepupa; $F_{1,168}$ = 27.1, P < 0.0001), prepupal masses ($F_{1,168}$ = 69.3, P < 0.0001), pupal masses ($F_{1,168}$ = 33.8, P < 0.0001), adult fresh ($F_{1,168}$ = 25.1, P < 0.0001) and adult dry masses ($F_{1,168}$ = 22.6, P < 0.0001; Table 1, Fig. 1 a, b) than those reared at the higher temperature. ANCOVA analyses with larval time as covariate revealed qualitatively identical patterns, and confirm that mass differences cannot be explained by differences in development time (all P-values > 0.3294, except final larval masses: P = 0.0178).

Sexes did not differ significantly in maximal larval mass ($F_{1,168}$ = 1.1, P = 0.3055), final larval mass ($F_{1,168}$ = 3.2, P = 0.0758), prepupal ($F_{1,168}$ = 1.5, P = 0.2226) or pupal mass ($F_{1,168}$ = 3.3, P = 0.0717), though there were some tendencies towards males being heavier. Regarding adults, however, females showed significantly higher fresh ($F_{1,168}$ = 11.8, P = 0.0007) and dry masses ($F_{1,168}$ = 90.7, P < 0.0001) compared to males (Table 1). A significant temperature by sex interaction for adult dry mass ($F_{1,158}$ = 4.44, P = 0.0367) indicates that the sexual size dimorphism was less pronounced at the higher (females 16.4 % heavier than males) than at the lower temperature (females 22.4 % heavier).

The contrasting results regarding the sexual differences in pre-adult versus adult masses are obviously caused by sex-specific differences in the mass conversion across the metamorphic boundary. While there is no difference between the sexes in MP ($F_{1,166}$ = 0.5, P = 0.4935; maximal larval mass as covariate n.s.) and PP ($F_{1,166}$ = 4.4, P = 0.0671; prepupal mass n.s.), females showed a higher PF ($F_{1,164}$ = 31.5, P < 0.0001; pupal mass $F_{1,164}$ = 4.2, P = 0.0415) and PD ($F_{1,166}$ = 276.6, P < 0.0001; pupal mass $F_{1,157}$ = 6.9, P = 0.0093; Table 1). Significant temperature effects on conversion rates were only found for MP ($F_{1,166}$ = 6.6, P = 0.0109) and PP ($F_{1,166}$ = 28.2, P < 0.0001), with proportional mass loss being lower at the higher temperature in MP, but higher at the higher temperature in PP. A significant temperature by sex interaction in MP ($F_{1,166}$ = 7.0, P = 0.0089) indicates that conversion rates in females were higher at 18°C but lower at 27°C compared to males, while the opposite pattern was observed in PP (interaction term $F_{1,166}$ = 6.1, P = 0.0143). Another significant

Table 2. Results of two-way AN(C)OVAs for the effects of temperature and sex on growth increment (GI), daily growth rate (DGR), consumption (CON), daily food consumption (DFC), relative growth rate (RGR), assimilation (AD), and efficiency of converting food into biomass (ECD) in *Lycaena tityrus* (for definitions see text). Throughout, initial mass was added as covariate. For AD and ECD (ratios) additionally 'CON' and 'CON-frass' were added as covariates. Minimum adequate models were constructed by removing non-significant interaction terms. Significant *P*-values are given in bold.

Trait and Source	DF	MS	F	P
GI [mg]				
temperature	1,167	5459.3	48.9	**< 0.0001**
sex	1,167	80.5	0.7	0.3970
Initial mass	1,167	38285.6	343.2	**< 0.0001**
DGR [mg/day]				
temperature	1,167	1827.9	233.2	**< 0.0001**
sex	1,167	260.7	33.3	**< 0.0001**
Initial mass	1,167	811.6	103.5	**< 0.0001**
CON [mg]				
temperature	1,166	359569.6	15.1	**0.0002**
sex	1,166	338580.3	14.2	**0.0002**
Initial mass	1,166	1216602.6	50.9	**< 0.0001**
DFC [mg/day]				
temperature	1.166	210386.8	181.6	**< 0.0001**
sex	1,166	4677.9	4.0	**0.0461**
Initial mass	1,166	2449.9	2.1	0.1478
RGR				
temperature	1,167	6924.1	262.8	**< 0.0001**
sex	1,167	492.3	18.7	**< 0.0001**
Initial mass	1,167	1106.5	42.0	**< 0.0001**
AD				
temperature	1,165	94185.5	31.6	**< 0.0001**
sex	1,165	20088.5	6.7	**0.0103**
Initial mass	1,165	647163.3	216.9	**< 0.0001**
CON	1,165	2771528.2	928.9	**< 0.0001**
ECD				
temperature	1,165	171331.0	47.3	**< 0.0001**
sex	1,165	3445.2	1.0	0.3310
initial mass	1,165	1210968.3	334.1	**< 0.0001**
CON-frass	1,165	3367077.8	928.9	**< 0.0001**

temperature by sex interaction suggests that the sex difference in PD was smaller at 27°C than at 18°C ($F_{1,157}$ = 4.5, P = 0.0361).

Body composition

High and low temperature butterflies differed significantly in relative protein ($F_{1,168}$ = 9.6, P = 0.0035) and fat content ($F_{1,159}$ = 135.1, P < 0.0001), both of which increased at the higher rearing temperature, but did not differ in water content ($F_{1,159}$ = 1.8, P = 0.1724; Fig. 1 c-e). Sexes, though, differed significantly in protein ($F_{1,168}$ = 20.8, P < 0.0001), fat ($F_{1,159}$ = 52.0, P < 0.0001), and water content ($F_{1,159}$ = 44.5, P < 0.0001). Protein content was higher in females, while fat and water content were higher in males (Fig. 1 c-e). A significant temperature by sex interaction for protein content ($F_{1,168}$ = 7.9, P = 0.0074) indicates a less pronounced sex difference at 27°C compared to 18°C (protein content by 14.7 or 48.1 % higher in females).

Nutritional indices

Temperature significantly affected GI, DGR, CON, DFC, RGR, AD and ECD, while only DGR, CON, DFC, RGR and AD differed significantly across sexes (Table 2). Mass increment (GI), consumption (CON), and conversion efficiency (ECD) were significantly higher at 18°C compared to 27°C, while the opposite pattern was observed for growth rates (DGR, RGR), daily food consumption (DFC), and assimilation (AD; Fig. 1 f-l). Regarding sex differences, growth rates (DGR, RGR) and daily food consumption (DFC) were higher in males than in females, while the opposite was true for total consumption (CON) and assimilation (AD, Fig. 1). With the exception of DFC, all nutritional indices were significantly affected by the covariate initial mass (Table 2). Further, while growth efficiency (ECD) was not affected by the covariate CON, the term 'CON-frass' had a significant impact on assimilation (AD; Table 2; positive correlation between AD and 'CON-frass': r = 0.66, P < 0.0001). Finally, adding ECD as a covariate when analyzing variation in body masses revealed that ECD had a significant positive effect on maximal larval mass ($F_{1,166}$ = 9.0, P = 0.0031) and final larval mass ($F_{1,165}$ = 5.2, P = 0.0239), but not on PM, pupal mass, adult fresh and dry mass (all P > 0.0711). Adding CON as a covariate revealed significant effects on all body masses (all P < 0.0001), but only adding CON and ECD together as covariates removed the temperature effects on most measures of body size (all P > 0.0575), except for maximal larval mass ($F_{1,164}$ = 10.5, P = 0.0015) and
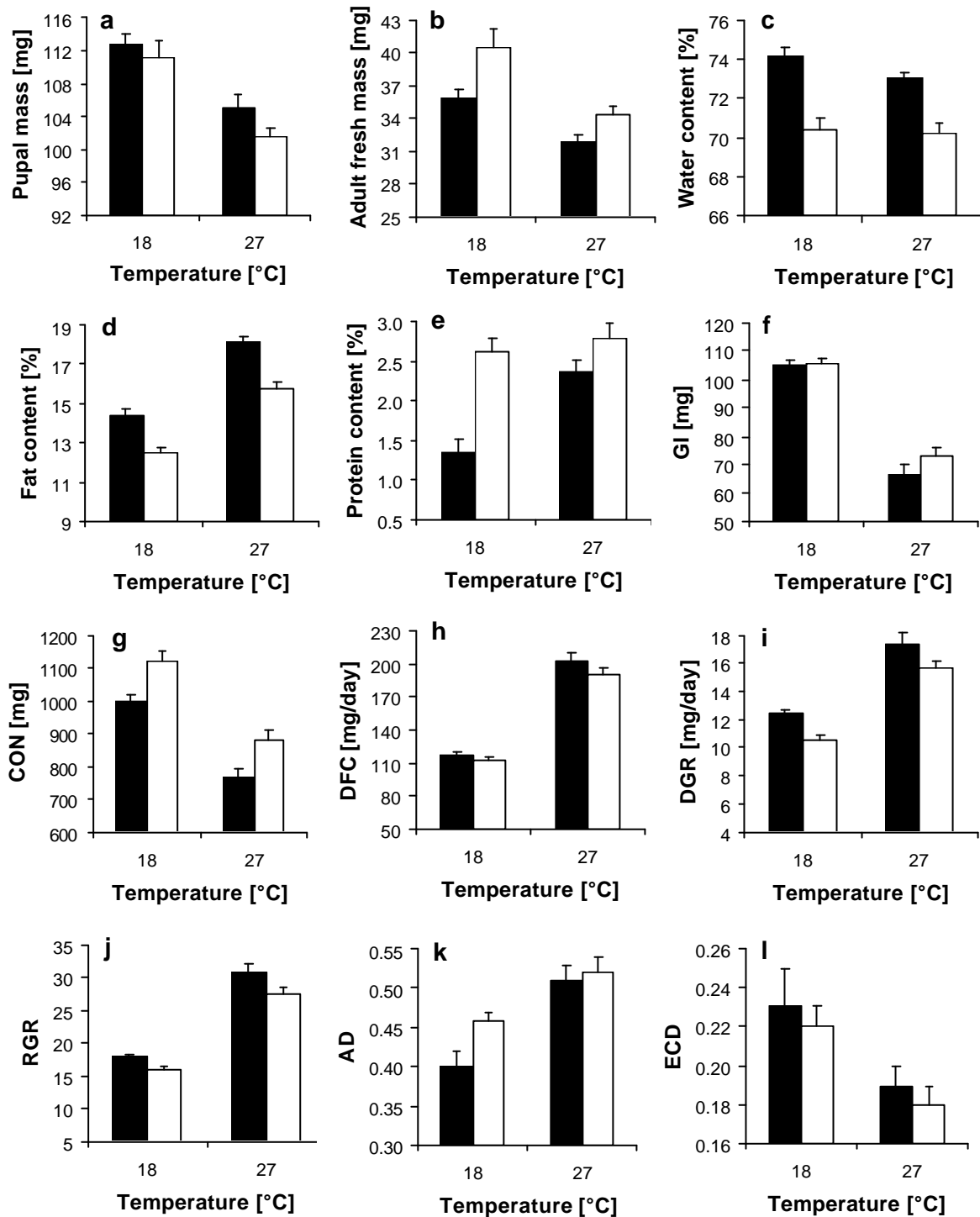
Fig. 1. Pupal mass (a), adult fresh mass (b), relative water (c), fat (d) and protein content (e), growth increment (GI; f), consumption (CON; g), daily food consumption (DFC; h), daily growth rate (DGR; i), relative growth rate (RGR; j), assimilation (AD; k) and efficiency of converting food into biomass (ECD; l) of *Lycaena tityrus* males (black bars) and females (white bars) reared at two different temperatures. Group means + 1 SE are shown. For definitions of nutritional indices see text.

prepupal mass ($F_{1,163}$ = 15.3, P = 0.0001). In these analyses, CON had quantitatively a larger impact than ECD throughout (being 0.3 times larger for maximal and final larval mass, 0.6 times larger for prepupal mass, 0.5 times larger for pupal mass, and 2-3 times larger for adult fresh and dry mass; as per effect sizes in ANCOVAs). Correlation analyses yielded qualitatively similar results (CON – body size: r_P = 0.363-0.531; ECD – body size: r_P = -0.011-0.318).

Discussion

Temperature and sex effects on life-history traits

As expected and as was previously found in *Lycaena tityrus* (Fischer and Fiedler 2000), this species conforms to the TSR (Bergmann's rule extended to ectotherms): lower developmental temperature caused a plastic increase in body size (e.g. Atkinson 1994, French et al. 1998, Stelzer 2002, Angilletta and Dunham 2003, Cabanita and Atkinson 2006; but see Kingsolver et al. 2007). The fact that larvae were actually heavier at the beginning of the last larval instar when reared at the higher temperature is considered an experimental artefact: at 27⁰C larvae molt and then grow quickly, and not all larvae could be weighed directly after ecdysis, thereby biasing body mass data towards higher values. Whether similar considerations also account for the sex difference at this stage or whether such patterns are caused by e.g. protandry selection, remains to be tested. Anyway, all later mass measurements showed the expected pattern of a larger body size at the lower temperature. The growth and feeding patterns underlying this rule, which are not merely byproducts of differences in development time (as per ANCOVA), will be analyzed in detail below.

Further as expected, larval development time was much shorter at the higher temperature and males showed generally shorter development times than females, both caused by higher growth rates at higher temperatures and in males, respectively (e.g. Atkinson 1994, Berrigan and Charnov 1994, Fischer and Fiedler 2000, 2001, 2002, Gibert and de Jong 2001, Fischer et al. 2003, Bochdanovits and de Jong 2003, Clarke and Fraser 2004). The latter is ultimately caused by protandry selection, thereby maximizing male mating opportunities (Fagerström and Wiklund 1982). The males' higher growth rates are evidently facilitated by a higher DFC, while total food

consumption (due to the females' longer developmental period) and assimilation was higher in females.

Other studies showed that males may take less time to grow to the same size as females (Gunnarsson and Johnsson 1990, Wiklund et al. 1991, Nylin 1992, Nylin et al. 1993). Such compensatory growth despite shorter development time was also observed here regarding preadult body mass, but not in adult body mass. Here, females were larger than males (see also Fischer and Fiedler 2000, 2001, 2002). The differential patterns in preadult and adult body size are caused by a higher mass loss in males during metamorphosis, which has been interpreted as a potential cost of the males' accelerated development (Fischer et al. 2004). In contrast to the sexual differences in conversion rates, temperature yielded only minor and inconsistent effects. While the temperature by sex interaction for development time basically reflects a smaller absolute sex difference at the higher temperature (see also Blanckenhorn et al. 2006), the one for adult dry mass suggests that the sexual size dimorphism is less pronounced at the higher temperature, challenging earlier results (Fischer and Fiedler 2000).

Temperature and sex effects on body composition
In *Lycaena tityrus*, rearing at different temperatures substantially affected body composition in adult butterflies (see also Hoffmann 1973, 1974, Woods et al. 2003). While water content was not influenced by temperature regime, fat and protein content increased in butterflies reared at the higher temperature (see also Fischer et al. 2003). Fat is the most efficient and most commonly used energy source in insects and is therefore indicative of condition (for example highly correlated with starvation resistance; Zwaan et al. 1991). Further, protein, which is not readily available from the butterflies' adult diet (Fischer et al. 2004, Bauerfeind and Fischer 2005), serves multiple functions including a prominent role in reproduction. The higher values found at 27°C suggests an advantage of developing at higher temperatures. These findings caution against using measures of body mass as the sole indicator of condition or energy content (cf. Angilletta and Dunham 2003). Regarding sex-related differences in body composition, water and fat content was higher, but protein content lower in males than in females. The higher protein content in females might be related to their high need for egg production, while enhanced fat reserves in males may serve as

flight fuel during mate location (e.g. Zera et al. 1998). Why males had a much lower protein content at 18°C compared to 27°C is unknown and difficult to interpret.

Getting big in the cold: how and why?

While the TSR as such is well documented, the mechanisms underlying this near-universal pattern as well as its potential adaptive significance are thus far largely unknown (e.g. Partridge and Coyne 1997, Arnett and Gotelli 1999, Gotthard et al. 2000, Frazier et al. 2001, Gibert and de Jong 2001, Angilletta et al. 2004, Weetman and Atkinson 2004, Atkinson et al. 2006, Cabanita and Atkinson 2006, Santos et al. 2006, Walters and Hassall 2006; but e.g. Moed et al. 1999). Here, we show that a larger body size at the lower temperature in *Lycaena tityrus* is proximately caused by a much higher weight gain within the last instar in larvae reared at the lower temperature (see also Kingsolver and Woods 1997, Petersen et al. 2000). Why this should be so, however, is not obvious upon first sight. Due to a detailed analysis of a variety of nutritional indices we can show that the increased weight gain is caused by contributions of different mechanisms, including a behavioral and a physiological component. The behavioural component involves a much increased food intake (i.e. consumption), the physiological one an increased efficiency in converting ingested food into body matter, despite a lower assimilation.

In line with results on the damselfly *Coenagrion hastulatum* (Van Doorslaer and Stoks 2005), we also found an increase in DFC at higher temperatures in order to maintain high growth rates (see also Kingsolver and Woods 1998 in a short-period experiment). Nevertheless, total food consumption (i.e. pooled across the last larval instar) was much higher at the lower temperature (cf. Neat et al. 1995, Lindgren and Laurila 2005), obviously caused by the prolonged development time. These results stress the notion that understanding variation in body size requires knowledge of both growth rates and development times. Interestingly though, assimilation was higher but growth efficiency lower at the higher temperature. This means that, on the one hand, a higher percentage of the food ingested was actually stored in the larvae's body, but that nevertheless a relatively smaller fraction of digested food could actually be converted into body matter. This seeming discrepancy is most likely related to higher metabolic losses at the higher temperature (Kingsolver and Woods 1997, Renault et al. 2002). Similarly, it could be shown in *Drosophila melanogaster*

that individuals reared at a lower temperature used limited food more efficiently than those reared at a higher temperature (Neat et al. 1995, Robinson and Partridge 2001). A causal relationship between growth efficiency / consumption and body mass was confirmed here by adding ECD and CON as covariates. Such factors fully explained the mass differences between temperatures, except for maximal larval mass and prepupal mass differences.

In a recent study it was shown that temperature-mediated changes in *Manduca* body size depend on temperature-sensitive endocrine control mechanisms (Davidowitz et al. 2004). Such processes regulate patterns of food intake and the timing of metamorphosis. We here investigated how such putatively hormonally-controlled differences in food intake (and growth efficiency) may directly affect differences in body size across temperatures. Interestingly, Davidowitz et al. (2004) found evidence that at 20°C fewer degree-days were required for development as compared to 25 and 30°C. The as-yet unknown mechanism behind this phenomenon may well be found in the differences in growth efficiency (efficiency in converting ingested food into body matter) reported here.

Our results do support the theoretical model developed by von Bertalanffy (1960) and Perrin (1995), assuming that the TSR arises as a consequence of differential effects of temperature on anabolism and catabolism (specifically, the Q_{10} of catabolism must be greater than the Q_{10} of anabolism). While those effects are difficult to measure directly, testable predictions can be derived, namely that growth efficiency (including anabolic and catabolic processes) should be negatively, but growth rate positively related to temperature (Angilletta and Dunham 2003). In other words, the temperature that maximizes growth efficiency is predicted to be lower than the temperature maximizing growth rate. Evidently, this is indeed the case in *Lycaena tityrus*. Unfortunately, however, a recent review failed to reveal conclusive support for the von Bertalanffy-Perrin model, as it only applied to a minority of the ectotherms studied so far (Angilletta and Dunham 2003).

These findings are currently based on a rather limited number of species, with a quite severe bias towards aquatic animals, mainly fish (Angilletta and Dunham 2003). Further, it is not actually known for most species included in the review whether they

actually follow the TSR or not (Angilletta and Dunham 2003). Therefore, it still seems possible that, at least for terrestrial arthropods, the von Bertalanffy-Perrin model does provide a suitable explanatory framework (see also Neat et al. 1995, Robinson and Partridge 2001). However, there are alternative explanations for the lack of support, for instance that the model may include some critical assumptions about thermal physiology that are invalid for most ectotherms, including neglect of acclimation through which growth efficiency may be adjusted to environmental needs (Angilletta and Dunham 2003). Atkinson (1997) proposed that the TSR could arise from a constraint on growth that arises late in ontogeny, i.e. from a decrease in the thermal optimum for growth efficiency with increasing body size. This may cause a smaller body size at higher temperatures independent of the Bertalanffy-Perrin model, and there is indeed some support in favour of this notion (Angilletta and Dunham 2003).

Another, yet closely related issue is whether the reaction norm represented by the TSR (i.e. is a negative relationship between temperature and body size) might be favoured by natural selection. A strong point of the von Bertalanffy-Perrin model, especially as it seems to apply to *Lycaena tityrus*, is that it provides not only a proximate mechanism, but also an adaptive explanation for the TSR. Given that a large body size is generally advantageous but that the costs of achieving large size increase with increasing temperature (due to a reduction in growth efficiency at higher temperatures), a negative relation based on diminishing returns should be optimal and thus adaptive.

Additional support for the notion that the TSR might, at least in some cases (and in particular in *Lycaena* butterflies), be adaptive stems from the observation of genetic variation in temperature reaction norms (Fischer and Fiedler 2000, 2001, 2002, Kingsolver et al. 2007), suggesting that such variation has been shaped by natural selection. In *Lycaena* butterflies, for instance, temperature reaction norms for body size differ across sexes and populations, being steeper in males than in females and in multi- compared to monovoltine populations (Fischer and Fiedler 2000, 2001, 2002). Both phenomena can be readily interpreted within an adaptive framework. The latter difference, for example, has been interpreted as resulting from multivoltine populations with short generation times gaining high compound interest benefits from reproducing early at high temperatures, indicating potential for extra generations,

even at the expense of being smaller. This should not apply for obligatorily monovoltine populations, showing shallow reaction norms (Fischer and Fiedler 2002). Thus, the compound interest hypothesis may also yield an adaptive explanation for the relationship between temperature and insect size at maturity, but has been poorly investigated thus far.

In summary, we here demonstrate that the butterfly *Lycaena tityrus* conforms to the TSR by getting bigger at lower temperatures. The higher mass increment in the last larval instar at the lower temperature was related to a higher food intake and an increased efficiency in converting ingested food into body matter. These findings do support the von Bertalanffy-Perrin model, but as yet it is rather unclear whether this comprises a rule or exception. In any case, such processes, in combination with temperature-mediated changes in cell size and number (Partridge et al. 1994, Blanckenhorn and Llaurens 2005, Atkinson et al. 2006), may explain the often reported increase in body size at lower developmental temperatures in at least some species, including *Drosophila*. However, based on the contradicting evidence summarized above, it seems most likely that the TSR may arise for different proximate as well as ultimate reasons in different organisms. It has long been known in life history studies that the same pattern of variation can be generated via different mechanisms (Partridge and French 1996). We suggest that our understanding of the factors underlying the TSR will greatly improve by performing detailed comparative case studies spanning a wide variety of taxa. The outcome may well be that there is no general explanation for the TSR, neither mechanistically nor ultimately. Clearly, much more empirical data is needed before any general conclusions can be drawn, and consequently this life-history puzzle will be solved.

Acknowledgements

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6. Altitudinal patterns in traits potentially related to thermal performance

6.1. Altitudinal life-history variation and thermal adaptation in the copper butterfly *Lycaena tityrus*

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Abstract

Understanding how organisms adapt to complex environments lies at the very heart of ecology and evolutionary biology. Clinal variation in traits related to fitness suggests a contribution of directional selection, and analyzing such variation has consequently become a key element in investigating adaptive evolution. In this study we examine climatic adaptation in the temperate-zone butterfly *Lycaena tityrus* across replicated populations from low-, (mid-) and high-altitudes, each reared at two different temperatures. In common garden experiments, high- compared to low-altitude populations showed a longer development time accompanied by reduced larval growth rates, increased cold- but decreased heat-stress resistance, and increased flight duration across a range of ambient temperatures. In contrast, differences in morphological traits such as pupal mass or wing size were negligible, suggesting that morphology is not necessarily indicative of flight performance. While patterns in stress resistance traits suggest adaptation to local temperatures, development times between populations were associated with differences in season length (enabling a second generation at lower altitudes, while high-altitude populations are monovoltine) rather than with temperature *per se*. Mid-altitude populations showed either intermediate patterns or patterns resembling low-altitude populations. Plastic responses to different rearing temperatures resulted, as expected, in reduced larval and pupal development times at higher temperatures accompanied by higher growth rates and decreased pupal mass. Further, butterflies reared at a lower temperature showed reduced chill-coma recovery times and decreased heat knock-down resistance as compared to those reared at a higher temperature. In summary, this study demonstrates local adaptations to regional climates, and that environmentally-induced plasticity can be as important as genetic factors in mediating adaptive responses.

Introduction

Facing a more or less wide range of environmental conditions through space and time, all organisms rely on the capability to adjust the expression of phenotypic values to environmental needs. Adjustment of phenotypic expression can be achieved via genetic differentiation (i.e. long-term genetic adaptation) or phenotypic plasticity (i.e. direct environmental effects on the phenotype as an adaptive strategy

to cope with short-term environmental variation; Bradshaw 1965, Pigliucci 2001). The latter is not necessarily an adaptation to variable environments, but may alternatively be merely a biochemical or physiological interaction of an organism with its environment (Bradshaw 1965). The question of how organisms adapt to complex environments (both genetically and plastically) lies at the very heart of ecology and evolutionary biology, and is of special concern in the face of rapidly changing climatic conditions at the global scale (e.g. Parmesan et al. 1999, Hitch and Leberg 2007).

Many species show genetically determined geographical variation in traits related to fitness (such as life history, stress resistance or behavioural traits). Such population-specific differences are thought to be the result of adaptive evolution (Hoffmann et al. 2002, Castañeda et al. 2005, Hoffmann et al. 2005, Collinge et al. 2006, Sambucetti et al. 2006). Particularly strong support for this notion comes from clinal variation, suggesting a contribution of directional selection to the differentiation among populations (Arnett and Gotelli 1999, Robinson and Partridge 2001, Chown and Klok 2003, Van Doorslaer and Stoks 2005). Consequently, analyzing clinal patterns in fitness-related traits has become a key element in investigating adaptive evolution (Bubliy and Loeschcke 2005, Sambucetti et al. 2006). Additionally, pronounced environmentally-induced variation in life-history traits is almost ubiquitous (Fischer and Fiedler 2000, Angilletta and Dunham 2003, Cabanita and Atkinson 2006). Though both sources of variation typically contribute jointly to adaptation, the relative importance of genetic adaptation versus phenotypic plasticity in shaping adaptive evolution is still a matter of a controversial discussion (e.g. Ayrinhac et al. 2004, Samietz et al. 2005).

To study climatic adaptation, geographic gradients along which climate strongly varies are of particular interest. Although several environmental factors may impact on the physiology of individuals and do vary geographically, temperature is thought to be one of the most important selective agents (Loeschcke et al. 2000). This is because of the strong covariance between temperature and geographic clines, and the fact that temperature is a key environmental factor for basically all terrestrial ectotherms, influencing virtually all aspects of their ecology and evolution (e.g. Hoffmann et al. 2003, Sinclair et al. 2003). Given the typically wide range of temperatures in space (along geographical ranges) and time (i.e. daily and seasonal

cycles) organisms are faced with, they are consequently expected to adapt genetically and/or plastically to different temperatures.

Studies on geographic gradients revealed some clear latitudinal patterns that are arguably related to changes in temperature or related factors, such as increased body size (e.g. Arnett and Gotelli 1999, Chown and Klok 2003), reduced developmental time (e.g. Robinson and Partridge 2001) and increased growth rates (e.g. Van Doorslaer and Stoks 2005) with increasing latitude. Despite much effort in investigating latitudinal variation particularly in insects, altitudinal patterns have been studied much less frequently, although high altitudes and latitudes share similarly extreme environmental conditions (Berner et al. 2004, Bubliy and Loeschcke 2005, Sørensen et al. 2005). This might be because altitudinal clines involve relatively short distances and thus represent very steep ecological gradients, with gene flow likely being important. Thus, for any differentiation being detectable one has to assume strong directional selection.

In this study, we investigate climatic adaptation of replicated populations in the temperate-zone butterfly *Lycaena tityrus* from low-, mid- and high-altitudes. We ran two similar experiments, one with populations from 2 altitudes reared at 2 temperatures, and one with populations from 3 altitudes reared at a single temperature. We focus on a variety of traits likely to be under thermal selection, ranging from life-history, stress resistance, morphology through to behaviour. For investigating thermal stress resistance, chill-coma recovery (i.e. the time until regaining mobility following chill-coma) and heat knock-down assays (i.e. the time until being knocked down when exposed to high temperatures) are used. Both indices are considered to be reliable proxies of climatic cold and heat adaptation, respectively (e.g. Hoffmann et. al. 2002, Ayrinhac et al. 2004, Castañeda et al. 2005, Sørensen et al. 2005). As actively flying organisms such as butterflies benefit from being able to track resources and escape from predators, and because butterflies initiate flight voluntarily only if body temperature is high (> 30°C, Shreeve 1984), we also investigated flight performance under suboptimal temperature conditions. The ability to fly even under such conditions should be highly relevant in terms of fitness (Barnes and Laurieahlberg 1986, Merckx et al. 2006). Presumed differences in flight

performance across populations may also be detectable at the morphological level (Berwaerts and van Dyck 2004), which is consequently also considered.

Based on the cooler and probably also less predictable environmental conditions found at higher altitudes, we predict climatic cold adaptation to result in shorter development times, increased growth rate, larger body size in combination with higher amounts of storage reserves, quicker recovery after a chill-coma but sooner knock-down under heat stress, better flight performance (at least at low temperatures) and possibly a lower wing loading, a higher wing aspect ratio and a higher investment into flight muscles facilitating better flight performance in high altitude animals (Dahlgaard et al. 2001, Norry et al. 2001, Berwaerts et al. 2002). By rearing the populations used at two different temperatures, we were also able to investigate plastic responses to temperature. This combination of approaches allows us to draw upon the following general questions: (1) Does climatic variation along an altitudinal gradient cause genetic differentiation in traits likely to be under thermal selection? (2) What is the relative importance of genetic as opposed to environmental factors in shaping adaptive reaction norms? (3) Do genotype and environment (rearing temperature) interact for certain traits? (4) How do our results on altitudinal variation relate to known latitudinal patterns?

Material and methods

Study organism and egg sampling

Lycaena tityrus (Poda, 1761) is a widespread temperate-zone butterfly, ranging from Western Europe to central Asia (Ebert and Rennwald 1991). The species is bivoltine with two discrete generations per year in most parts of its range, although populations with one or three generations per year occur (Ebert and Rennwald 1991, Tolman and Lewington 1998). The principal larval host-plant is *Rumex acetosa* L., but some congeneric plant species such as *R. acetosella* L. and *R. scutatus* L. are utilised as well (Ebert and Rennwald 1991, Tolman and Lewington 1998).

Mated females from replicated low-, mid- and high-altitude *Lycaena tityrus* populations (see further below) were captured in 2005 (*experiment 1*) and 2006 (*experiment 2*) in Rhineland-Palatinate (Germany), in Bavaria (Germany), and at

different localities in the Austrian and Italian Alps, respectively, and were transferred to Bayreuth University for egg laying. Butterflies were kept in a climate chamber at 27°C, high humidity (ca. 70%), and Light 18h: Dark 6h (24 h light cycles). For oviposition, females were placed individually in translucent plastic pots (1 L) covered with gauze, and were provided with *R. acetosa* (oviposition substrate), fresh flowers (*Crepis spec.*, *Achillea millefolium*, *Polygonum bistorta*, *Leucanthemum vulgare*) and a highly concentrated sucrose solution (for adult feeding). Eggs were collected daily, then pooled across females and kept, separated by population, in small glass vials until hatching. Two experiments were carried out as detailed below.

Experimental design

Experiment 1

In the first experiment four populations of *Lycaena tityrus* were used, with two replicates originating from lowland populations [Bavaria I, Germany: 450 above see level, a.s.l. (49° 53' N, 11° 37' E); Bavaria II, Germany: 500 a.s.l. (49° 57' N, 11° 46' E)] and two from highland populations [South Tyrol, Italy: 2010 a.s.l. (46° 43' N, 10° 52' E); Tyrol, Austria: 2050 a.s.l. (46° 52' N, 11° 01' E)]. Caught females were allowed to deposit eggs as described above. After hatching, larvae were randomly divided among two rearing temperatures (18°C and 27°C, L18:D6 throughout). They were placed individually in translucent plastic boxes (125 ml), containing moistened filter paper and fresh cuttings of *R. acetosa* in ample supply. Boxes were checked daily and supplied with new food when necessary. For all larvae development time (from hatching to pupation), pupal mass (to the nearest 0.01 mg; Sartorius microscale MC 210 P), pupal development time and growth rate (calculated as the ratio of pupal mass and larval developmental time) were recorded.

Following adult eclosion, butterflies were kept individually in translucent plastic pots (1 L) covered with gauze at their respective rearing temperature, and were provided with fresh flowers (*Crepis spec.*, *Achillea millefolium*, *Polygonum bistorta*, *Leucanthemum vulgare*) and a highly concentrated sucrose solution. On day two after eclosion, chill-coma recovery times were recorded. For testing, the butterflies from the different temperature groups and populations were placed individually in small translucent plastic cups (125 ml), which were arranged on a tray in a randomized block design. The tray was then exposed for 6 min to -20°C. Six minutes

were selected because preliminary studies showed that longer cold exposure induced significant mortality, and shorter exposure lead to very quick recovery. The trays were then transferred to an environmental cabinet with a constant temperature of 20°C. Recovery time was defined as the time elapsed between taking the tray out of the freezer until a butterfly was able to stand on its legs.

After having recovered from the cold (mortality: 15 out of 419 individuals = 3.6 %), butterflies were transferred back to their respective rearing temperature. The following day heat stress resistance was determined by using a knock-down assay (mortality between assays was random across populations). Butterflies were placed in small, sealed glass vials, which were submerged in a water bath kept at a constant temperature of 47°C, again in a randomized block design. Butterflies were continuously monitored and heat knock-down time (defined as the time until a butterfly was no longer able to stand upright) for each individual was recorded.

Experiment 2
Experiment 2 used two low- [Bavaria, Germany: 600 a.s.l. (47° 42' N, 11° 24' E); Rhineland-Palatinate, Germany: 250 a.s.l., (50° 30' N, 7° 58' E)]; two mid- [Tyrol, Austria: 1500 a.s.l. (47° 13' N, 10° 56' E); South Tyrol, Italy: 1350 a.s.l., (46° 58' N, 11° 20' E)], and two high-altitude *Lycaena tityrus* populations[South Tyrol, Italy: 2010 a.s.l. (46° 43' N, 10° 52' E); Tyrol, Austria: 2050 a.s.l. (46° 52' N, 11° 01' E)]. Mean July temperatures are ca. 19-20°C (or higher) for the low-, ca. 13-15°C for the mid-, and ca. 10°C (or below) for the high-altitude populations (also for experiment 1). As in *experiment 1* population by temperature interactions were either non-significant or had very little effect on overall patterns, all larvae were reared at a single temperature of 27°C (L18:D6) to reduce work load. For all individuals larval development time, pupal mass, pupal development time and larval growth rate were recorded as detailed above. Further, chill-coma recovery and heat knock-down times were determined as above, however, butterflies were randomly assigned to either the cold or heat stress treatment, not both.

While heat-stressed butterflies were not used any further (note that heat stress induces significant mortality), cold-stressed butterflies (though exclusively from the low and high altitudes to reduce work load) were used further for investigating flight

duration and morphological traits. Flight duration was measured for males and females in relation to population and ambient temperatures (i.e. at 5, 12, 19 and 26°C) on day 3 of adult life (i.e. on the day following the cold stress). Prior to the experiments, butterflies were allowed to acclimate at the respective test temperature for 30 min (Van Dyck and Matthysen 1998). Each individual was tested at all 4 temperatures within one day. For testing, a butterfly sitting with closed wings was picked up with a pair of tweezers and was released from a standard height of 2 m. Flight duration was timed from the release of the butterfly until it alighted (Merckx et al. 2006). Only flights of at least 2 m in length were considered (thus excluding occasions when a butterfly alighted on the testing person). Each individual was tested three times at each temperature, and the corresponding means were used for further analyses.

For morphological analyses all butterflies were frozen on day 3 of adult life following the above experiments. Later, they were dried to a constant mass for 24 h at 70°C. Wings, heads and legs were removed, and thorax and abdomen mass were determined separately to the nearest 0.01 mg (Sartorius microscale MC 210 P). Relative fat content was determined as the mass difference between adult dry mass and the remaining dry mass after two fat extractions. In each extraction, fat was extracted for 48 hours using 2 ml of dichloromethane (CH_2Cl_2) / methanol (CH_3OH) (2:1) solution for each butterfly (cf. Fischer et al. 2003). Forewing area and length (from basis to apex) was measured using digital images of left forewings (captured from ventral with a digital camera, Leica DC300, mounted on a stereo microscope, Leica MZ 7.5) and Scion Image software (release Beta 4.0.2). Wing loading was calculated as total dry body mass divided by forewing area, and wing aspect ratio as $4 \times$ forewing length^2 divided by forewing area (Berwaerts et al. 2002). Wing loading is closely related to flight performance (lower wing loading means better flight performance, Berwaerts et al. 2002), while a higher aspect ratio specifies a narrower wing, resulting in an enhanced acceleration capacity (Norry et al. 2001).

Statistical analyses

Life history, morphology and stress resistance data were analysed using nested analyses of (co-)variance (AN(C)OVAs) with replicate population nested within altitude. Replicates were treated as random effects, whilst altitude, sex and

temperature (the latter in *experiment 1* only) were considered fixed effects. Pupal mass was added as covariate when appropriate. Throughout, minimum adequate models were constructed by removing non-significant interaction terms. Prior to analyses, data on flight duration, body dry mass, total fat content, forewing area, wing loading, and wing length were transformed as appropriate to meet ANOVA requirements. Pair-wise comparisons were performed employing Tukey's HSD. All statistical tests were performed by using JMP (4.0.0) or Statistica (6.1). Unless otherwise stated, least square means ± 1 SE are given in text.

Results

Experiment 1

Altitudinal and temperature-related variation in life-history traits

Larval development time varied significantly between altitudes (high: 23.5 ± 0.3 days > low: 21.7 ± 0.3 days), replicates, sexes (males: 21.8 ± 0.1 days < females: 23.4 ± 0.2 days) and temperatures (18°C: 30.0 ± 0.2 days > 27°C: 15.2 ± 0.1 days; Table 1, Fig. 1a). The absolute difference between altitudes was higher at 18 (high: 31.5 ± 0.3 days vs. low: 28.7 ± 0.2 days) compared to 27°C (high: 15.9 ± 0.2 days vs. low: 14.5 ± 0.2 days), causing a significant altitude by temperature interaction. Results for growth rates showed qualitatively a very similar pattern (though there was no significant variation across replicates), being significantly higher in the low- versus high-altitude populations (low: 6.0 ± 0.1 mg/day > high: 5.2 ± 0.1 mg/day), in males versus females (males: 5.9 ± 0.1 mg/day > females: 5.4 ± 0.1 mg/day), and at the higher versus the lower temperature (27°C: 7.4 ± 0.1 mg/day > 18°C: 3.8 ± 0.1 mg/day; Fig. 1b). For pupal time no difference was detected between altitudes and replicates, but development was significantly shorter at the higher (7.4 ± 0.03 days) than at the lower (13.9 ± 0.05 days) rearing temperature, and was shorter in males (10.5 ± 0.04 days) than females (10.8 ± 0.05 days). A significant altitude by temperature interaction indicates that the temperature effect was slightly less pronounced in high- than in low-altitude butterflies (being by 45.7 % compared to 47.3 % shorter in individuals reared at 27°C; Fig. 1c). Pupal mass was significantly higher at the lower (114.7 ± 1.1 mg) compared to the higher (110.5 ± 0.7 mg) rearing temperature and also varied across replicate populations, but did not differ between altitudes or sexes (Fig. 1d).

Table 1. Nested AN(C)OVAs for the effects of altitude, replicate population (nested within altitude), sex and temperature on life-history and stress-resistance traits in *Lycaena tityrus*. Pupal mass (covariate) was added as appropriate. Minimum adequate models were constructed by removing non-significant interaction terms. Sign. *P*-values are given in bold.

Trait and source	DF	MS	F	P
Larval time [days]				
Altitude	1,2	387.7	22.8	**0.0340**
Replicate [Altitude]	2,448	19.5	4.0	**0.0183**
Sex	1,448	286.7	59.5	**< 0.0001**
Temperature	1,448	20262.3	4204.4	**< 0.0001**
Altitude x Temperature	1,448	47.6	9.9	**0.0018**
Error	448	4.8		
Growth rate [mg/day]				
Altitude	1,3	52.3	31.8	**0.0168**
Replicate [Altitude]	2,448	1.7	1.5	0.2150
Sex	1,448	23.3	20.6	**< 0.0001**
Temperature	1,448	1166.1	1029.9	**< 0.0001**
Altitude x Temperature	1,448	6.7	5.9	**0.0155**
Error	448	1.1		
Pupal time [days]				
Altitude	1,2	3.4	4.0	0.1637
Replicate [Altitude]	2,448	0.9	2.5	0.0816
Sex	1,448	11.4	30.5	**< 0.0001**
Temperature	1,448	3834.8	10252.4	**< 0.0001**
Altitude x Temperature	1,448	3.8	10.2	**0.0015**
Error	448	0.4		
Pupal mass [mg]				
Altitude	1,2	2924.2	2.1	0.2882
Replicate [Altitude]	2,449	1440.9	8.9	**0.0002**
Sex	1,449	438.7	2.7	0.1004
Temperature	1,449	1494.4	9.2	**0.0025**
Error	449	161.9		
Chill-coma recovery time [sec]				
Altitude	1,2	1165252	88.6	**0.0055**
Replicate [Altitude]	2,409	12186.4	0.3	0.7787
Sex	1,409	467.3	0.01	0.9220
Temperature	1,409	958735	19.7	**< 0.0001**
Pupal mass	1,409	274.9	0.01	0.9401
Error	409	48699		
Heat knock-down time [sec]				
Altitude	1,3	797360	13.2	**0.0430**
Replicate [Altitude]	2,393	63231.4	1.3	0.2658
Sex	1,393	227112	4.8	**0.0295**
Temperature	1,393	1733202	36.4	**< 0.0001**
Altitude x Temperature	1,393	700543	14.7	**0.0001**
Pupal mass	1,393	81605.6	1.7	0.1910
Error	393	47556		
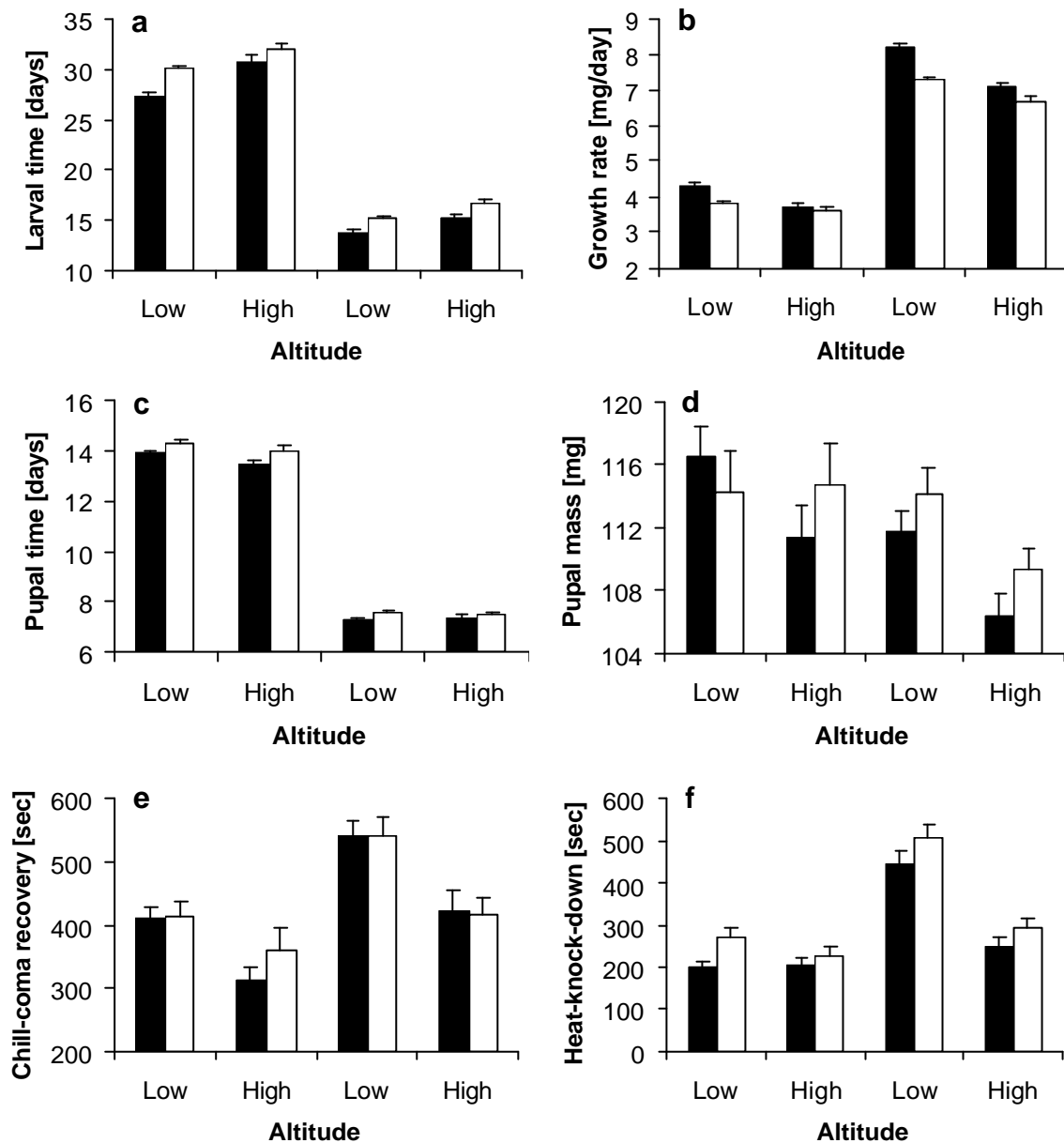
Fig. 1. Means (+ 1 SE) for larval time (a), larval growth rate (b), pupal time (c), pupal mass (d), chill-coma recovery time (e) and heat knock-down time (f) for *Lycaena tityrus* males (black bars) and females (white bars) across two rearing temperatures (18°C and 27°C) and altitudes (low- and high-altitude). Data were pooled across two replicates each. Group sample sizes range between 20 and 83 individuals.

Altitudinal and temperature-related variation in thermal stress resistance

Chill-coma recovery time varied significantly between altitudes and temperatures, but was not affected by replicate, sex or the covariate pupal mass (Fig. 1e, Table 1). High-altitude animals (374.1 ± 11.9 sec) showed faster recovery compared to low-

altitude ones (475.8 ± 10.8 sec), as did animals reared at the lower (372.0 ± 20.9 sec) compared to the higher (477.9 ± 13.0 sec) temperature.

Heat knock-down time was significantly shorter in high- (243.6 ± 18.2 sec) than in low-altitude butterflies (350.2 ± 16.5 sec), in males than in females (271.5 ± 15.5 sec vs. 322.3 ± 17.6 sec), and in animals reared at the lower (224.4 ± 20.7 sec) versus the higher rearing temperature (369.3 ± 13.2 sec; Fig. 1f), while there was no significant impact of replicates or the covariate pupal mass. Low-altitude butterflies showed a bigger difference in heat knock-down time across temperatures than high-altitude butterflies, as evidenced by a significant altitude by temperature interaction (knock-down time increased by 51.2 % at 27°C compared to 20°C in low-, but only by 18.1 % in high-altitude butterflies). Heat knock-down time and chill-coma recovery time were marginally correlated ($R = 0.12$, $P = 0.02$, $N = 404$).

Experiment 2

Altitudinal variation in life-history traits

Altitude significantly affected larval (high: 15.0 ± 0.07 days = mid: 14.8 ± 0.07 days > low: 14.0 ± 0.07 days) and pupal development time (low: 7.29 ± 0.02 days = mid: 7.36 ± 0.02 days > high: 6.76 ± 0.02 days), but not growth rate or pupal mass (Fig. 2 a-d; Table 2), although pupal mass tended to increase in the higher-altitude populations. Sexes differed significantly in larval time (males: 13.9 ± 0.07 days < females: 15.3 ± 0.08 days), pupal development time (males: 7.0 ± 0.03 days < females: 7.3 ± 0.03 days) and growth rate (males: 8.2 ± 0.07 mg/day > females: 7.6 ± 0.07 mg/day), but not in pupal mass (Fig. 2 a-d). A significant altitude by sex interaction for pupal mass indicates some variation in sexual size dimorphism, with females being by about 1.7 % and 3.9 % heavier in mid- and high-altitude populations, but by about 2.4 % lighter in the low-altitude populations as compared to males (Fig. 2d). Further, sexual differences in growth rates were most pronounced in low- (13.3 % higher in males) followed by mid- (8.0 % higher) and high-altitude populations (4.5 % higher in males; significant altitude-by-sex interaction; Fig. 2b). Throughout, there was no significant variation between replicates.

Table 2. Nested AN(C)OVAs for the effects of altitude, replicate population (nested within altitude) and sex on life-history and stress-resistance traits in *Lycaena tityrus*. Pupal mass (covariate) was added as appropriate. Minimum adequate models were constructed by removing non-significant interaction terms. Significant *P*-values are given in bold.

Trait and source	DF	MS	F	P
Larval time [days]				
Altitude	2,3	59.6	50.2	**0.0045**
Replicate [Altitude]	3,678	1.2	0.6	0.6207
Sex	1,678	333.9	167.4	**< 0.0001**
Error	678	2.0		
Growth rate [mg/day]				
Altitude	2,3	5.8	1.9	0.2850
Replicate [Altitude]	3,676	3.0	1.8	0.1447
Sex	1,676	69.6	41.6	**< 0.0001**
Altitude x Sex	2,676	5.7	3.4	**0.0341**
Error	676	1.7		
Pupal time [days]				
Altitude	2,3	21.9	195.9	**0.0005**
Replicate [Altitude]	3,678	0.1	0.4	0.7575
Sex	1,678	18.7	66.4	**< 0.0001**
Error	678	0.3		
Pupal mass [mg]				
Altitude	2,3	3805.4	8.7	0.0551
Replicate [Altitude]	3,676	442.1	2.4	0.0690
Sex	1,676	277.8	1.5	0.2222
Altitude x Sex	2,676	705.9	3.8	**0.0230**
Error	676	186.2		
Chill-coma recovery time [sec]				
Altitude	2,3	1786980	17.4	**0.0216**
Replicate [Altitude]	3,274	104895	4.0	**0.0078**
Sex	1,274	2350	0.1	0.7638
Pupal mass	1,274	39321	1.5	0.2197
Error	274	7120073		
Heat knock-down time [sec]				
Altitude	2,5	3969545	246.9	**< 0.0001**
Replicate [Altitude]	3,276	12580	0.2	0.9188
Sex	1,276	1665873	22.1	**< 0.0001**
Pupal mass	1,276	98.9	0.0001	0.9712
Error	276	20831702		
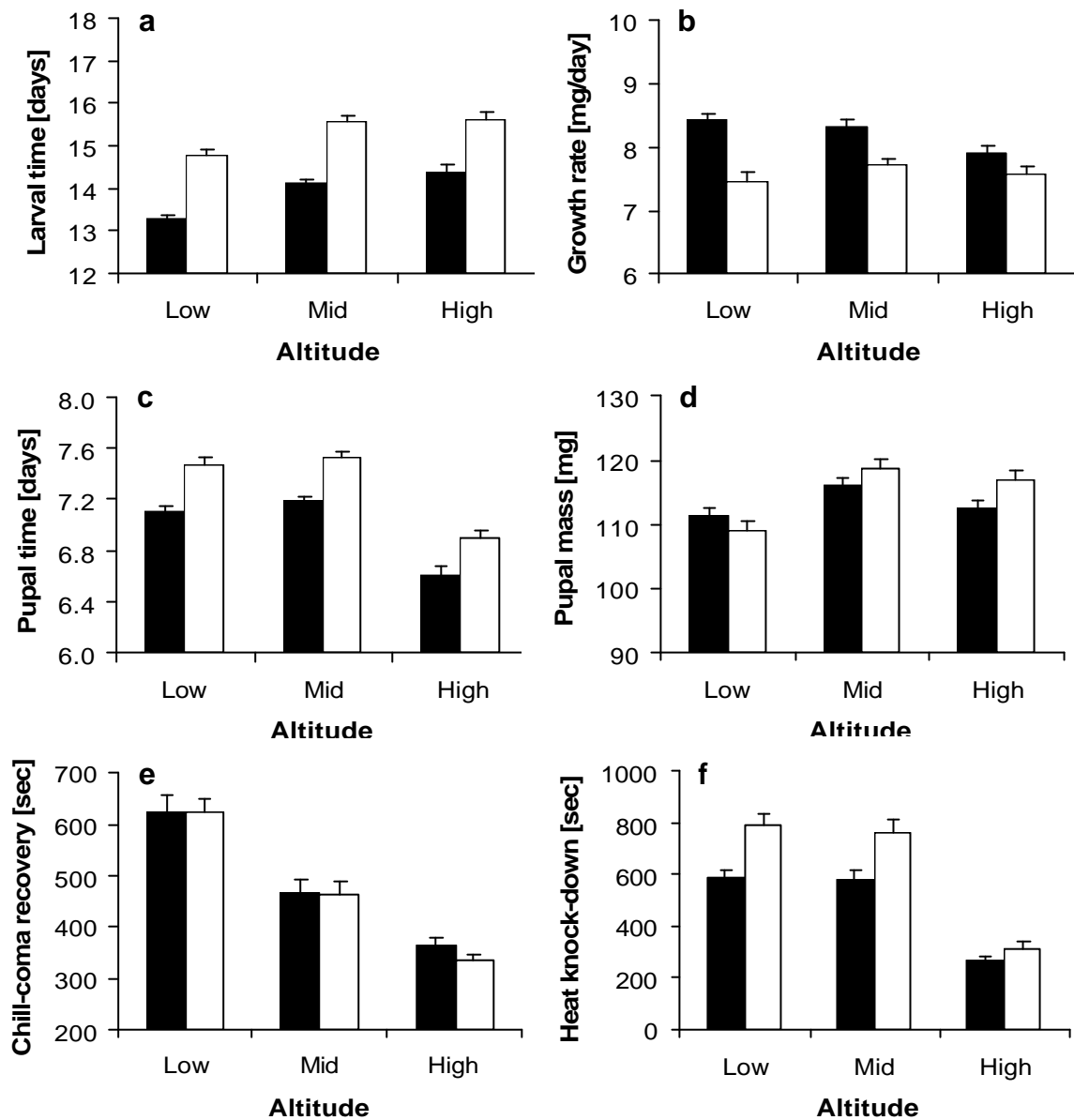
Fig. 2. Means (+ 1 SE) for larval time (a), larval growth rate (b), pupal time (c), pupal mass (d), chill-coma recovery time (e) and heat knock-down time (f) of *Lycaena tityrus* males (black bars) and females (white bars) from three different altitudes (low-, mid- and high-altitude) at 27°C. Data were pooled across two replicates each. Group sample sizes range between 96 and 146 individuals for life-history traits and between 33 and 72 individuals for stress resistance traits.

Altitudinal variation in thermal stress resistance

Chill-coma recovery time varied significantly across altitudes (low: 621.2 ± 32.1 sec > mid: 464.4 ± 35.6 sec > high: 349.5 ± 33.4 sec; Fig. 2e; Table 2) and replicates, but not between the sexes. Heat knock-down time also differed significantly across

altitudes (low: 686.9 ± 12.3 sec = mid: 665.5 ± 13.5 sec > high: 282.3 ± 15.3 sec; Fig. 2f) and between the sexes (females: 623.5 ± 25.0 sec > males: 466.2 ± 22.6 sec), but not among replicates. Pupal mass did not significantly affect either stress resistance trait.

Altitudinal variation in morphology

Butterflies from high- versus low-altitude differed significantly in thorax, abdomen and total fat content, but not in total dry mass, thorax-abdomen ratio, wing length, wing area, wing loading and wing aspect ratio (see Table 3). Thoracic (low: 14.2 ± 0.02 % > high: 13.9 ± 0.02 %), abdomen (low: 28.4 ± 0.9 % > high: 22.8 ± 0.9 %) and total fat content (low: 21.3 ± 0.3 % > high: 18.5 ± 0.3 %; Table 4) were higher in low- compared to high-altitude butterflies. Differences across replicates were present for thorax-abdomen ratio and wing area, but not for any other trait. Sexes differed significantly in total dry mass, thorax-abdomen ratio, abdomen fat content, wing area, wing loading and wing aspect ratio, but not in thorax fat content, total fat content and wing length (Table 3). Males showed a higher thorax-abdomen ratio (males: 1.53 ± 0.03 > females: 0.59 ± 0.03), wing aspect ratio (males: 9.2 ± 0.2 > females: 8.1 ± 0.2) and abdomen fat content (males: 28.7 ± 0.8 % > females: 22.4 ± 0.8 %), but a lower total body mass (males: 8.8 ± 0.3 mg < females: 12.7 ± 0.3 mg), a smaller wing area (males: 1.05 ± 0.02 cm² < females: 1.19 ± 0.02 cm²) and a lower wing loading (males: 8.9 ± 0.3 mg/cm² < females: 11.2 ± 0.3 mg/cm²; Table 4) compared to females. All interactions were non-significant.

Altitudinal and temperature-related variation in flight capacity

Flight duration varied significantly across altitudes at 5°C ($F_{1,2} = 37.1$, $P = 0.0246$; high: 0.89 ± 0.003 sec > low: 0.86 ± 0.003 sec) and 12°C ($F_{1,2} = 25.0$, $P = 0.0377$; high: 1.59 ± 0.04 sec > low: 1.31 ± 0.04 sec), but not at 19°C ($F_{1,2} = 3.4$, $P = 0.2061$) and 26°C ($F_{1,2} = 15.9$, $P = 0.0567$), though the tendency for high-altitude butterflies to fly longer remained (see Table 4). Males flew significantly longer than females at 5°C ($F_{1,212} = 76.8$, $P < 0.0001$), 12°C ($F_{1,212} = 5.7$, $P = 0.0175$) and 19°C ($F_{1,212} = 13.3$, $P = 0.0003$), but not at 26°C ($F_{1,212} = 3.1$, $P = 0.0797$; Table 4). Further, sex differences at 12°C were more pronounced in the high- than in the low-altitude populations (altitude by sex interaction: $F_{1,211} = 3.9$, $P = 0.0495$; for all other interactions $P > 0.18$).

Table 3. Nested AN(C)OVAs for the effects of altitude, replicate population (nested within altitude) and sex on morphological traits in *Lycaena tityrus*. Minimum adequate models were constructed by removing non-significant interaction terms. Sign. *P*-values are given in bold.

Trait and source	DF	MS	F	P
Total dry mass [mg]				
Altitude	1,2	< 0.1	< 0.1	0.9506
Replicate [Altitude]	2,228	0.4	2.1	0.1250
Sex	1,228	20.0	114.4	**< 0.0001**
Error	228	39.8		
Thorax fat content [%]				
Altitude	1,3	5.3	107.7	**0.0031**
Replicate [Altitude]	2,218	< 0.1	< 0.1	0.9966
Sex	1,218	15.2	1.2	0.2758
Error	218	2772.9		
Abdomen fat content [%]				
Altitude	1,2	1745.7	20.4	**0.0457**
Replicate [Altitude]	2,218	85.8	1.3	0.2679
Sex	1,218	2245.1	34.7	**< 0.0001**
Error	218	14111.3		
Total fat content [%]				
Altitude	1,2	0.33	22.5	**0.0414**
Replicate [Altitude]	2,218	0.01	< 0.1	0.5436
Sex	1,218	0.01	0.3	0.5737
Error	218	5.12		
Thorax-abdomen ratio				
Altitude	1,2	0.2	0.5	0.5704
Replicate [Altitude]	2,218	0.5	4.3	**0.0144**
Sex	1,218	48.7	418.2	**< 0.0001**
Error	218	25.4		
Wing length [cm]				
Altitude	1,2	0.13	1.7	0.3216
Replicate [Altitude]	2,217	0.08	1.9	0.1579
Sex	1,217	0.08	1.8	0.1830
Error	217	9.14		
Wing area [cm^2]				
Altitude	1,2	0.07	1.2	0.3746
Replicate [Altitude]	2,217	0.05	3.9	**0.0198**
Sex	1,217	0.14	10.9	**0.0011**
Error	217	2.79		
Wing loading [mg/cm^2]				
Altitude	1,2	0.08	3.6	0.1964
Replicate [Altitude]	2,217	0.02	1.1	0.3255
Sex	1,217	0.54	28.9	**< 0.0001**
Error	217	4.09		
Aspect ratio				
Altitude	1,2	13.0	1.1	0.4027
Replicate [Altitude]	2,217	11.7	2.3	0.0978
Sex	1,217	75.2	15.1	**0.0001**
Error	217	1080.5		

Throughout, neither replicate population (all $P > 0.1853$) nor pupal mass significantly affected flight duration (all $P > 0.0550$).

Altitudinal and temperature-related variation in flight capacity

Flight duration varied significantly across altitudes at 5°C ($F_{1,2} = 37.1$, $P = 0.0246$; high: 0.89 ± 0.003 sec > low: 0.86 ± 0.003 sec) and 12°C ($F_{1,2} = 25.0$, $P = 0.0377$; high: 1.59 ± 0.04 sec > low: 1.31 ± 0.04 sec), but not at 19°C ($F_{1,2} = 3.4$, $P = 0.2061$) and 26°C ($F_{1,2} = 15.9$, $P = 0.0567$), though the tendency for high-altitude butterflies to fly longer remained (see Table 4). Males flew significantly longer than females at 5°C ($F_{1,212} = 76.8$, $P < 0.0001$), 12°C ($F_{1,212} = 5.7$, $P = 0.0175$) and 19°C ($F_{1,212} = 13.3$, $P = 0.0003$), but not at 26°C ($F_{1,212} = 3.1$, $P = 0.0797$; Table 4). Further, sex differences at 12°C were more pronounced in the high- than in the low-altitude populations (altitude by sex interaction: $F_{1,211} = 3.9$, $P = 0.0495$; for all other interactions $P > 0.18$). Throughout, neither replicate population (all $P > 0.1853$) nor pupal mass significantly affected flight duration (all $P > 0.0550$).

Discussion

Altitudinal and temperature-related variation in life-history traits

As expected, most of the life-history traits investigated varied across *Lycaena tityrus* populations from different altitudes, presumably due to genetic differentiation. Note, however, that these differences may include maternal effects, as all animals were reared from wild-caught females. Unfortunately, *Lycaena tityrus* does not mate in captivity, and consequently we could not rear the paternal generation under standardized laboratory conditions to control for such effects. However, usually maternal effects are particularly pronounced early in life (e.g. affecting egg size and early larval development), but typically play an ever-smaller role as individuals mature (Blanckenhorn 1997; but see Räsänen and Kruuk 2007). Further, population genetic analyses proved substantial genetic differentiation, especially between the low- and the higher-altitude populations (Karl et al. in prep.). While interpreting the following results it should be kept in mind that low- and mid-altitude populations are geographically much closer to each other than the low-altitude to any of the other populations (cf. Methods).

In *Lycaena tityrus* altitudinal variation was found in larval and pupal development time, both being longer at higher altitudes (accompanied by reduced growth rates). This contrasts with the prediction of intrinsically higher growth rates and/or shorter development times at higher altitudes as an adaptation to the shorter season (Atkinson 1994, Abrams et al. 1996). The pattern can be easily explained by a change in voltinism: while high-altitude populations are monovoltine, low-altitude ones are bivoltine (Tolman and Lewington 1998). Obviously, the time stress imposed by fitting in an additional generation a year is more severe than that imposed by the shorter growing season length in higher altitudes (Roff 1980). In accordance, prolonged development times in monovoltine as compared to bivoltine populations have been reported for other butterfly species as well (*L. hippothoe*, Fischer and Fiedler 2002, *Aricia agestis*, Burke et al. 2005).

There was virtually no evidence for altitudinal variation in pupal mass in *Lycaena tityrus*, except for a slight tendency towards higher pupal masses in high-altitude populations in *Experiment 2.* Recent studies show that the associations between temperature or environmental clines and body size are complex ranging from positive to negative (Blanckenhorn and Demont 2004, Chown and Klok 2003). Again, our results are likely attributable to differences in voltinism, suggesting that not only temperature regime but also its interactions with generation time, voltinism, and season length are likely to have strong impacts on insect body size (Roff 1980, Blanckenhorn 1997, Chown and Gaston 1999). In studies involving latitudinal variation in *Drosophila* individuals were frequently found to be larger and to grow faster at higher latitudes (James and Partridge 1995, James et al. 1995, Van't Land et al. 1999).

Males generally showed shorter larval and pupal development times accompanied by higher larval growth rates than females (e.g. Fischer and Fiedler 2001, 2002). This is likely to be ultimately caused by protandry selection (i.e. an earlier emergence of males), thereby maximizing male mating opportunities (Fagerström and Wiklund 1982). The variation in sex-related differences in growth rates across altitudes is striking, especially as the same trend can be found in both experiments (though attaining significance in *experiment 2* only). The pattern of decreased differences in

Table 4. Fat content, morphological traits and flight duration at different temperatures (means ± 1 SE) for male and female *Lycaena tityrus* from low- and high-altitude populations. Data were pooled across two replicates each. Note that, as only two altitudes were considered here (in contrast to the traits analysed in Table 2), statistical results are presented in the text.

Trait	Low altitude		High altitude	
	Males (*N* = 55-60)	Females (*N* = 54-55)	Males (*N* = 55-59)	Females (*N* = 59)
Total dry mass [mg]	9.19 ± 0.25	12.40 ± 0.45	8.58 ± 0.29	13.08 ± 0.45
Thorax fat content [%]	13.75 ± 0.55	14.58 ± 0.48	13.75 ± 0.51	13.98 ± 0.35
Abdomen fat content [%]	32.34 ± 1.26	24.47 ± 1.01	25.15 ± 1.15	20.34 ± 0.87
Total fat content [%]	21.56 ± 0.81	20.98 ± 0.70	18.87 ± 0.78	18.21 ± 0.63
Thorax-abdomen ratio	1.57 ± 0.07	0.62 ± 0.02	1.48 ± 0.06	0.51 ± 0.01
Wing length [cm]	1.51 ± 0.01	1.49 ± 0.01	1.52 ± 0.01	1.51 ± 0.01
Wing area [cm²]	1.01 ± 0.03	1.14 ± 0.05	1.10 ± 0.04	1.24 ± 0.04
Wing loading [mg/cm²]	9.57 ± 0.40	11.38 ± 0.50	8.29 ± 0.36	11.04 ± 0.48
Aspect ratio	9.45 ± 0.31	8.34 ± 0.32	9.04 ± 0.33	7.79 ± 0.25
Flight duration at 5°C [sec]	0.90 ± 0.01	0.83 ± 0.01	0.93 ± 0.01	0.85 ± 0.01
Flight duration at 12°C [sec]	1.30 ± 0.05	1.32 ± 0.05	1.73 ± 0.05	1.46 ± 0.05
Flight duration at 19°C [sec]	3.82 ± 0.24	3.40 ± 0.25	4.76 ± 0.24	3.59 ± 0.24
Flight duration at 26°C [sec]	5.04 ± 0.24	4.93 ± 0.25	6.18 ± 0.24	5.45 ± 0.24

higher-altitude populations might be related to their monovoltine lifecycle, potentially resulting in relaxed selection for faster growth in males due to a decoupling of growth rate and development time (as males may slightly later enter/terminate diapause; Nylin et al. 1993). An alternative explanation would be that in monovoltine populations there is a higher selection pressure on synchronized adult emergence to maximize mating opportunities. Although females are typically the larger sex in insects (Roff 2002), we were not able to detect a consistent sex difference in pupal mass. On the contrary, in the low-altitude populations females even tended to be lighter than males. However, in the adult stage we found the expected sexual size dimorphism with females being larger than males (see also Fischer and Fiedler 2000, 2001, 2002). These different patterns in preadult and adult body size are caused by a greater mass loss in males during metamorphosis, which has been interpreted as a potential cost of the males' accelerated development (Fischer et al. 2004).

As expected for an ectothermic organism, growth and development depended strongly on temperature (Fischer and Fiedler 2000, Van Doorslaer and Stoks 2005). Larval and pupal development times were considerably shorter at the higher temperature, accompanied by increased growth rates (see also Blanckenhorn 1997, Burke et al. 2005). Interactions between temperature and altitude for larval and pupal time reflect some marginal variation in the responses to temperature, with differences in larval time being less but differences in pupal time being more pronounced in low-altitude populations. However, based on their rather small effect these effects are probably of marginal relevance.

Also as expected, and as was previously found (Fischer and Fiedler 2000), *Lycaena tityrus* conforms to the temperature-size rule (Bergmann's rule extended to ectotherms): the lower developmental temperature caused a plastic increase in body size (e.g. Atkinson 1994, Angilletta and Dunham 2003; but see Kingsolver et al. 2007). Though the ultimate factors underlying this almost universal pattern are not understood, potential mechanisms may include an increase in food intake as well as an increase in the efficiency in converting ingested food into body matter (Karl and Fischer 2008), in combination with temperature-mediated changes in cell size and/or number (Partridge et al. 1994, Blanckenhorn and Llaurens 2005, Atkinson et al. 2006).

Altitudinal and temperature-dependent variation in thermal stress resistance

Chill-coma recovery and heat knock-down time are both considered reliable indicators of climatic adaptation, the former being related to cold, the latter to heat stress tolerance (Sørensen et al. 2001, Hallas et. al. 2002, Hoffmann et. al. 2002, David et al. 2003, Castañeda et al. 2005). Our results show rather large variation in these stress resistance traits along an altitudinal gradient, indicating genetic differentiation across populations. The decrease in chill-coma recovery time with increasing altitude suggests an enhanced cold stress resistance of higher-altitude animals. This may facilitate earlier activity in the morning and later activity in the evening, allow for generally higher levels of activity under colder conditions due to a lower thermal threshold, and potentially enhance over-winter survival (Gibert et. al. 2001, Watt et al. 2003, Haag et al. 2005). Similar results along latitudinal and altitudinal clines obtained in e.g. the common woodlouse *Porcellio laevis* (Castañeda et al. 2005), *Drosophila serrata* (Hallas et al. 2002) and *Drosophila melanogaster* (Hoffmann et al. 2002, Hoffmann et al. 2005, Collinge et al. 2006).

Conversely, and consistent with the chill-coma results, heat knock-down resistance decreased with increasing altitude, indicating lower heat stress resistance of high-altitude animals. Low heat tolerance has been suggested to be a common feature in high-altitude populations of *Drosophila* (Kimura and Beppu 1993, Sørensen et al. 2005), reflecting that such animals may rarely be exposed to exceedingly high temperatures. Butterflies from warmer habitats at low altitudes, in contrast, exhibited a much increased heat tolerance. A similar pattern in knock-down resistance along a latitudinal cline was detected for *Drosophila melanogaster* in Australia (Hoffmann et al. 2005). Our results thus support the notion that heat knock-down time is an ecologically relevant trait reflecting adaptive variation (Sørensen et al. 2001, for review see Hoffmann et al. 2003).

Thermal stress resistance was additionally influenced by prevailing environmental conditions. A lower developmental and early adult temperature (note the butterflies were tested on day 2 of adult life) caused reduced chill-coma recovery times (cf. Zeilstra and Fischer 2005) and a decrease in heat knock-down resistance at the higher temperature. Similar results were obtained in studies on *Drosophila* (Chen and Walker 1994, Ayrinhac at al. 2004, Hoffmann et al. 2005). The lack of evidence for an

interaction between genotype (populations from different altitudes) and environment (rearing temperature) for chill-coma recovery time means that the plastic response to temperature was similar across populations, that is, clinal patterns were independent of rearing temperatures (see also Hoffmann et al. 2005). In contrast, such an interaction could be observed with regard to heat knock-down time: Butterflies from low-altitude populations showed a much more pronounced plastic response to temperature than high-altitude ones. The latter leaves substantial potential to quickly adjust heat stress resistance under hot conditions for low-altitude butterflies, which is apparently not needed in populations from higher altitudes.

The sexes did not differ in chill-coma recovery times, a result also being reported for *Drosophila* (Chen and Walker 1994, Gilchrist et al. 1997, Jensen et al. 2007). This might be related to the importance of cold tolerance for over-winter survival (Gibert et. al. 2001), which should be equally relevant to both males and females. In contrast, there was a sex difference in heat tolerance, being reduced in males compared to females (see also Sørensen et al. 2001, Folk et al. 2006, Jensen et al. 2007 for *Drosophila*). Whether this pattern might be related to a higher expression of heat shock proteins in females (Sørensen et al. 2001) remains to be tested. The pattern itself might be related to the fact that males are generally more stressed than females due to sexual selection being usually more intense than is fecundity selection on females, potentially resulting in lower stress resistance in males (Reim et al. 2006). In any case, the females' enhanced heat stress resistance is unlikely to be related to their larger body size frequently assumed to promote temperature resistance, as we have generally found no impact of pupal mass on stress resistance in *Lycaena tityrus*.

Altitudinal variation in morphology and flight performance
The ability to fly even under sub-optimal conditions like low temperatures or strong winds is likely to be closely related to fitness in flying organisms such as butterflies (Barnes and Laurieahlberg 1986, Merckx et al. 2006). Additionally, better flight performance can be beneficial in mountainous areas (Norry et al. 2001, Hodkinson 2005). Accordingly, *Lycaena tityrus* from high-altitude populations exhibited generally increased flight durations compared to butterflies from lower altitudes. Differences were most pronounced at the lowest temperatures tested (5°C and 12°C), suggesting

an adaptation to the generally cooler conditions in the high–altitude populations. Similarly, Merckx et al. (2006) showed stronger selection for flight ability in cool-adapted woodland populations of *Pararge aegeria* as compared to populations from warmer, agricultural landscapes.

In butterflies, differences in flight performance have often been inferred from morphological variation (Berwaerts and van Dyck 2004). For instance, higher relative thorax (and hence flight muscle) masses were found to be related to greater flight capacity (e.g. Berwaerts et al. 2002), and differences in wing shape have been interpreted to reflect divergent demands on aerodynamic performance (Wickman 1992, Van Dyck and Wiklund 2002). Further, a higher wing loading in highland *Drosophila buzzatii* populations is believed to be caused by a stronger selection on flight performance (Norry et al. 2001). Interestingly though, in *Lycaena tityrus* we found no differences in thorax mass, thorax/abdomen-ratio, wing length, wing area, wing loading or wing aspect ratio across altitudes, despite pronounced differences in flight performance. These findings contrast with studies on *Drosophila buzzatii* showing both higher wing loadings and increased wing lengths at higher altitudes (Dahlgaard et al. 2001, Norry et al. 2001). However, in *Drosophila birchii* variation in wing shape components along a latitudinal cline were also lacking (Griffiths et al. 2005).

Flight performance without morphological differentiation across altitudes strongly suggests that low- and high-altitude populations differ in physiological traits. Accordingly, we found a higher amount of fat stored in butterflies from low- as compared to higher-altitudes in *Lycaena tityrus*. Previous studies on butterflies have shown that fat content increases at higher rearing temperatures (Fischer et al. 2003, Karl and Fischer 2008). These results are somewhat counter-intuitive. Based on the fact that fat is the most efficient and most commonly used energy source in insects and therefore indicative of condition (as it is, for example, highly correlated with starvation resistance in *Drosophila*, Zwaan et al. 1991), the reverse pattern with high-altitude butterflies showing an increased fat content as an adaptation to the harsher environmental conditions was expected. Apparently, fat stores do not seem to play a decisive role for the differences in flight performance described above. One plausible, though currently speculative explanation would be a better flight performance in high-

altitude butterflies caused by changes in allele frequencies, e.g. at the PGI (phosphoglucose isomerase) locus. This enzyme is known to profoundly affect flight metabolic rate in butterflies, enabling higher levels of activity under cooler conditions (Watt et al. 2003, Haag et al. 2005). Of course this needs to be tested in *Lycaena tityrus*, but population genetic analyses showed substantial change in allele frequencies at the locus in question in the populations considered here (Karl et al. in prep).

Finally, flight duration also differed between males and females, with males showing generally increased flight performance. In contrast to clinal patterns, these differences can be easily linked to differences in morphology. Males showed a higher thorax/abdomen-ratio, wing aspect ratio and abdomen fat content, and a reduced wing area and wing loading than females. Similarly, in *Pararge aegeria* sexual differences in several aspects of their flight morphology have been shown, such as an increased wing loading in females, likely reflecting divergent selective pressures on male and female flight performance (Van Dyck et al. 1998, Berwaerts et al. 2002, Van Dyck and Wiklund 2002). As male butterflies spend most of their active time locating mates, a higher level of acceleration capacity is of particular significance, while female butterflies perform longer and more persistent flights in search for oviposition sites.

Conclusions

Most efforts to understanding temperature stress tolerance so far have focussed on *Drosophila* as the model organism. We have investigated genetic differentiation and environmentally-induced plasticity in the Copper butterfly *Lycaena tityrus*, by comparing an array of traits across populations from different altitudes, each reared at two different temperatures. We found clear evidence for genetic differentiation (supported by molecular data; Karl et. in prep.), at least some of which can be easily interpreted within an adaptive evolutionary framework. Likely adaptations to the low-temperature environment experienced by high-altitude butterfly populations include an increased cold but decreased heat stress resistance as well as enhanced flight performance, particularly at lower temperatures. Morphological traits, on the other hand, showed weak if any variation across populations from different altitudes,

suggesting weak thermal selection on these traits. Development times, finally, were associated with seasonal time constraints rather than with temperature per se.

Most importantly, however, stress resistance traits also showed strong plastic responses, likely reflecting adaptive phenotypic plasticity. Consequently both sources of variation need to be considered when trying to predict responses to short- (such as particularly hot or cold days/nights) or long-term temperature variation (such as global warming). Animals clearly do possess the ability to respond to temperature changes, both by means of genetic adaptation and short-term physiological adjustment (plasticity). To explore the limits within which such mechanisms can help buffer predictable changes in global temperatures remains an important task for future research (e.g. Van Doorslaer et al. 2007). In this respect the reduced heat resistance of high-altitude populations is striking, especially since this is associated with reduced flexibility (i.e. a comparably small plastic response). Whether this is a common feature in high-altitude populations remains to be tested.

Acknowledgements

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6.2. HSP70 expression in the Copper butterfly *Lycaena tityrus* depends on altitude and temperature

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Abstract

The ability to express heat-shock proteins under thermal stress is an essential mechanism for ectotherms to cope with unfavourable conditions. In this study we investigate if Copper butterflies originating from different altitudes and / or being exposed to different rearing and induction temperatures show differences in HSP expression. HSP expression increased substantially at the higher rearing temperature in low-altitude butterflies, which might represent an adaptation to occasionally occurring heat spells. On the other hand, high-altitude butterflies showed little plasticity and seem to rely more on genetically fixed stress resistance. Therefore, high-altitude populations may be more vulnerable to global warming than low-altitude populations. Further, HSP70 expression was generally higher at the higher rearing temperature (indicating mildly stressful conditions), and increased with both colder and warmer induction temperatures, respectively.

Introduction

Variation in environmental conditions is a significant source of mortality in nature (Willmer et al. 2000), and in this context temperature is thought to be a key factor and consequently considered an important selective agent (Clarke 2003, Hoffmann et al. 2003). Most organisms experience variable thermal environments, posing substantial challenges for key elements of fitness such as survival and reproduction (Dahlhoff and Rank 2007). Consequently, the evolution of behavioural, physiological and molecular mechanisms to cope with stressful conditions is expected and generally found (Hoffmann et al. 2003, Sørensen et al. 2003). Facing rapidly changing climatic conditions at the global scale (e.g. Parmesan et al. 1999, Hitch and Leberg 2007), organisms will have to adapt to the changing environment to avoid extinction (Angilletta et al. 2002, Helmuth 2002, Dahlhoff and Rank 2007). However, the ability to cope with temperature extremes rather than different mean temperatures is probably of much greater relevance for species survival and thermal adaptation (Anderson et al. 2003).

One well-known mechanism to cope with extreme temperatures is the expression of stress-inducible heat-shock proteins (HSPs), which are thought to play an important ecological and evolutionary role in thermal adaptation (Sørensen et al. 2003). Most

HSPs function as molecular chaperones participating in protein folding and unfolding, and they are essential in the cell's response to a variety of damaging conditions (Parsell and Lindquist 1994). However, with respect to terrestrial arthropods, our knowledge on the role of HSPs is almost entirely restricted to a few model organisms (mainly *Drosophila*; Krebs and Loeschcke 1994 a, b, Lansing et al. 2000, Krebs and Holbrook 2001 Sørensen et al. 2005). Studies using such model organisms have yielded much insight into the complex relationships between temperature stress resistance and particularly HSP70 expression (e.g. Sørensen et al. 2005, Dahlhoff and Rank 2007).

Although the up-regulation of stress-inducible HSPs may help organisms to cope with stress thus enhancing survival (Sørensen et al. 2003, Dahlhoff 2004, Dahlhoff and Rank 2007), this may involve substantial costs. HSP expression consumes much cellular energy and competes with the housekeeping metabolism, causing reduced cell growth rates and a reduction in productivity (Krebs and Loeschcke 1994 a, b, Krebs and Holbrook 2001, Robertson 2004). Consequently, HSP induction may increase vulnerability to other stresses (Feder and Hofmann 1999, Morales et al. 2006). Continuous or frequent exposure to stress may therefore reduce the expression of HSP70 through evolution, as the associated costs may outweigh its benefits (Sørensen et al. 1999, Lansing et al. 2000, Sørensen et al. 2001). Such variation in the expression of HSPs may limit the distribution and abundance of organisms along steep ecological (e.g. thermal) gradients in nature (Roberts et al. 1997, Dahlhoff et al. 2001, Dahlhoff 2004, Hofmann 2005). If variation in physiological responses is found over short geographical distances such as altitudes, this strongly supports the notion of adaptive evolution via directional selection (Dahlgaard et al. 2001).

Here, we transfer *Drosophila* expertise (expression of stress-inducible heat-shock proteins) to a non-model organism, the temperate-zone butterfly *Lycaena tityrus*. We compare expression patterns across replicated populations originating from different altitudes, and at the same time across different ambient temperatures. We specifically addressed the following questions: (1) Does the expression of HSP70 vary across populations from different altitudes? In high-altitude populations, being exposed to harsher environmental conditions in their natural environment, a lower

level of HSP expression might be expected at any given stress level, being experienced as less of an emergency situation. (2) Does HSP70 expression vary across developmental/acclimation and induction temperatures (with the expectation that high as well as low temperatures induce increased HSP expression)?

Material and methods

Study organism and butterfly rearing

Lycaena tityrus (Poda, 1761) is a widespread temperate-zone butterfly, ranging from Western Europe to central Asia (Ebert and Rennwald 1991). The species is bivoltine with two discrete generations per year in most parts of its range, although populations with one or three generations per year occur (Ebert and Rennwald 1991, Tolman and Lewington 1998). The principal larval host-plant is *Rumex acetosa* L., but some congeneric plant species such as *R. acetosella* L. and *R. scutatus* L. are utilised as well (Ebert and Rennwald 1991, Tolman and Lewington 1998). Mated females from replicated low- [Rhineland-Palatinate, Germany: 250 a.s.l., (50° 30' N, 7° 58' E; *N* = 13); Bavaria, Germany: 600 a.s.l. (47° 42' N, 11° 24' E; *N* = 6)] and high-altitude [South Tyrol, Italy: 2010 a.s.l. (46° 43' N, 10° 52' E, *N* = 23); Tyrol, Austria: 2050 a.s.l. (46° 52' N, 11° 01' E; *N* = 21)] populations were caught in July/August 2007 in the field and transferred to Bayreuth University.

For egg laying, butterflies were kept in a climate chamber at 27°C, high humidity (ca. 70%), and a photoperiod of light 18h: dark 6h (24 h light cycle). Females were placed group-wise, separated by population, into translucent plastic boxes (15 L) and provided with *R. acetosa* (oviposition substrate), fresh flowers (*Crepis spec.*, *Achillea millefolium*, *Bistorta officinalis*, *Leucanthemum vulgare*) and a highly concentrated sucrose solution (for adult feeding). Eggs were collected daily, pooled within populations, and transferred to small glass vials. After hatching, larvae were randomly divided among two rearing temperatures (20°C and 27°C; L18:D6 and 70% relative humidity throughout). Larvae were first reared in groups of ten individuals, but during the last two larval stages individually in translucent plastic boxes (125 ml), containing moistened filter paper and fresh cuttings of *R. acetosa* in ample supply. Boxes were checked daily and supplied with new food when necessary. Following adult eclosion, butterflies were separated by eclosion day and population and

transferred to cylindrical hanging cages kept at their respective rearing temperature. They were provided with fresh flowers (*Crepis spec.*, *Achillea millefolium*, *Polygonum bistorta*, *Leucanthemum vulgare*) and a highly concentrated sucrose solution for adult feeding.

Experimental design and sample preparation

On day two after eclosion, the butterflies from both rearing temperatures and each population were randomly divided among five treatments, being either exposed for 1 h to 1, 10, 20, 27 or 37°C. Thereafter, butterflies were back-transferred to their respective rearing temperature for 1 h to allow for the possible up-regulation of HSP, after which they were frozen at -78°C for later analysis. In total, 560 individuals in 40 groups (2 altitudes x 2 replicates x 2 rearing temperatures x 5 test temperatures) were exposed to the different temperature treatments. Samples were prepared for measuring HSP expression by removing heads, legs, wings and abdomen. Thorax fresh mass was determined to the nearest 0.01 mg (Sartorius microscale MC 210 P). Thereafter, thoraxes were homogenized in 400 µl ice-cold phosphate-buffered saline (PBS), containing 200mM PEFAbloc and a 1 vol.% antiprotease cocktail (100 µl/ml pepstatin A, 50 µl/ml leupeptin, 10 mM benzamidine, 10mM sodium metabisulfite), and then centrifuged for 30 min at 13000 rpm at 4°C. The supernatant was divided into three replicate samples of 100 µl each, transferred to 0.5 ml Eppendorf tubes, and frozen again at -78°C.

ELISA

As for *L. tityrus* HSP expression patterns were never investigated before and consequently no specific antibodies were known, western blotting was used to confirm measuring a protein of the predicted size (70kDa) for the antibody used (Fig. 1). HSP70 expression was measured by ELISA (Enzyme-Linked ImmunoSorbent Assay) following the protocol of Dahlgaard et al. (1998) with some modifications, i.e. by using an HSP70-specific monoclonal antibody (Clone 5A5, mouse-anti rabbit, 1:750) and an HRP-conjugated secondary antibody (Polyclonal Rabbit Anti-Mouse IgG, DAKO A/S). The primary antibody detects both, the constitutive and induced HSP70 family members (referred to as HSP70 for simplicity here). Linearity was verified by testing signal responses with increasing HSP70 concentration (data not shown). On each plate all 80 groups (40 groups by 2 sexes) were represented, and

all samples were measured on four replicate plates. The resulting signal was measured by a spectrophotometric microplate reader (EL$_x$ 800, Bio-Tek instruments) at 562 nm. To standardize between plates, all data were adjusted to plate means. HSP70 expression is given as mean value of the four replicate plates relative to standardized protein content of 30 µl/ml [by means of BCA assays (Pierce Biochemicals) according to manufacturer's instructions]. With the same method total protein content of thoraxes was determined.
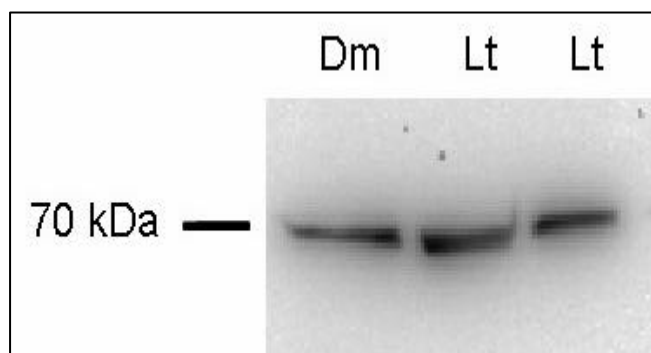
Fig. 1. Western blot showing bands of similar sizes around 70 kDa in *Drosophila melanogaster* (Dm) and *Lycaena tityrus* (Lt). The antibody recognized Hsp70 in *Lycaena tityrus* with comparable affinity as in *Drosophila melanogaster*.

Statistical analyses

HSP70 expression were analysed using nested analyses of co-variance (ANCOVAs), with altitude, rearing temperature, induction temperature and sex as fixed effects, and replicate population (nested within altitude) as random effect. For analysis of HSP70 expression thorax mass and total protein content (in % of thorax mass) were added as covariates. Throughout, minimum adequate models were constructed by removing non-significant interaction terms. Pair-wise comparisons were performed employing Tukey's HSD. All statistical tests were performed by using JMP (4.0.0) or Statistica (6.1). Unless otherwise stated, least square means ± 1 SE are given in the text.

Results

Variation in HSP70 expression

All factors except altitude significantly affected HSP70 expression in *Lycaena tityrus* (Table 1). HSP expression was higher for individuals reared at 27°C (0.486 ± 0.005) compared to 20°C (0.454 ± 0.004) and in females (0.477 ± 0.005) compared to males (0.463 ± 0.004). It was further highest at an induction temperature of 1°C and lowest

at 20°C (1°C: 0.504 ± 0.009 = 37°C: 0.484 ± 0.010 = 10°C: 0.467 ± 0.006 > 27°C: 0.448 ± 0.006 = 20°C: 0.446 ± 0.005; Tukey HSD after ANCOVA; Fig. 2a).

Table 1. Nested ANCOVAs for the effects of altitude, replicate population (nested within altitude), rearing temperature, induction temperature and sex on HSP70 expression. Thorax mass and thorax protein content (covariates) were added as appropriate. Minimum adequate models were constructed by removing non-significant interaction terms. Significant *P*-values are given in bold.

Trait and source	DF	MS	F	P
Hsp70 expression				
Altitude	1,2	0.08	0.5	0.5499
Replicate [Altitude]	2,545	0.16	37.9	**< 0.0001**
Rearing Temperature	1,545	0.14	33.6	**< 0.0001**
Induction Temperature	4,545	0.06	15.4	**< 0.0001**
Sex	1,545	0.02	6.4	**0.0116**
Altitude x Rearing Temperature	1,545	0.05	11.2	**0.0009**
Altitude x Sex	1,545	0.12	27.3	**< 0.0001**
Rearing Temperature x Sex	1,545	0.04	9.6	**0.0020**
Thorax mass	1,545	0.001	0.3	0.5904
Protein content	1,545	0.002	0.4	0.5248
Error	545	0.004		

A significant interaction between altitude and rearing temperature indicates that low-altitude animals strongly responded to rearing temperature, while a comparable response in high-altitude animals was almost entirely absent (Fig. 2b). Further, low-altitude females showed much higher levels of HSP70 expression compared to high-altitude females and males in general (significant altitude by sex interaction; Fig. 3a; Table 1). The increase in expression at the higher rearing temperature was much more pronounced in females than in males (significant rearing temperature by sex interaction; Fig. 3b). Neither covariate significantly affected HSP70 expression.
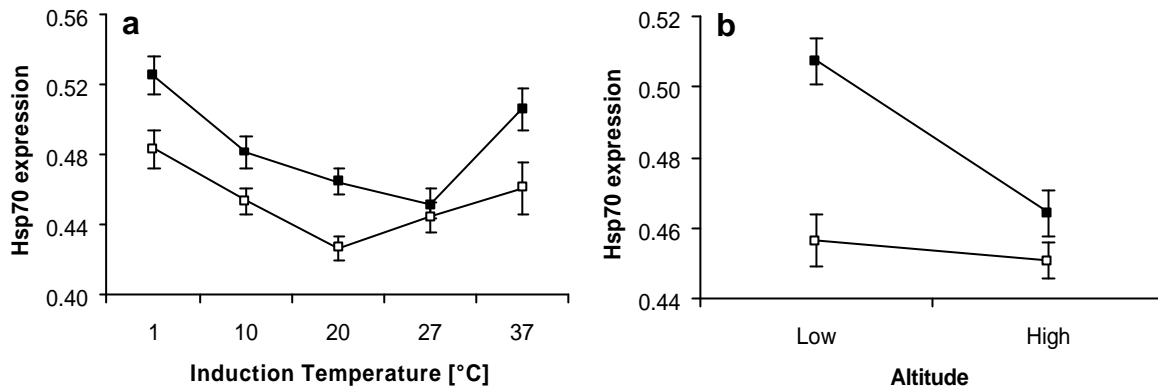
Fig. 2. Means (± 1 SE) of HSP70 expression for *Lycaena tityrus* across rearing temperatures (20°C: white symbols; 27°C: black symbols) and induction temperatures (A) or altitudes (B).
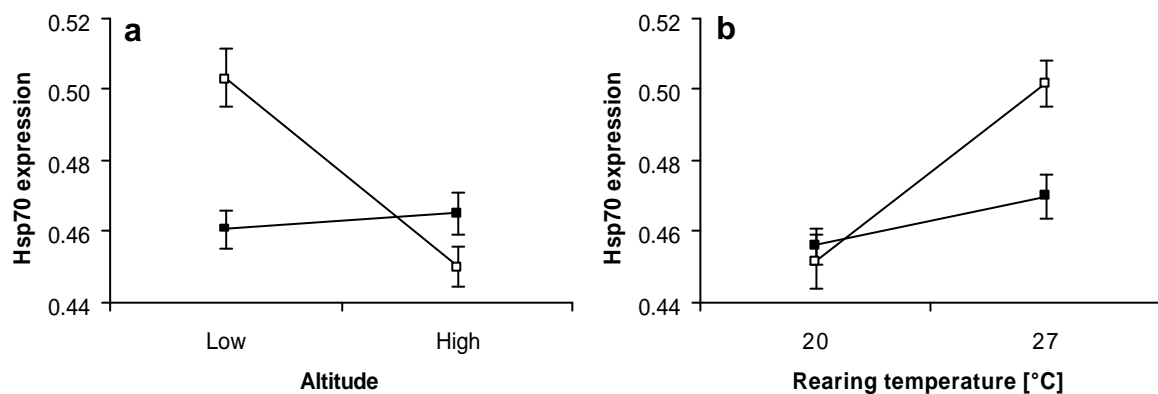
Fig. 3. Means (± 1 SE) of Hsp70 expression for *Lycaena tityrus* males (black symbols) and females (white symbols) across altitudes (A) and rearing temperatures (B).

Discussion

Facing rapid human-induced climate change, understanding the mechanisms by which organisms respond to environmental variation received increasing attention (Dahlhoff and Rank 2007). In this context interest in the molecular and physiological functions of heat-shock proteins increased over recent years (Parsell and Lindquist 1993, Sørensen et al. 2003). However, very few data are presently available for non-model organisms such as the butterfly species studied here. Such data, however, are needed for assessing the generality of findings on the role of the heat shock response for thermal adaptation obtained from model species.

The ability to express HSPs under thermal and other stresses is an essential mechanism to cope with unfavourable conditions for ectothermic organisms (Sørensen et al. 2003, Dahlhoff 2004, Dahlhoff and Rank 2007). Though differentiation in HSP expression across altitudinal and latitudinal clines can be expected (Garbuz et al. 2003, Sørensen et al. 2005), there were no overall effects of altitude in *L. tityrus* detectable. However, while high-altitude butterflies responded only marginally to differences in rearing temperature, HSP expression increased substantially at the higher temperature in low-altitude butterflies. Consequently, the latter genotype is considerably more plastic compared to the former. Note in this context that 27°C is a relatively high rearing temperature for a temperate-zone butterfly, and that such conditions may amplify otherwise obscured phenotypic differences between genotypes (Hoffmann and Parsons 1991, Hoffmann and Merilä 1999, Blanckenhorn and Heyland 2004). Nevertheless, the high-altitude environment is certainly generally cooler and probably also less predictable than the low-altitude environment (Franz 1979). Thus, the reduced plasticity in high-altitude animals is likely to be related to a chronic exposure to thermal stress (cf. Sørensen et al. 1999, Lansing et al. 2000, Sørensen et al. 2001).

Under such conditions, the heat shock response would consume a large amount of cellular energy, which may cause an energy dept (Gething and Sambrook 1992, Krebs and Loeschcke 1994 a, b, Rank and Dahlhoff 2007). Such energy losses may favour alternative mechanisms to cope with high-altitude conditions. In *L. tityrus*, one mechanisms seems to be allelic variation at the PGI locus, with a particular genotype, being associated with increased cold stress resistance, dominating in high-altitude populations (Karl et al. in prep.). Thus, these populations seem to rely in the first place on genetically fixed resistance helping to conserve energy under the harsher environmental conditions. Concomitantly and owing to their relative lack of plasticity (see above), high-altitude populations of *L. tityrus* might be more vulnerable to global warming than are low-altitude populations. The pronounced plastic response in low-altitude populations, in contrast, might be related to occasionally occurring fast increases in daily temperatures, warranting a molecular system which is able to respond rapidly (Dahlgaard and Loeschcke 1997).

Overall, environmentally-induced plasticity (through different rearing temperatures) had a stronger effect on HSP70 expression than genetic factors (across populations). First, HSP expression was generally higher at the higher rearing (and adult) temperature, suggesting that a temperature of 27°C imposes already a mild stress to *L. tityrus*. This was expected as this butterfly will rarely encounter such a high temperature permanently in nature, though it is well within the range of daily highs in its natural habitats. Similarly, HSP expression increased during warm seasons in other ectotherms (Fader et al. 1994, Roberts et al. 1997).

Second, butterflies clearly responded to the different induction temperatures used. Although often only high temperatures are used for HSP induction (e.g. Dahlgaard et al. 1998, Sørensen et al. 2001, Sørensen et al. 2005), low temperatures are also known to upregulate HSPs (Hoffmann et al. 2003, Michaud and Denlinger 2005, Yocum 2001). Accordingly, in *L. tityrus* the expression of HSP70 increased both towards high and low temperatures. Most interestingly, lowest expression levels were found at the same temperature the respective individuals were reared at (i.e. for individuals reared at 27°C at an induction temperature of 27°C; and for individuals reared at 20°C at 20°C). This may indicate that a change in the thermal environment generally induces some stress, supporting the beneficial acclimation hypothesis (Huey et al. 1999, Woods and Harrison 2002).

In addition to the patterns discussed above, sexes differed in HSP expression with females showing higher values than males. Particularly high expression levels were found in females from low-altitudes or females reared at the higher temperature. Differences between sexes were also found in *Drosophila* species (Dahlgaard et al. 1998, Sørensen et al. 2005). Contrary to our findings, it was shown that the level of HSP70 in *Drosophila* males exceeded that of females (Dahlgaard et al. 1998). However, HSP70 expression in our study was measured one hour after induction, and thus we have only data for one time point. This might be important as in *Drosophila* males the increase in HSP70 expression is less steep than for females, and highest values were found around two hours after hardening (Dahlgaard et al. 1998, Sørensen et al. 2001). Nevertheless, as sexes also varied across rearing temperatures with females showing increased HSP expression (accompanied by an increased heat resistance; Karl et al. 2008), there might be a true difference in the

heat shock response across sexes. As for example in *Drosophila ananassae* heat stress strongly affected survival and reduced female fecundity (Sisodia and Singh 2006), such differences in HSP expression may reflect the females' higher need for protection against thermal stress.

Conclusions

As most of our knowledge on patterns of HSP expression stems from studies using *Drosophila* as a model organism, we here investigate genetic and environmental effects on HSP70 expression in the Copper butterfly *L. tityrus*. Environmental effects according to different rearing and induction temperatures were more pronounced than genetic effects. The latter were largely restricted to low-altitude butterflies reared at the higher temperature. These results suggest that high-altitude butterflies generally rely more on fixed resistance to environmental variation than lowland ones, while low-altitude animals showed much higher levels of plasticity. Consequently, high-altitude populations appear more vulnerable to rapid human-induced climatic change than low-altitude ones. HSP70 expression was generally higher at the higher rearing temperature (indicating mildly stressful conditions), and increased with increasingly colder and warmer induction temperatures, respectively. The latter finding gave some support for the beneficial acclimation hypothesis, as the lowest levels of HSP expression coincided with the respective rearing temperature. This is the first study on HSP70 expression in a Copper butterfly, laying the fundament for future investigations within a comparative context.

Acknowledgements

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7. The genetic background of altitudinal variation in life-history and temperature stress resistance traits

7.1. Genetic differentiation between alpine and lowland populations of a butterfly is caused by variation at the PGI locus

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Abstract

Understanding the ecological process of population differentiation and identifying the molecular changes that contributes to adaptation lies at the heart of evolutionary biology. In this study, analyzing geographic variation in allozyme allele frequencies (based on 15 enzyme systems representing 18 loci) across 18 populations of the butterfly *Lycaena tityrus* from different altitudes, we tried to detect enzymes that are likely under natural selection. Population genetic analyses showed that intrapopulational genetic diversity, namely the mean number of alleles per loci (1.8) and the expected heterozygosity (12 %), was within the range of values typical for the Lepidoptera. The populations of *Lycaena tityrus* investigated showed a remarkable genetic differentiation (F_{ST}: 0.065), being clearly divided into an alpine (highland) and a non-alpine (lowland) cluster. This differentiation was almost entirely caused by variation at a single locus, PGI. Although the involvement of historical events cannot be ruled out, several lines of evidence strongly suggest that the specific pattern of allozyme (PGI) variation found is caused by thermal selection. First, genetic variation was highly locus-specific rather than relatively uniform, as is expected for effects of natural selection. Second and more importantly, the PGI 2-2 genotype dominating in alpine (in contrast to lowland) populations is known to exhibit increased cold stress resistance and other features typical of alpine populations. Thus, PGI is an obvious target for thermal selection in *Lycaena tityrus* and probably a variety of other insects.

Introduction

Geographic variation in traits related to fitness along latitudinal (Robinson et al. 2000, Van Doorslaer and Stoks 2005) and altitudinal clines (Chown and Klok 2003, Karl et al. 2008) is widespread. Given a genetic basis, such differences may be caused by adaptive evolution (i.e. adaptation to different climates; Hoffmann et al. 2005, Collinge et al. 2006, Sambucetti et al. 2006) and/or by random processes like genetic drift or isolation by distance (e.g. Endler 1977, Ibrahim et al. 1996). Though the selective forces underpinning such variation were not explicitly investigated in the majority of cases, a contribution of directional selection to the differentiation among populations is often supposed (e.g. Chown and Klok 2003, Van Doorslaer and Stoks 2005).

The ability to adapt to different environments throughout a given species' range depends on the existence of variation at ecologically relevant loci (Veliz et al. 2004). Understanding the ecological processes influencing the maintenance of such variation, thereby dissolving the genetic framework of adaptive evolution, is one of the major goals of evolutionary biology (Fry et al. 2007). Thus, adaptive phenotypes or traits are convenient starting points for investigating adaptation at the genetic or genome level (Bonin et al. 2006), and identifying the precise molecular changes that contribute to adaptation remains one of the principal challenges (Hoekstra and Coyne 2007).

Several environmental factors impact on the physiology of individuals and pose selective pressures; however, temperature is thought to be one of the most important selective agents (Loeschcke et al. 2000). Clinal variation is of particular interest with respect to temperature adaptation as it provides an opportunity to disentangle the traits and genes associated with environmental conditions (Hoffmann and Weeks 2007). Some studies based on allozymes or DNA sequences already revealed associations between gene frequencies and clinal variation in environmental factors, such as temperature and salinity (reviewed in Eanes 1999, Watt 2000). Examples include the worldwide cline for ADH in *Drosophila melanogaster* presumably caused by thermal selection (Berry and Kreitman 1993, McKenzie et al. 1994). As another example, a tendency towards a higher genetic diversity was found in the fly *Scathophaga cynipsea* at higher altitudes, caused mainly by variation at the MDH locus potentially involved in stress adaptation (Kraushaar et al. 2002).

For the temperate-zone Copper butterfly *Lycaena tityrus* recent studies documented pronounced altitudinal differences in life-history traits, flight performance, temperature stress resistance and the expression of stress-inducible heat-shock proteins (Karl et al. 2008, in prep.), and associations between part of these traits and variation at the PGI locus (Karl et al. in press) above findings offer an outstanding opportunity to identify the molecular basis of thermal adaptation, by investigating the geographic distribution of PGI genotypes of known temperature stress resistance across a thermal gradient in nature.

We selected allozymes as molecular markers because the association between PGI variation and stress resistance is known in *Lycaena tityrus* (see above), because these markers are powerful tools for unravelling the molecular biogeography of butterflies (Schmitt and Seitz 2001a, Habel et al. 2005, Schmitt et al. 2005), and because there is evidence that at least some allozyme loci are under natural selection while other may be neutral (Berry and Kreitman 1993, Eanes 1999, Storz and Nachman 2003). We analysed 15 enzyme systems (representing 18 loci) in 18 populations of *Lycaena tityrus* from different altitudes, trying to determine the genetic structure across populations and detecting enzymes that are likely to be under natural selection. Specifically we investigate whether (i) PGI genotypes of greater cold stress tolerance show higher frequencies with increasing altitude, whether (ii) variation at this locus exceeds variation at other loci and / or whether (iii) PGI shows clinal variation with altitude not found in other loci.

Material and methods

Study organism and sampling populations

Lycaena tityrus (Poda, 1761) is a widespread temperate-zone butterfly ranging from Western Europe to central Asia (Ebert and Rennwald 1991). The species is bivoltine with two discrete generations per year in most parts of its range, although populations with one or three generations per year occur (Ebert and Rennwald 1991, Tolman and Lewington 1998). *Lycaena tityrus* hibernates as half-grown larva. The principal larval host-plant is *Rumex acetosa* L., but some congeneric plant species such as *R. acetosella* L. and *R. scutatus* L. are accepted as well (Ebert and Rennwald 1991, Tolman and Lewington 1998). For this study, *Lycaena tityrus* males were collected between 2004 and 2006 at 18 localities varying in altitude (for details see Table 1), and were afterwards stored in liquid nitrogen until electrophoresis. Populations were sampled in Germany (populations 1-5), North Tyrol / Austria (populations 6-11) and South Tyrol / Italy (populations 12-18). Samples were defined as low- (1-5), mid- (6, 8, 9, 12-15, 17) and high-altitude populations (7, 10, 11, 16, 18) according to altitude.

Table 1. Name, location and altitude of 18 collection sites of *Lycanea tityrus* in Germany (G), North Tyrol / Austria (NT) and South Tyrol / Italy (ST).

Population	Country	Area	Name	Altitude [m a.s.l]	Coordinates
1	G	Mecklenburg-Western Pomerania (MW)	Greifswald	0	54°02'59"N – 13°21'06"E
2	G	Rhineland-Palatinate (RP)	Salz	350	50°30'22"N – 07°57'39"E
3	G	Rhineland-Palatinate (RP)	Pottum	420	50°35'32"N – 08°00'06"E
4	G	Bavaria (BY)	Bayreuth	440	49°54'18"N – 11°37'26"E
5	G	Bavaria (BY)	Benediktbeuern	600	47°42'58"N – 11°22'41"E
6	NT	Pitz Valley (PiV)	Mittelberg	1730	46°57'29"N – 10°52'31"E
7	NT	Pitz Valley (PiV)	Taschachtal	2020	46°57'22"N – 10°51'17"E
8	NT	Oetz Valley (OV)	Kühtai	1500	47°13'43"N – 10°56'19"E
9	NT	Oetz Valley (OV)	Winterstall	1710	46°53'52"N – 10°57'26"E
10	NT	Oetz Valley (OV)	Vent	1930	46°51'29"N – 10°54'31"E
11	NT	Oetz Valley (OV)	Obergurgl	2050	46°51'41"N – 11°01'21"E
12	ST	Pflersch Valley (PflV)	Innerpflersch	1350	46°58'05"N – 11°19'46"E
13	ST	Pfossen Valley (PfoV)	Jägerruh	1680	46°44'02"N – 10°55'37"E
14	ST	Pfossen Valley (PfoV)	Central Valley	1780	46°44'27"N – 10°55'58"E
15	ST	Schnals Valley (SV)	Gerstberger Hof	1750	46°43'75"N – 10°48'42"E
16	ST	Schnals Valley (SV)	Kurzras	2010	46°45'13"N – 10°46'47"E
17	ST	Matsch Valley (MV)	Glieshof	1780	46°43'26"N – 10°40'39"E
18	ST	Matsch Valley (MV)	Matsch Alp	2010	46°44'35"N – 10°41'53"E

Electrophoresis

A total of 602 individuals were analysed with sample sizes per population ranging from 16 to 45 individuals. Half of the abdomen of the imagos were homogenised in Pgm-buffer (Harris and Hopkinson 1978) by ultrasound and centrifuged at 8,000 g for 5 min. Cellulose acetate plates were used for allozyme electrophoresis applying standard protocols (Hebert and Beaton 1993). We analysed 15 enzyme systems representing 18 loci (see Table 2 for loci studied and electrophoretic conditions).

Table 2. Electrophoretic conditions for the enzyme systems analysed in *Lycaena tityrus*. TC: Tris-citrate pH 8.2 (Richardson et al. 1986), TG: Tris-glycine pH 8.5 (Hebert and Beaton 1993), TM: Tris-maleic acid pH 7.0 (adjusted from TM pH 7.8; Richardson et al. 1986). All buffers were run at 200 V.

Enzyme	EC-No.	Number of loci	Buffer	Homogenate applications	Running time [min]
GOT	2.6.1.1	2	TC	3	60
FUM	4.2.1.2	1	TC	3	45
GAPDH	1.2.1.12	1	TC	4	40
ME	1.1.1.40	1	TC	3	40
G6PDH	1.1.1.49	1	TC	3	60
ACON	4.2.1.3	1	TC	3	45
MDH	1.1.1.37	2	TC	3	40
PK	2.7.1.40	1	TC	2	45
PEP$_{PhePro}$	3.4.11/13	1	TG	3	20
HBDH	1.1.1.1	1	TG	4	30
PGI	5.3.1.9	1	TG	1	40
PGM	5.4.2.2	1	TG	1	40
6PGDH	1.1.1.44	1	TM	3	60
IDH	1.1.1.42	2	TM	3	60
APK	2.7.3.3	1	TM	1	30

Statistics

The alleles were labelled according to their relative mobility, starting with "1" for the slowest. The allele frequencies were calculated with the package G-Stat (Siegismund 1993). Hardy-Weinberg equilibrium (Louis and Dempster 1987), genetic disequilibrium (Weir 1991), locus by locus *F*-statistics and analyses of molecular variance (AMOVAs) were calculated with the package Arlequin 2.000 (Schneider et

al. 2000), with F_{IS} representing the genetic variance component among individuals within populations, F_{ST} the genetic variance component among populations, F_{CT} the genetic variance component among groups of populations, and F_{SC} the genetic variance component among populations within groups of populations. The neighbour joining phenograms (Saitou and Nei 1987) were calculated from Nei's (1972) genetic distances, using the package PHYLIP (Felsenstein 1993). Bootstraps based on 1000 iterations were calculated with the same software. Differences in genetic parameters across population groups were analysed by Man-Whitney U-tests using Statistica 6.1. Differences in PGI allele frequencies across different altitudes were analyzed using analyses of variance (ANOVAs; JMP 4.0.0) with altitude as fixed effect. If not otherwise stated, means are given ± 1 SD throughout the text.

Results

Thirteen of the analysed loci were polymorphic for allele frequencies (see Appendix), whilst 5 loci (IDH2, MDH1, GAPDH, FUM, APK) were monomorphic. No significant deviations from Hardy-Weinberg equilibrium were detected after Bonferroni correction, and no significant linkage disequilibrium was observed for any locus over more than two populations (results not shown). For all polymorphic loci across all 18 populations the mean number of alleles per locus (A) was 1.76 ± 0.11 (ranging from 1.61 to 2.11), the expected heterozygosity (H_e) was 12.3 ± 1.5 % (ranging from 9.7 % to 15.5 %), the percentage of observed heterozygosity (H_o) was 12.2 ± 1.8 % (ranging from 9.0 % to 15.0 %), the total percentage of polymorphic loci (P_{tot}) was 49.7 ± 6.5 % (ranging from 33.3 % to 61.1 %), and the percentage of polymorphic loci with the most common allele not exceeding 95 % (P_{95}) was 35.5 ± 5.1 % (ranging from 27.9 % to 44.4 %; see Table 3 for details).

The total genetic variance was 1.1801, 93.3 % (1.0888) of which being found within individuals. Of the remaining among-individual genetic variance (0.0913), 83.8 % (0.0765) was found among populations, and 16.2 % (0.0148) within populations. The overall F_{ST} calculated across all 18 populations was 0.0648 ($P < 0.0001$), and the average F_{IS} across all polymorphic loci was 0.0134 ($P = 0.19$).

Table 3. Expected heterozygosity (H_e), observed heterozygosity (H_o), mean number of alleles per locus (A), percentage of polymorphic loci (P_{tot}), percentage of loci with the most common allele not exceeding 95% (P_{95}), and sample size (N) across 18 populations of *Lycaena tityrus.* For details on the populations analyzed see Table 1.

Population	H_e [%]	H_o [%]	A	P_{tot} [%]	P_{95} [%]	N
1	13.5	14.5	1.8	50.0	38.9	35
2	15.5	14.8	2.1	50.0	33.3	36
3	13.7	13.2	1.7	50.0	33.3	34
4	13.7	14.5	1.9	61.1	44.4	16
5	14.7	15.0	1.6	33.3	33.3	38
6	10.0	10.1	1.7	50.0	38.9	25
7	11.4	11.3	1.8	55.6	27.8	34
8	11.9	12.2	1.7	55.6	38.9	33
9	12.7	12.3	1.7	55.6	44.4	34
10	13.1	14.1	1.8	55.6	38.9	34
11	9.7	9.0	1.7	38.9	33.3	45
12	12.4	12.0	1.8	44.4	38.9	33
13	10.6	10.1	1.7	50.0	33.3	34
14	11.5	11.4	1.7	44.4	38.9	34
15	12.4	11.5	1.7	50.0	27.8	34
16	11.5	10.3	1.8	50.0	33.3	37
17	11.2	11.5	1.7	50.0	33.3	34
18	11.8	11.6	1.8	50.0	27.8	34
Means ± SD	12.3 ± 1.5	12.2 ± 1.8	1.76 ± 0.11	49.7 ± 6.5	35.5 ± 5.1	34 ± 6

A neighbour joining tree based on genetic distances (Nei 1972) showed a division into two main clusters, the alpine and the non-alpine populations (Fig. 1; F_{CT}: 0.0841; F_{SC}: 0.0297; both $P < 0.0001$). Expected heterozygosity (H_e) and observed heterozygosity (H_o) were significantly higher in non-alpine than in alpine populations, whilst A, P_{tot} and P_{95} did not differ across these two groups (Table 4). No significant differences in genetic parameters were found comparing north- (populations 6-11) and south-alpine populations (populations 12-18), mid- (6, 8, 9, 12-15, 17) and high-altitude populations (7, 10, 11, 16, 18), and different sample sites within valleys (all $P > 0.05$).

Fig. 1. Neighbour joining dendrogram based on the genetic distances (Nei 1972) of 18 populations of *Lycaena tityrus*. Abbreviations: G: Germany, A: Austria, I: Italy, BY: Bavaria, RP: Rhineland-Palatinate, MV: Mecklemburg-Western Pomerania, OV: Oetz Valley, PflV: Pflersch Valley, PiV: Pitz Valley, MV: Matsch Valley, SV: Schnals Valley and PfoV: Pfossen Valley (cf. Table 1). Values at the nodes of the branches indicate bootstrap percentages from 1000 iterations. Only values above 40% are given.

The overall differentiation between alpine and non-alpine populations was strongly related to variation in one single locus (PGI; F_{CT}: 0.4362, $P < 0.0001$). A neighbour joining tree based only on the genetic distances of PGI showed similar patterns to those obtained from all loci (see Fig. 1 and 2). Four PGI genotypes differed in their frequency significantly across low- (L), mid- (M) and high- (H) altitude populations, namely genotype 1-1 (L > M = H; Tukey HSD after ANOVA; $F_{2,18} = 19.2$, $P < 0.0001$), 1-2 (L > M = H; $F_{2,18} = 131.1$, $P < 0.0001$), 1-3 (L > M = H; $F_{2,18} = 14.3$, $P = 0.0003$) and 2-2 (L < M = H; $F_{2,18} = 84.2$, $P < 0.0001$; Fig. 3), while PGI genotypes 3-3, 1-4, 2-3, 2-4, 2-5 and 3-5 (all $P > 0.05$) did not.

Table 4. Means ± SD for genetic parameters across alpine and non-alpine populations of *Lycanea tityrus*. Differences were tested for significance with Mann-Whitney U-tests (significant *P*-values in bold). Abbreviations: H_e: percentage of expected heterozygosity, H_o: percentage of observed heterozygosity, *A*: number of alleles per locus, P_{tot}: total percentage of polymorphic loci, P_{95}: percentage of polymorphic loci with the most common allele not exceeding 95%, *N*: number of individuals. For definition alpine and non-alpine populations see text.

	Alpine populations	Non-alpine populations	P
H_e	11.6 ± 1.0	14.2 ± 0.9	**0.0014**
H_o	11.3 ± 1.3	14.4 ± 0.7	**0.0019**
P_{tot}	50.0 ± 5.1	48.9 ± 9.9	0.8825
A	1.74 ± 0.05	1.87 ± 0.25	0.4902
P_{95}	35.0 ± 5.3	36.6 ± 5.0	0.6574
N	34 ± 4	32 ± 9	0.5542

Within the alpine populations we found an F_{ST} of 0.0198 ($P < 0.0001$) based on differences among populations in 6PGDH (0.0489, $P < 0.0001$), ACON (0.0443, $P < 0.0001$) and PEP (0.0253, $P = 0.0020$). Within the non-alpine populations the F_{ST} was 0.0558 ($P < 0.0001$), mostly due to differences in PGM (0.1030, $P < 0.0001$), IDH1 (0.0753, $P = 0.0010$) and PGI (0.0506, $P < 0.0001$). We found no genetic differentiation between populations from the northern (North Tyrol) and southern Alps (South Tyrol; F_{CT}: 0.052, $P = 0.0518$) or between mid- and high-altitude populations ($F_{CT} < 0.001$, $P = 0.6500$). There was no significant differentiation within valleys (all $P > 0.05$), except from the Oetz Valley (F_{ST}: 0.0166, $P = 0.0176$).

Discussion

Genetic diversity and differentiation between populations

In the analysed *Lycaena tityrus* populations intrapopulational genetic diversity, namely the mean number of alleles per loci and the expected heterozygosity, was comparable to the values typically found in the Lepidoptera (Graur 1985). However, for lycaenids genetic diversity is known to be high (Marchi et al. 1996, Schmitt and Seitz 2001a, Schmitt et al. 2002), and in particular the mean number of alleles per locus (1.74) is lower than in other common lycaenids (e.g. *Polyommatus icarus*: 2.98, Schmitt et al. 2003; *Polyommatus coridon*: 2.72, Schmitt et al. 2002). High levels of intra-populational diversity are characteristic of species having an open population
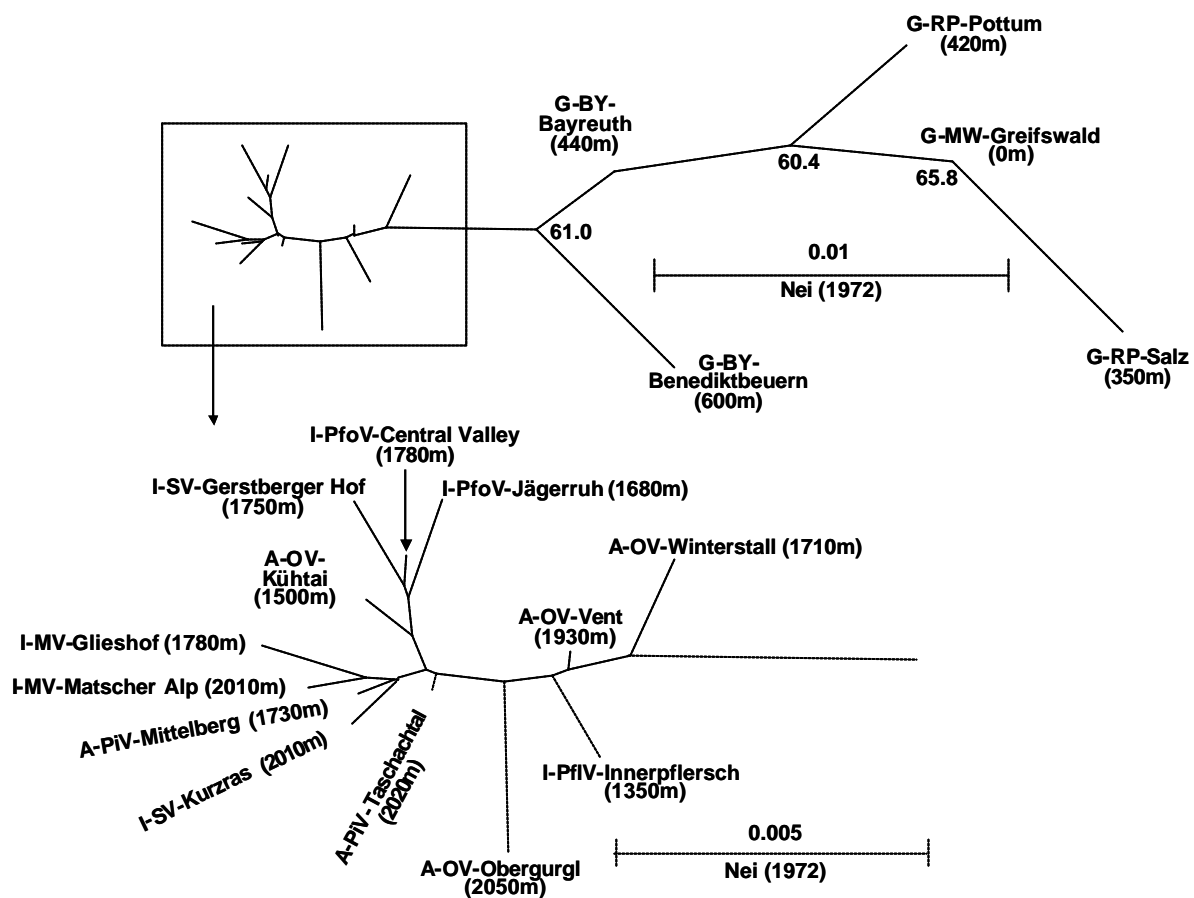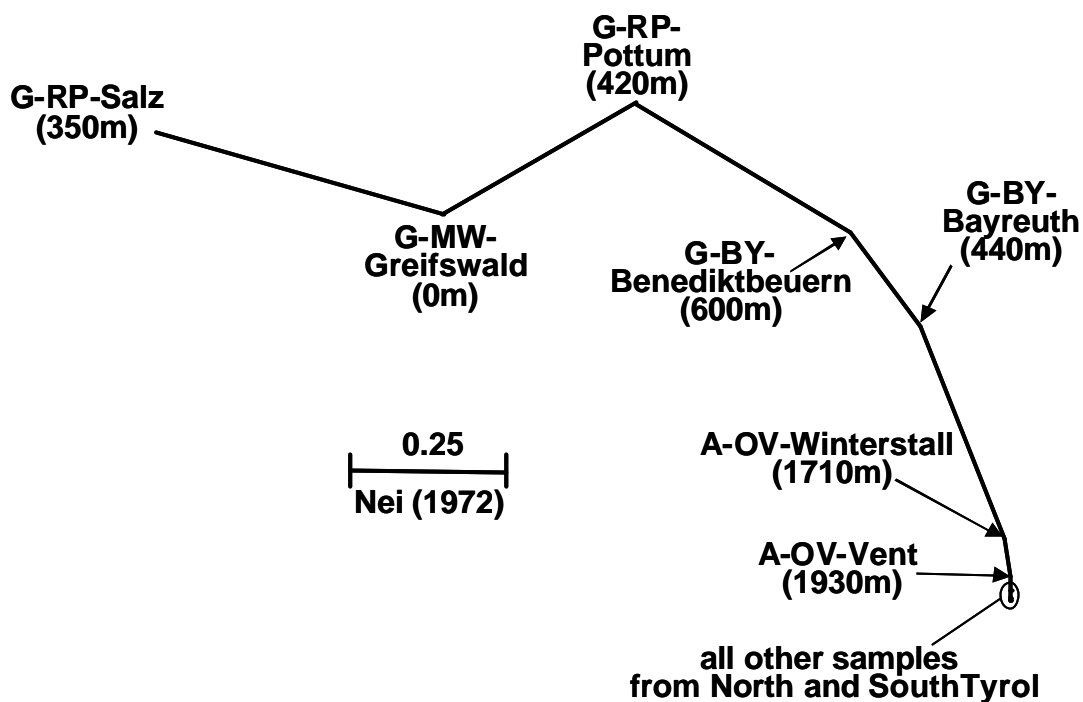
Fig. 2. Neighbour joining dendrogram based on the genetic distances (Nei 1972) of 18 populations of *Lycaena tityrus*, using only the PGI locus. For abbreviations see Fig. 1.
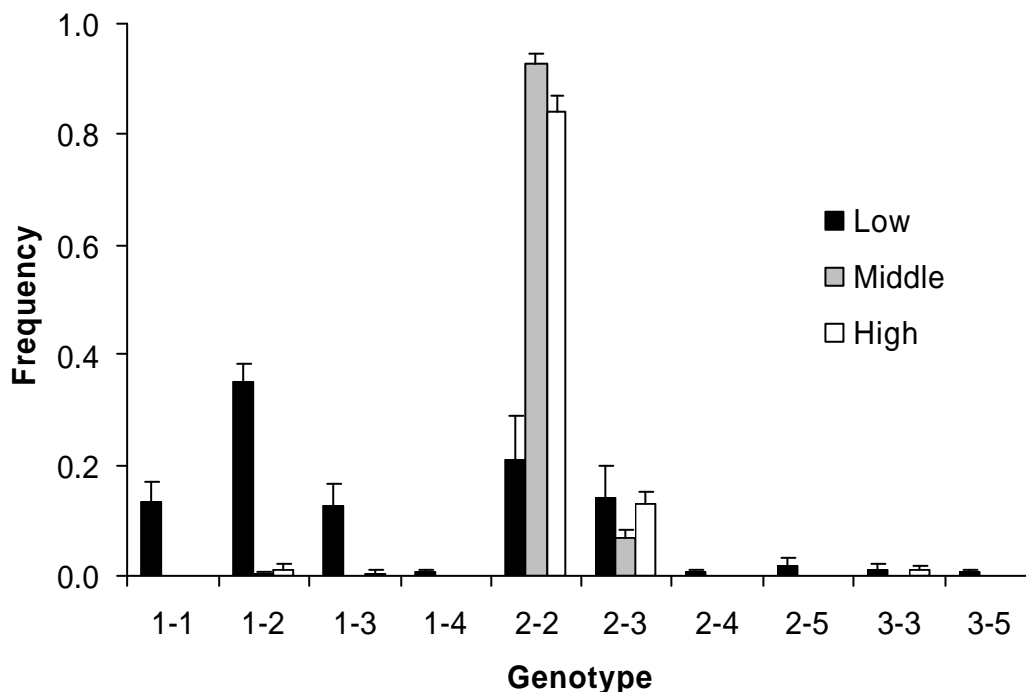
Fig. 3. Frequencies of PGI genotypes for low-, mid- and high-altitude populations of *Lycaena tityrus*. Given are means ± 1 SE. Data were pooled across replicate populations.

structure (Vanderwoestijne et al. 1999) and / or high local population densities (Schmitt et al. 2002). On the other hand, extremely low diversities as e.g. found in *Polyommatus coridon gennargentii* (Marchi et al. 1996) are a common feature of peripheral and isolated populations. The values found for *Lycaena tityrus* in comparison with other butterflies allow no general conclusions concerning size and isolation of populations, except that they appear intermediate between the above mentioned extremes (thus broadly matching the species' ecology; Ebert and Rennwald 1991, Tolman and Lewington 1998).

Although not as high as for example in *Erebia medusa* throughout large parts of Europe (F_{ST}-value 0.149; Schmitt and Seitz 2001b), the genetic differentiation among the studied *Lycaena tityrus* populations (F_{ST}-value 0.0648) is relatively high and within the range of other strongly differentiated species like *Euphydryas gilettii* (Debinski 1994) or *Polyommatus coridon* (Schmitt and Seitz 2001a). The neighbour joining tree for *Lycaena tityrus* based on genetic distances (Fig. 1) revealed a clear distinction of populations into two main clusters, an alpine and a non-alpine (lowland) one. Although there is still a fairly high variability among populations within the lowland cluster, the division into those clusters is clearly defined (see further below).

Interestingly, the expected and observed heterozygosity in *Lycaena tityrus* is lower in alpine than in lowland populations. Low heterozygosity is often associated with fitness costs (Ochando and Ayala 1999, Hotz and Semlitsch 2000). The results in *Lycaena tityrus* are in contrast with altitudinal patterns in the dung fly *Scathophaga cynipsea* showing a higher genetic diversity at higher altitudes (Kraushaar et al. 2002), and also with the intuitive assumption of a positive rather than a negative correlation with altitude, based on the harsher environmental conditions warranting high individual fitness. However, the pattern found in *Lycaena tityrus* seems to be related to variation at the PGI locus, with one homozygote genotype, PGI-2-2, dominating in all alpine populations, while lowland populations showed much more heterogeneous distributions with many heterozygotes.

Possible reasons for population differentiation in Lycaena tityrus
Geographical variation in allele frequency is often taken as evidence for the action of natural selection; however such patterns can also arise from population processes

and historical factors (Hoffmann and Weeks 2007). Good evidence for an impact of selective forces can be obtained from studies investigating fitness effects of certain alleles in a range of environmental conditions (Huestis and Marshall 2006). One of the best such examples is clinal variation of ADH alleles in *Drosophila melanogaster*, paralleled on different continents (Fry et al. 2007, Hoffmann and Weeks 2007). Further, the frequencies of IDH1 alleles in the cricket *Allonemobius socius* follow a gradient in mean annual temperature, indicating a contribution of natural selection (Huestis and Marshall 2006). On the other hand, geographic clines in allele frequencies may be related to post-glacial expansions (Schmitt and Seitz 2001a), and the strong differentiation of three lineages in the alpine-endemic butterfly *Erebia melampus* indicates a discontinuous distribution during the last ice age (Haubrich and Schmitt 2007).

Geographic differentiation in the *Lycaena tityrus* populations analysed is primarily caused by variation at the PGI locus. PGI is an enzyme involved in important glycolytic pathways being thus at the central point of all ATP-based energy supplies (Watt 1985). It has received much attention over the last decades and evidence for a covariance between environmental variables (especially temperature) and PGI allelic variation is accumulating (Watt 1992, Rank and Dahlhoff 2002, Watt et al. 2003, McMillan et al. 2005). As mentioned above, a recent study using *Lycaena tityrus* showed strong effects of PGI genotype on life-history traits and thermal tolerance (Karl et al. in press). Most importantly, the PGI 2-2 genotype, dominating in alpine in contrast to lowland populations, showed increased cold stress resistance and other features typical of high-altitude populations (Karl et al. in press), suggesting that PGI is under thermal selection in *Lycaena tityrus*.

This notion is further supported by the fact that effects of natural selection are generally locus-specific, whereas effects of migration, drift or inbreeding are expected to have relatively uniform effects across the entire genome (Storz and Nachmann 2003). The latter, however, is clearly not the case in *Lycaena tityrus*. Additionally, a literature survey for over 75 species indicated strong selection on the PGI locus, with the alleles moving faster in the electrophoresis often being associated with more stressful conditions (Riddoch 1993). In accordance, the PGI 2-2 genotype, containing the faster of the two most common alleles 1 and 2, increased

from 44 % in *Lycaena tityrus* lowland populations to up to 94 % in high-altitude populations. However, we found no significant differences in PGI genotypes between mid- and high-altitude populations within the Alps (range 1350-2050 m a.s.l.). Thus, given that geographic variation in PGI genotypes is caused by selection, a substantial change in selective forces is expected to occur between 600 m a.s.l. (highest lowland population) and 1350 m a.s.l. Anyway, investigating genetic differentiation without PGI does not reveal a division into an alpine and a lowland cluster anymore, and within the lowland populations there is a relatively high genetic differentiation, with no indication of continuous lineages. Thus, although our evidence is correlative in nature, there is quite strong support for the notion that the PGI locus is under thermal selection in *Lycaena tityrus*, and that this is the ultimate reason underlying the altitudinal genetic differentiation found. While such variation in allele frequencies may be caused by many ecological factors, temperature is a key environmental factor influencing virtually all aspects of the ecology and evolution of ectotherms, and is therefore thought to be one of the most important selective agents (e.g. Loeschcke et al. 2000, Hoffmann et al. 2003).

Conclusions

Understanding the ecological process of adaptation and population differentiation, in combination with unraveling the genes targeted by selection, lies at the heart of evolutionary biology (Fry et al. 2007). In the Copper butterfly *Lycaena tityrus*, we found a clear differentiation into two main clusters, a lowland and an alpine one, caused mainly by variation at a single locus, PGI. Consequently, it appears rather unlikely that the pattern found is caused by random processes (Hoffmann and Weeks 2007). This, however, does not rule out the involvement of historical events, which have probably impacted on the patterns found and may be responsible for the lack of differentiation within the alpine cluster. Thus, there may well be a distinct alpine lineage having evolved in allopatry. To our mind, though, the specific pattern of variation with altitude at the PGI locus found in *Lycaena tityrus* can hardly be explained by factors others than natural selection. The PGI genotype dominating in alpine populations is known to exhibit increased cold tolerance and other features typical of alpine populations, such that the PGI locus is an obvious target for selection (Karl et al. in press).

Acknowledgements

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Appendix: Allele frequencies for the 13 polymorphic (out of 18 analysed) loci across 18 populations of *Lycaena tityrus*. For further information on collection sites see text and Table 1.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
6Pgdh																		
1	0	0	0	0	0	0	0.015	0.091	0	0	0	0	0	0	0	0	0	0
2	1.000	0.941	1.000	0.969	1.000	1.000	0.985	0.909	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.986	1.000	0.985
3	0	0	0	0.031	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0.015
4	0	0.059	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IDH1																		
1	0	0	0	0	0	0.020	0	0	0	0.015	0	0	0.015	0	0	0	0	0
2	0.200	0.147	0.306	0.219	0.474	0.320	0.368	0.303	0.338	0.221	0.200	0.318	0.485	0.368	0.426	0.378	0.294	0.456
3	0.800	0.853	0.694	0.781	0.526	0.660	0.618	0.697	0.647	0.765	0.789	0.667	0.500	0.632	0.574	0.622	0.706	0.544
4	0	0	0	0	0	0	0.015	0	0.015	0	0.011	0.015	0	0	0	0	0	0
MDH2																		
1	0.031	0	0	0	0.029	0	0	0	0	0	0	0	0.029	0.015	0.015	0.014	0.044	0.074
2	0.969	1.000	0.972	0.938	0.943	0.935	0.956	0.953	0.956	1.000	1.000	0.924	0.971	0.941	0.956	0.865	0.941	0.882
3	0	0	0.028	0.063	0.029	0.065	0.044	0.047	0.044	0	0	0.976	0	0.044	0.029	0.122	0.015	0.044
G6PDH																		
1	0.086	0.242	0.222	0.167	0.346	0.160	0.221	0.121	0.235	0.338	0.222	0.242	0.132	0.118	0.162	0.125	0.279	0.221
2	0.914	0.758	0.750	0.833	0.654	0.840	0.779	0.879	0.765	0.662	0.778	0.758	0.868	0.882	0.838	0.875	0.721	0.779
3	0	0	0.028	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PK2																		
1	0	0	0	0.031	0	0	0	0	0.015	0.015	0	0	0	0	0.015	0	0	0
2	1.000	1.000	1.000	0.969	1.000	1.000	1.000	1.000	0.985	0.985	1.000	1.000	1.000	1.000	0.985	1.000	1.000	1.000

Continuation Appendix 1

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Hbdh																		
1	0.029	0	0.014	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0.086	0	0.056	0	0	0	0	0	0	0.029	0	0	0	0	0	0	0	0
3	0	0	0	0.031	0	0	0.015	0	0	0	0	0	0	0	0	0	0	0
4	0.886	0.985	0.931	0.969	1.000	1.000	0.985	1.000	1.000	0.971	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
5	0	0.015	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACON1																		
1	1.000	1.000	1.000	1.000	1.000	0.980	1.000	0.909	0.926	0.882	1.000	1.000	0.882	0.912	0.838	1.000	0.971	0.971
2	0	0	0	0	0	0.020	0	0.091	0.074	0.118	0	0	0.118	0.088	0.162	0	0.029	0.029
GOT1																		
1	0	0	0.014	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0.929	1.000	0.958	0.906	1.000	0.940	0.971	0.971	0.941	0.985	0.978	0.879	0.985	0.971	1.000	0.986	0.985	1.000
3	0.071	0	0.014	0.094	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0
4	0	0	0.014	0	0	0	0	0	0	0	0.011	0.045	0	0	0	0	0	0
5	0	0	0	0	0	0.060	0.029	0.029	0.059	0.015	0.011	0.076	0.015	0.029	0	0	0.015	0
GOT2																		
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0
2	0	0	0	0	0	0.040	0.044	0.074	0.088	0.088	0.167	0.045	0.074	0.118	0.015	0.014	0	0.029
3	0.957	0.985	0.958	0.906	1.000	0.960	0.956	0.926	0.912	0.912	0.833	0.939	0.912	0.868	0.985	0.959	0.956	0.956
4	0	0	0	0.063	0	0	0	0	0	0	0	0.015	0.015	0	0	0	0.044	0
5	0.043	0.015	0.042	0.031	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0.015

Continuation Appendix 1

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
ME																		
1	1.000	0.971	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
2	0	0.029	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pep																		
1	0.141	0.106	0.375	0.133	0.237	0.060	0.162	0.172	0.221	0.206	0.078	0.273	0.044	0.206	0.242	0.108	0.106	0.103
2	0.688	0.742	0.583	0.800	0.658	0.940	0.765	0.781	0.779	0.765	0.900	0.667	0.853	0.735	0.682	0.838	0.879	0.868
3	0.172	0.152	0.042	0.067	0.105	0	0.074	0.047	0	0.029	0.022	0.061	0.103	0.059	0.076	0.054	0.015	0.029
PGI																		
1	0.443	0.353	0.556	0.313	0.208	0	0	0	0	0.088	0	0	0	0	0.015	0.041	0	0
2	0.414	0.471	0.264	0.656	0.528	0.940	0.941	0.985	0.868	0.882	0.933	0.955	0.985	1.000	0.956	0.865	0.941	0.956
3	0.143	0.176	0.153	0.031	0.208	0.060	0.059	0.015	0.132	0.029	0.067	0.045	0.015	0	0.029	0.095	0.059	0.044
4	0	0	0.028	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0.056	0	0	0	0	0	0	0	0	0	0	0	0	0
PGM																		
1	0	0	0.014	0.031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0.061	0.059	0.069	0.031	0.231	0.040	0.015	0.088	0.118	0.191	0.122	0.091	0.029	0.029	0.059	0.056	0.088	0.118
3	0.803	0.471	0.708	0.813	0.718	0.700	0.721	0.691	0.809	0.721	0.744	0.803	0.750	0.750	0.676	0.681	0.559	0.618
4	0.015	0	0.014	0	0	0	0	0	0	0	0.011	0	0	0	0	0	0	0
5	0.106	0.471	0.125	0.125	0.051	0.220	0.235	0.206	0.059	0.088	0.111	0.076	0.221	0.221	0.265	0.264	0.338	0.235
6	0	0	0	0	0	0	0	0.015	0	0	0.011	0	0	0	0	0	0.015	0
7	0.015	0	0.069	0	0	0.040	0.029	0	0.015	0	0	0.030	0	0	0	0	0	0.029

7.2. PGI genotype affects life history traits and cold stress resistance in a Copper butterfly

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Abstract

Accumulating evidence suggests that the phosphoglucose isomerase (PGI) locus is under thermal selection. In the Copper butterfly *Lycaena tityrus* PGI allele frequencies show altitudinal variation, with a single genotype occurring in ca. 90 % of high-altitude animals. In low-altitude populations variation at this locus is much higher. Here, we investigate variation in life-history traits and temperature stress resistance across PGI genotypes in *Lycaena tityrus* from different lowland populations reared at two temperatures (19°C and 24°C). PGI genotype significantly affected larval and pupal development time, growth rate, pupal mass and chill-coma recovery time, but had no effect on heat knock-down resistance. The latter suggests that heat and cold stress resistance are based on differential mechanisms. As expected temperature also influenced all traits under investigation, its effect being more pronounced compared to that of PGI genotype (except for pupal mass). Patterns found for the PGI genotype dominating in high-altitude populations were consistent with those found for high-altitude animals. Therefore, and because of the direct link between PGI genotype and cold stress resistance, we conclude that PGI is likely to contribute to thermal adaptation in *Lycaena tityrus*. Genotypes promoting rapid development and largest body size were rather rare, suggesting weak selection on both traits and / or rather high associated costs.

Introduction

For several ectotherms it has been shown that allozyme variation correlates with variation in an array of fitness-related traits including morphological and physiological ones (e.g. Watt 1992, Neargarder et al. 2003, McMillan et al. 2005, Dahlhoff and Rank 2007, Saastamoinen 2007). Usually, those studies supposed the involvement of environmental variables as selective agents causing a given allozyme variation. Where alternative forms of enzymes exist (allelic products of a single locus), selection through temperature is particularly likely because of its effects on enzyme function and metabolic rates, in turn affecting fitness (Borrell et al. 2004, Ward et al. 2004). In recent years, the importance of environmental effects on the phenotypic expression of individual genes became increasingly clear (DeWitt and Scheiner 2004), and it is furthermore now generally accepted that genetic variation at single

loci may affect multiple phenotypic characters (Pigliucci 2004, Pigliucci and Preston 2004).

One possibility to detect interactions between the forces shaping phenotypic expression and those maintaining its underlying genetic variation is to look for variation at a specific gene locus and to identify its effects on the phenotype (Krause and Bricelj 1995). In this context, enzymes that are involved in important metabolic pathways, e.g. glycolytic enzymes, are of special interest, as glycolysis is the centre point of all ATP-based energy supplies (Watt 1985). One of these enzymes, phosphoglucose isomerase (PGI), has received much attention since the 1980s, and evidence for a covariance between environmental variables (especially temperature) and PGI is accumulating (Watt 1983, 1992, Rank and Dahlhoff 2002, Neargarder et al. 2003, McMillan et al. 2005). In *Colias* butterflies, for example, PGI genotypes differ dramatically in their enzyme-kinetic properties, thus affecting glycolytic fluxes and thereby flight performance (Watt 1983, 1992). Likewise, in the Glanville fritillary butterfly, PGI genotype has a significant effect on flight metabolic rate and concomitantly on dispersal rate (Haag et al. 2005). As another example, directional changes in PGI allele frequencies, coinciding with variation in HSP70 expression, temperature stress resistance, running speed, survival and fecundity were found in the leaf beetle *Chrysomelia aeneicollis* (Dahlhoff and Rank 2000, Rank and Dahlhoff 2002, Neargarder et al. 2003, McMillan et al. 2005, Dahlhoff and Rank 2007, Rank et al. 2007). However, apart from these studies, our knowledge of the consequences of variation at the PGI locus on life history and stress resistance traits is still very limited.

In the present study, we quantified variation in life-history and temperature stress resistance traits across different PGI genotypes in the butterfly *Lycaena tityrus*. For this species, we have found altitudinal variation in PGI allele frequencies, causing significant genetic differentiation between high- and low-altitude populations (Karl et al. unpublished). In the high-altitude populations, a single PGI genotype (PGI 2-2) was found in about 90 % of all animals, while low-altitude butterflies showed a much larger variation at the PGI locus (Karl et al. unpublished). Because of the strong covariance between temperature and geographic clines, temperature is generally believed to be one of the most important selective agents causing such clinal

variation (Loeschcke et al. 2000), which is supposedly also the case in *Lycaena tityrus*. Assuming that the dominance of the PGI 2-2 genotype in *Lycaena tityrus* high-altitude populations is causally related to thermal selection in these low-temperature environments, we test here whether PGI 2-2 butterflies (from lowland populations) show features typical of high-altitude butterflies, namely whether they show shorter development times, increased growth rates (but see Karl et al. 2008), larger body size, increased cold stress resistance, but decreased heat stress resistance as compared to other genotypes.

Material and methods

Study organism

Lycaena tityrus (Poda, 1761) is a widespread temperate zone butterfly, ranging from Western Europe to central Asia (Tolman and Lewington 1998). The species is bivoltine with two discrete generations per year in most parts of its range, although populations with one or three generations per year occur (Ebert and Rennwald 1991, Tolman and Lewington 1998). *Lycaena tityrus* hibernates as L3-larvae. The principal larval host-plant is *Rumex acetosa* L., but some congeneric plant species such as *R. acetosella* L. and *R. scutatus* L. are utilised as well, and may in some regions represent the main hosts (SBN 1987, Ebert and Rennwald 1991, Tolman and Lewington 1998). For this experiment, 18 freshly eclosed, mated females were caught in June 2007 in different bivoltine lowland populations in Germany (western Germany - 'Westerwald', north-eastern Germany - near the city of Greifswald, southern Germany – near Benediktbeuern) and transferred to Bayreuth University. Females from different populations were included to promote a high genetic diversity at the PGI locus in the offspring generation.

Experimental design

Oviposition and butterfly rearing

Field-caught females were kept in a climate chamber at 24°C and L18:D6 (24h light cycle) throughout. For oviposition they were placed individually in translucent plastic pots (1 L) covered with gauze, and were provided with *R. acetosa* (oviposition substrate), fresh flowers (*Crepis sp.*, *Achillea millefolium*, *Bistorta officinalis*, *Leucanthemum vulgare*) and a highly concentrated sucrose solution (for adult

feeding). Eggs were collected daily, pooled across females and transferred to small glass vials. After hatching, larvae were randomly divided among two climate chambers differing in rearing temperature (19°C and 24°C, respectively). Both climate chambers used are located within the same building next to each other. They are identical in terms of construction, lightning and air conditioning. Throughout, photoperiod was set at L18:D6, and relative humidity at 70 %. Larvae were reared in groups (10 individuals each) in translucent plastic boxes (500 ml), containing moistened filter paper and fresh cuttings of *R. acetosa* in ample supply. Boxes were checked daily and supplied with new food when necessary. Resulting pupae were weighed (to the nearest 0.01 mg; Sartorius microscale MC 210 P) and afterwards kept individually in numbered plastic pots (125 ml). Following adult eclosion, butterflies were kept individually in translucent plastic pots (250 ml) covered with gauze, and were provided with a highly concentrated sucrose solution for adult feeding until the start of stress tolerance assays (see below).

Data acquisition

Larval development time (from hatching to pupation), pupal mass, pupal development time and growth rate (calculated as quotient of pupal mass and larval developmental time) was recorded for all individuals. On day 2 after eclosion, butterflies were randomly assigned to either a cold or heat stress resistance assay. Cold stress resistance was determined as chill-coma recovery time (Hoffmann et al. 2002, Ayrinhac et al. 2004, Castañeda et al. 2005). Butterflies were placed individually in small translucent plastic cups (125 ml), which were arranged on a tray in a randomized block design. The tray was then exposed for 6 min to -20°C. This period was selected as preliminary studies showed that longer cold exposure induced significant mortality, and a shorter one very quick recovery. After cold exposure the trays were transferred to an environmental cabinet with a constant temperature of 20°C. Recovery time was defined as the time elapsed between taking the tray out of the freezer until a butterfly was able to stand on its legs. Only butterflies that had recovered within one hour were included in further analyses, as mortality is very high for butterflies with longer recovery times. Following recovery, butterflies were frozen at -80°C for later allozyme analyses.

Heat stress resistance was determined by using a knock-down assay (Sørensen et al. 2005). Butterflies were placed in small, sealed glass vials, which were submerged in a water bath kept at a constant temperature of 47°C (again in a randomized block design). Butterflies were continuously monitored and heat knock-down time (defined as the time until a butterfly was no longer able to stand upright) was recorded for each individual. To reduce mortality, knocked-down butterflies were immediately taken out of the water bath and, after a short recovery time (to be sure that the butterfly was still alive), they were frozen at -80°C for further analyses.

Electrophoresis

PGI genotype was analyzed for 1381 individuals. Therefore, half the butterflies' abdomen was homogenised in Pgm-buffer (Harris and Hopkinson 1978) by ultrasound and centrifuged at 8,000 g for 5 min. Cellulose acetate plates were used for electrophoresis applying standard protocols (Hebert and Beaton 1993). Samples were run in TG-buffer (Tris-glycine pH 8.5; see Hebert and Beaton 1993) at 200 V. The alleles were labelled according to their relative mobility, starting with '1' for the slowest.

Statistical analyses

Due to the presence of rare genotypes and concomitantly low sample sizes in some groups, life history and stress resistance data were only analyzed for the four most common PGI genotypes (1-1; 2-2; 1-2; 2-3). Despite this restriction, sample sizes were highly unbalanced owing to the differential frequency of genotypes. Nevertheless, all respective individuals were included in the statistical analyses, as analyses using similar group sizes (by drawing random samples from the two most frequent genotypes) yielded qualitatively identical results. Data were analysed using analyses of (co-)variance (AN(C)OVAs) with PGI genotype, temperature and sex as fixed effects. Pupal mass was added as covariate when analyzing stress resistance traits. Throughout, minimum adequate models were constructed by removing non-significant interaction terms. Pair-wise comparisons were performed employing Tukey's HSD. All statistcal tests were performed using JMP (4.0.0) or Statistica (6.1). Unless otherwise stated, least square means ± 1 SE are given throughout the text.

Results

The butterflies analyzed (*N* = 1381) represent 7 different PGI genotypes. The two most common genotypes, PGI 1-2 (*N* = 606) and PGI 2-2 (*N* = 486), represent 79.1 % of all individuals. They are followed by PGI 2-3 (*N* = 127), PGI 1-1 (*N* = 86), PGI 1-3 (*N* = 49), PGI 1-4 (*N* = 21), and PGI 3-3 (*N* = 6; cf. S1 for sample sizes and trait values across all genotypes and treatment groups). All results below are based on *N* = 1306 representing the four most common genotypes.

Effects of PGI genotype on life-history traits

All four life-history traits investigated showed significant variation across genotypes, rearing temperatures, and sexes, the only exception being that there was no sex difference in pupal mass (Table 1). Larval development time varied significantly between genotypes (PGI 1-2: 22.7 ± 0.1 days = PGI 2-2: 22.5 ± 0.1 days > PGI 1-1: 21.9 ± 0.2 days = PGI 2-3: 21.7 ± 0.1 days), rearing temperatures (19°C: 26.7 ± 0.1 days > 24°C: 17.7 ± 0.1 days), and sexes (females: 23.3 ± 0.1 days > males: 21.1 ± 0.1 days; Fig. 1a). The significant temperature by sex interaction for larval time indicates that the sex difference was slightly less pronounced at the higher (males 2.0 days faster) than at the lower temperature (males 2.4 days faster).

Patterns in growth rates were largely opposite to those found in larval time (genotypes - PGI 2-3: 6.40 ± 0.06 mg/day > PGI 1-1: 6.09 ± 0.08 mg/day = PGI 2-2: 5.91 ± 0.03 mg/day > PGI 1-2: 5.76 ± 0.03 mg/day; temperatures - 24°C: 7.3 ± 0.03 mg/day > 19°C: 4.8 ± 0.04 mg/day, sexes - males: 6.3 ± 0.03 mg/day > females: 5.7 ± 0.03 mg/day; Fig. 1b). The sex difference across temperatures was less pronounced at 19°C (males by 0.37 mg/day faster) than at 24°C (males by 0.80 mg/day faster). Further, a significant interaction between genotype and temperature indicates a more pronounced effect of temperature on growth rate in PGI 2-3 butterflies compared to other genotypes.

Regarding pupal time, PGI 2-3 butterflies (11.6 ± 0.07 days) showed a significantly shorter development time compared to PGI 1-1 (12.0 ± 0.08 days) and PGI 1-2-butterflies (11.9 ± 0.04 days), while PGI 2-2 butterflies (11.8 ± 0.04 days) did not differ from any other group. Further, development was significantly shorter at the higher (8.99 ± 0.04 days) than at the lower (14.68 ± 0.04 days) rearing temperature,

Table 1. AN(C)OVAs for the effects of PGI genotype, temperature and sex on life-history and stress-resistance traits in *Lycaena tityrus*. Pupal mass (covariate) was added as appropriate. Significant *P*-values are given in bold.

Traits and source	DF	MS	F	P
Larval time				
Genotype	3	109.6	17.3	**< 0.0001**
Temperature	1	13355.1	6321.1	**< 0.0001**
Sex	1	789.6	373.7	**< 0.0001**
Genotype x Temperature	3	3.3	0.5	0.6627
Genotype x Sex	3	5.5	0.9	0.4569
Temperature x Sex	1	8.2	3.9	**0.0492**
Genotype x Temp. x Sex	3	3.6	0.6	0.6358
Error	1289	2.1		
Growth rate				
Genotype	3	45.9	32.7	**< 0.0001**
Temperature	1	1076.0	2296.2	**< 0.0001**
Sex	1	56.2	119.9	**< 0.0001**
Genotype x Temperature	3	7.0	4.9	**0.0019**
Genotype x Sex	3	0.6	0.4	0.7186
Temperature x Sex	1	7.3	15.7	**< 0.0001**
Genotype x Temp. x Sex	3	1.6	1.1	0.3444
Error	1289	0.5		
Pupal time				
Genotype	3	12.6	5.6	**0.0009**
Temperature	1	5458.5	7202.3	**< 0.0001**
Sex	1	34.7	45.8	**< 0.0001**
Genotype x Temperature	3	3.9	1.7	0.1591
Genotype x Sex	3	0.7	0.3	0.8228
Temperature x Sex	1	3.2	4.2	**0.0392**
Genotype x Temp. x Sex	3	4.8	2.1	0.0988
Error	1289	0.8		
Pupal mass				
Genotype	3	5967.4	16.4	**< 0.0001**
Temperature	1	720.6	5.9	**0.0150**
Sex	1	105.7	0.9	0.3511
Genotype x Temperature	3	254.0	0.7	0.5540
Genotype x Sex	3	123.7	0.3	0.7968
Temperature x Sex	1	23.2	0.2	0.6623
Genotype x Temp. x Sex	3	237.6	0.7	0.5819
Error	1289	121.5		

Continuation Table 1

Traits and source	DF	MS	F	P
Chill-coma recovery time				
Genotype	3	909206.8	4.1	**0.0070**
Temperature	1	237327.6	3.2	**0.0399**
Sex	1	182290.0	2.5	0.1180
Genotype x Temperature	3	167288.3	0.7	0.5229
Genotype x Sex	3	41226.4	0.2	0.9068
Temperature x Sex	1	106805.7	1.4	0.2313
Genotype x Temp. x Sex	3	158236.8	0.7	0.5469
Pupal mass	1	9563.1	0.1	0.7201
Error	633	74399.0		
Heat knock-down time				
Genotype	3	88437.2	0.6	0.5919
Temperature	1	126212.8	2.7	**0.0494**
Sex	1	3848598.6	83.0	**< 0.0001**
Genotype x Temperature	3	78752.2	0.6	0.6373
Genotype x Sex	3	121983.1	0.9	0.4524
Temperature x Sex	1	337502.8	7.3	**0.0071**
Genotype x Temp. x Sex	3	99442.5	0.7	0.5431
Pupal mass	1	942221.3	20.3	**< 0.0001**
Error	638	46341.0		

and was shorter in males (11.63 ± 0.04 days) than in females (12.04 ± 0.04 days; Fig. 1c). Sex differences were slightly smaller at 19°C (males by 0.3 days faster) than at 24°C (males by 0.5 days faster; indicated by a significant temperature-by-sex interaction). Pupal mass finally was more than 5 % higher in PGI 2-3 than in PGI 1-2 butterflies (PGI 2-3: 130.9 ± 0.9 mg > PGI 1-1: 128.2 ± 1.2 mg ≥ PGI 2-2: 126.2 ± 0.5 mg > PGI 1-2: 123.9 ± 0.4 mg), was higher at 24°C than at 19°C (24°C: 128.4 ± 0.5 mg > 19°C: 126.3 ± 0.5 mg), but did not differ between the sexes (Fig. 1d).

Effects of PGI genotype on stress resistance

Chill-coma recovery time varied significantly between genotypes and temperatures, but was not affected by sex or the covariate pupal mass (Fig. 2a, Table 1). PGI 2-2 animals (406.8 ± 17.7 sec) showed by more than 15 % reduced recovery times compared to PGI 1-2 animals (484.6 ± 15.7 sec), while PGI 1-1 (508.7 ± 39.4 sec) and PGI 2-3 butterflies (489.7 ± 34.5 sec) did not differ significantly from any other
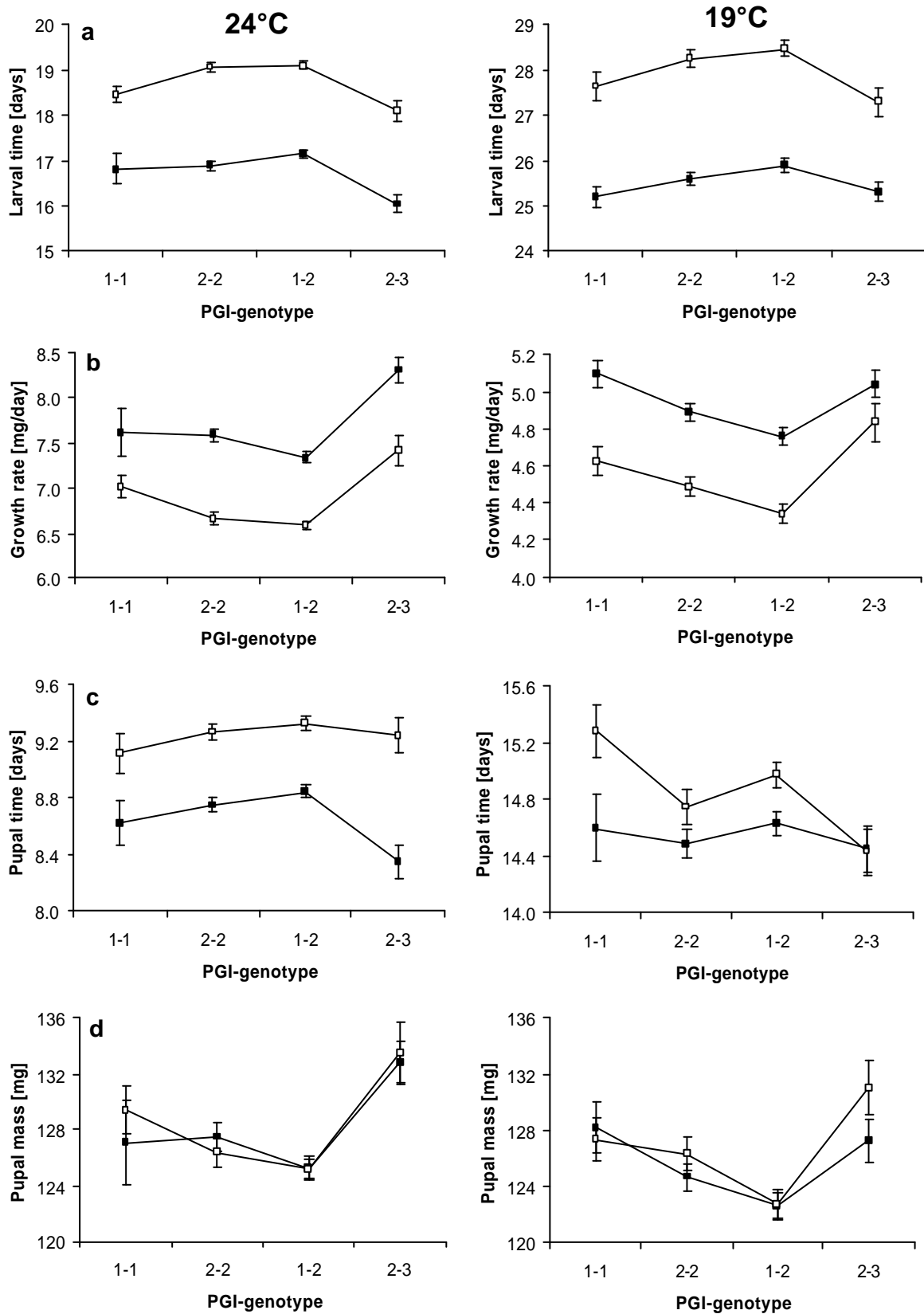
Fig. 1. Means (± 1 SE) for larval time (a), larval growth rate (b), pupal time (c) and pupal mass (d) for *Lycaena tityrus* males (black symbols) and females (white symbols) across four PGI genotypes and two rearing temperatures (19°C and 24°C). Note the partly different scales on the Y-axis.

group despite of having the longest mean recovery times (but note the substantial within-group variation, obviously caused by relatively low sample size; see S1). Further, the animals reared at the lower temperature (430.8 ± 17.6 sec) showed a reduced recovery time (> 15 %) compared to the ones reared at the higher temperature (514.0 ± 18.3 sec).
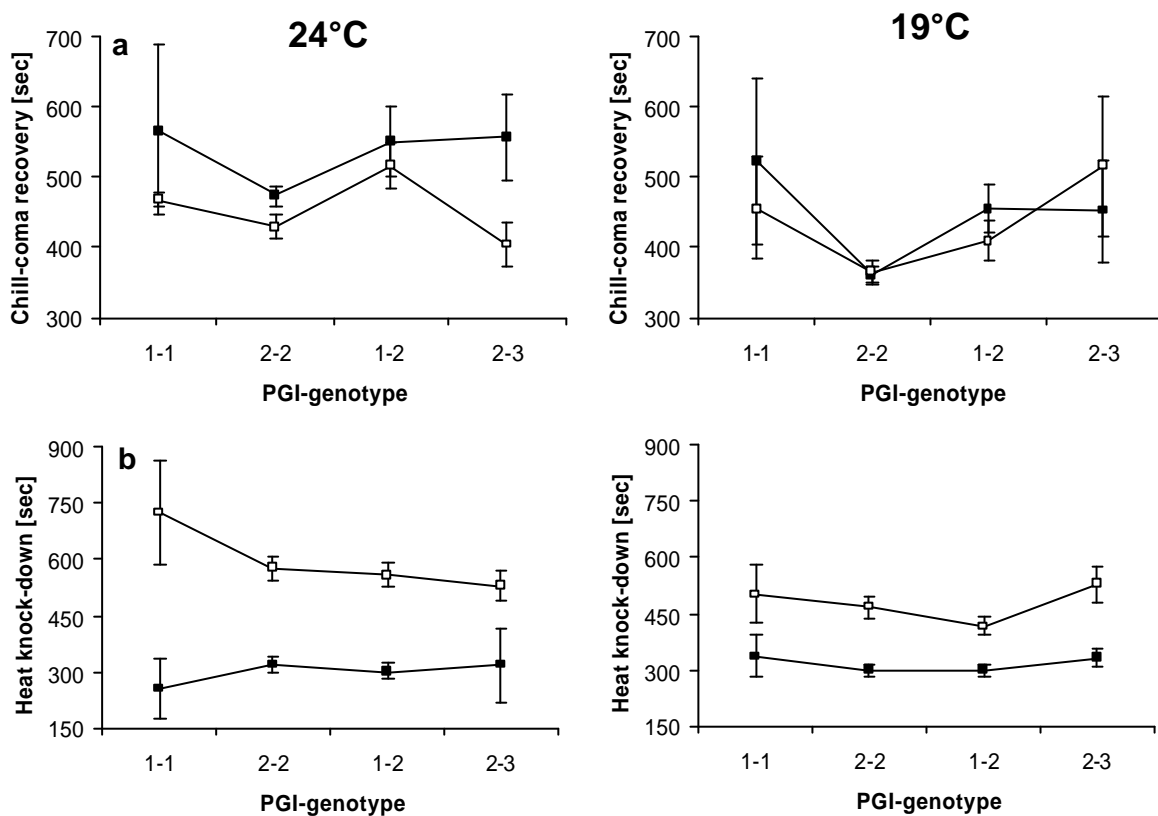

Fig. 2. Means (± 1 SE) for chill-coma recovery time (a) and heat knock-down time (b) for *Lycaena tityrus* males (black symbols) and females (white symbols) across four PGI genotypes and two rearing temperatures (19°C and 24°C).

In contrast, heat knock-down time was not affected by PGI genotype, but was more than 10 % shorter in animals reared at the lower (393.0 ± 14.5 sec) versus the higher temperature (444.0 ± 14.9 sec; Fig. 2b), and nearly 40 % shorter in males than in females (315.9 ± 14.9 sec vs. 521.1 ± 14.5 sec). The latter difference was much more pronounced at the higher compared to the lower temperature, as evidenced by a significant temperature by sex interaction (knock-down time was 260.0 sec longer in females compared to males at the higher temperature, but only 151.3 sec longer at the lower temperature). Further, there was a significant impact of the covariate pupal

mass, with larger individuals being on average more resistant to heat stress than smaller ones ($R = 0.186$, $P < 0.0001$, $N = 655$).

Discussion

A wealth of studies shows correlations between variation in life history traits and individual fitness (e.g. Munch and Conover 2003, Fischer et al. 2004, Berger et al. 2006), and at least some of such fitness-related traits vary with PGI genotype (e.g. Neargarder et al. 2003, McMillan et al. 2005, Dahlhoff and Rank 2007, Saastamoinen 2007). If a single locus shows patterns of variation being discordant with other loci, natural selection is likely acting on that locus (Slatkin 1987). Here, we demonstrate a direct link between variation in life-history and stress resistance traits and PGI genotypes in the Copper butterfly *Lycaena tityrus*. Note in this context that hardly any data have been published to date on the potential association between developmental traits and PGI genotype.

Effects of PGI genotype on life-history traits

PGI genotype considerably affected larval and pupal development time, growth rate, and pupal mass in *Lycaena tityrus*. As PGI 2-2 is the dominant genotype found in high-altitude butterflies (Karl et al. unpublished), a reduced development time with concomitantly higher growth rates was expected for this compared to other genotypes. Such results were for instance obtained for *Drosophila buzzatii* populations from the coolest highland localities (Bubliy and Loeschcke 2005). Our results on *Lycaena tityrus*, however, show that the high-altitude genotype had intermediate to long development times. This can be explained by the fact that indeed high-altitude populations do exhibit longer development times in *Lycaena tityrus*, presumably caused by a change in voltinism (low-altitude populations are bivoltine, while high-altitude ones are monovoltine; Karl et al. 2008). Of course, developmental traits may be affected by a large number of different genes (Chippindale et al. 2003), but the trend towards relatively long development times in addition to the pronounced overall variation across genotypes clearly suggests that PGI does affect developmental pathways (including growth rates, see below). Similarly, variation in development time among PGI genotypes was shown in the leaf beetle *Chrysomelia aeneicollis*, though such differences were thought to be related to

differences in the expression of heat shock proteins rather than PGI genotype per se (McMillan et al. 2005).

It is a common belief that "faster is better" in ecology and evolutionary biology, and accumulating evidence suggests that growth rate in itself is a target of natural selection (Arendt 1997, Nylin and Gotthard 1998, Munch and Conover 2003). Although it is intuitively appealing that consequently growth rates should be maximized, recent studies convincingly implicate that growth rates are optimized rather than maximized (Arendt 1997, Nylin and Gotthard 1998). This in turn implies that fast growth carries costs such as a lower viability (Chippindale et al. 1997), a higher weight loss during metamorphosis (Fischer et al. 2004), or a higher predation risk (Gotthard 2000, Munch and Conover 2003). Against this background it is interestingly to note that in *Lycaena tityrus* growth rates in PGI 1-1 and PGI 2-3 butterflies, having low frequencies of 6 and 9 %, were clearly higher than in PGI 1-2 (43 %) and PGI 2-2 butterflies (35 %). This may suggest, in line with the above considerations, that selection is not favoring genotypes promoting fast development.

A large number of studies, many of which were concerned with clinal variation, suggest that ectotherms tend to be larger in colder environments (James and Partridge 1995, James et al. 1995). However, recent work indicates that this may not necessarily be the case, and that many different outcomes are possible (Blanckenhorn 1997, Blanckenhorn and Demont 2004). In Copper butterflies, different studies could not find an association between the temperature conditions at the place of population origin and body size (Karl et al. 2008). Accordingly, high-altitude genotypes (PGI 2-2) were not of particularly large size in this study.

Another interesting pattern is that there was no trade-off between fast growth and body size, i.e. slow-growing individuals did not become large, and fast-growing ones not small (cf. Blanckenhorn 1999, Davidowitz et al. 2004). In contrast, PGI 2-3 individuals with the highest growth rates also showed the highest pupal masses, while PGI 1-2 butterflies with the lowest growth rates were smallest. It remains unclear though why the (under laboratory conditions) obviously highly efficient genotype PGI 2-3 is relatively rare (9 %) in nature. Furthermore, the two most common genotypes were smallest in size, suggesting that the costs associated with

achieving and/or maintaining large body size largely outweigh any potential benefit such as increased fecundity or mating success (Roff 1992, Blanckenhorn 2000).

Effects of PGI genotype on temperature stress resistance

In addition to life-history traits, PGI genotype also affected cold but not heat stress resistance. The genotype dominating in high-altitude populations (PGI 2-2) exhibited the shortest chill-coma recovery times. This is consistent with an increased cold stress resistance in high-altitude *Lycaena tityrus* populations (Karl et al. 2008), and suggests that the PGI locus is under thermal selection (see also Watt 1994, Dahlhoff and Rank 2000, McMillan et al. 2005). The mechanisms underlying the association between PGI variation and cold resistance are not yet known for *Lycaena tityrus*. In the willow leaf beetle, however, allelic variation at the PGI locus across a latitudinal gradient is linked to variation in the expression of HSP70 (Dahlhoff and Rank 2000). This may also apply to *Lycaena tityrus*, as significant differences in HSP70 expression between high- and low-altitude individuals were recently found (Karl et al. unpublished).

In contrast, despite variation in heat stress resistance across high- and low-altitude *Lycaena tityrus* populations (Karl et al. 2008), no significant variation across PGI genotypes in heat resistance were detected in this study. Thus far, however, there is no evidence for a causal link between knockdown resistance and chill-coma recovery, and further our findings corroborate the notion that the mechanisms underlying increased cold tolerance are at least partly uncoupled from the mechanisms increasing heat tolerance (see also Chown 2001, Klok and Chown 2003, Sørensen et al. 2005). Certainly, PGI is not the only locus under thermal selection, but other yet unknown loci may also contribute to thermal adaptation, some of which may cause the reduced heat resistance in high-altitude populations. Finally, an association between PGI and heat resistance cannot be excluded, as different methods to assess thermo-tolerance may involve different genetic pathways (Folk et al. 2006).

Effects of rearing temperature and sex on life-history traits and temperature stress resistance

The differences across sexes and rearing temperatures were largely consistent with previous results (Fischer and Fiedler 2000, Karl and Fischer 2008, Karl et al. 2008). As expected for an ectothermic organism, development times decreased with increasing temperature, accompanied by increased growth rates (see also Blanckenhorn 1997, Fischer et al. 2003, Burke et al. 2005, Van Doorslaer and Stoks 2005). In contrast to earlier findings (Fischer and Fiedler 2000, Karl and Fischer 2008, Karl et al. 2008), individuals reared at the lower temperature did not achieve a higher pupal mass, though this effect can be restricted to the adult phase due to differential weight losses during metamorphosis (Fischer et al. 2004). As predicted from protandry theory (Fagerström and Wiklund 1982), males showed generally shorter development times than females. Further, lower temperatures caused increased cold, but reduced heat stress tolerance. Such plastic responses to the prevailing temperature conditions were also found in a number of other studies (e.g. Ayrinhac et al. 2004, Hoffmann et al. 2005, Zeilstra and Fischer 2005, Karl and Fischer 2008). While there was no sex difference in chill-coma recovery time, heat tolerance was reduced in males as compared to females. Similar patterns, again indicating different underlying mechanisms, were also obtained in other studies (e.g. Chen and Walker 1994, Gilchrist et al. 1997, Sørensen et al. 2001, Folk et al. 2006, Jensen et al. 2007).

Conclusions

Our study adds to the accumulating evidence that PGI is a locus under thermal selection (Watt 1983, 1994, Dahlhoff and Rank 2000, McMillan et al. 2005). In extension to previous studies, we show that PGI not only affects cold tolerance, but also all life-history traits under investigation here (viz. development time, growth rate, pupal mass). Given the large variation in such traits associated with variation at the PGI locus, PGI can be considered a pleiotropic gene of large effect (although genes linked to the PGI locus may also contribute to the variation found). Most interestingly, the patterns caused by variation in PGI genotype are in broad agreement with those across high- and low-altitude populations, i.e. the PGI genotype dominating in high-altitude populations showed increased cold tolerance and rather long development times associated with rather low growth rates (cf. Karl et al. 2008). This genotype is

also present in low-altitude populations (~ 35%). Its increase to ca. 90 % in the high-altitude populations is, based on the current results, likely to be caused by thermal selection on the PGI (and possibly associated) locus (see also Rank and Dahlhoff 2002). This strongly supports the notion that the PGI locus is heavily involved in thermal adaptation in arthropods (Neargarder et al. 2003, McMillan et al. 2005), although it is not related to heat stress tolerance in *Lycaena tityrus*. Future studies will focus on the mechanisms underlying the association between PGI genotypes and cold tolerance, involving the expression of stress-inducible heat shock proteins.

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Supplementary material:

Appendix S1: Means (± 1 SE) for life-history and stress resistance traits for *Lycaena tityrus* males and females across PGI genotypes and two rearing temperatures. PGI 3-3 is not shown because of low sample size.

		24°C						19°C					
		Males			Females			Males			Females		
	PGI	N	Mean	SE	N	mean	SE	N	mean	SE	N	mean	SE
Larval time [days]	1-1	16	16.81	0.33	17	18.47	0.17	25	25.20	0.24	28	27.64	0.31
	2-2	146	16.88	0.08	125	19.07	0.11	117	25.61	0.14	98	28.26	0.17
	1-2	167	17.14	0.09	179	19.10	0.09	137	25.90	0.15	123	28.48	0.18
	2-3	23	16.04	0.19	29	18.10	0.22	38	25.32	0.22	37	27.30	0.32
	1-3	13	17.69	0.50	14	18.57	0.27	12	25.33	0.40	10	28.90	0.53
	1-4	5	17.20	0.37	9	18.89	0.39	5	26.60	0.93	2	28.00	0.00
Growth rate [mg/day]	1-1	16	7.62	0.27	17	7.01	0.12	25	5.09	0.07	28	4.63	0.08
	2-2	146	7.59	0.07	125	6.66	0.07	117	4.89	0.05	98	4.49	0.05
	1-2	167	7.34	0.06	179	6.58	0.05	137	4.76	0.05	123	4.34	0.05
	2-3	23	8.31	0.15	29	7.42	0.17	38	5.04	0.07	37	4.83	0.10
	1-3	13	7.10	0.27	14	6.94	0.19	12	5.07	0.11	10	4.54	0.14
	1-4	5	7.28	0.42	9	6.38	0.17	5	4.32	0.20	2	4.10	0.32
Pupal mass [mg]	1-1	16	127.06	3.04	17	129.36	1.74	25	128.23	1.79	28	127.36	1.57
	2-2	146	127.48	1.01	125	126.34	0.99	117	124.67	0.99	98	126.32	1.20
	1-2	167	125.27	0.82	179	125.20	0.78	137	122.63	0.94	123	122.76	1.11
	2-3	23	132.85	1.48	29	133.48	2.18	38	127.26	1.53	37	131.05	1.93
	1-3	13	124.38	2.94	14	128.28	1.85	12	128.15	2.32	10	130.64	2.46
	1-4	5	124.75	5.73	9	120.16	2.44	5	114.42	4.52	2	114.81	5.93

Continuation Appendix S1

	PGI	24°C Males N	Mean	SE	24°C Females N	mean	SE	19°C Males N	mean	SE	19°C Females N	mean	SE
Pupal time [days]	1-1	16	8.63	0.15	17	9.12	0.15	25	14.60	0.24	28	15.29	0.18
	2-2	146	8.75	0.05	125	9.27	0.06	117	14.49	0.11	98	14.74	0.12
	1-2	167	8.84	0.05	179	9.33	0.05	137	14.63	0.09	123	14.98	0.09
	2-3	23	8.35	0.12	29	9.24	0.13	38	14.45	0.17	37	14.43	0.17
	1-3	13	8.46	0.14	14	9.00	0.18	12	14.42	0.29	10	14.80	0.33
	1-4	5	9.00	0.33	9	9.00	0.00	5	14.60	0.24	2	13.50	0.50
Heat knock-down [sec]	1-1	6	255.0	80.6	9	725.0	139.0	12	337.1	53.5	11	502.7	77.3
	2-2	77	318.5	19.5	64	576.7	33.4	61	300.0	16.8	48	467.4	30.9
	1-2	88	300.5	22.8	85	556.2	33.0	66	298.8	15.2	64	417.7	27.2
	2-3	8	317.5	98.0	19	529.7	41.2	16	330.6	24.4	21	528.6	48.9
	1-3	6	284.2	41.2	9	583.3	121.7	7	260.0	62.5	4	367.5	82.5
	1-4	2	100.0	10.0	3	736.7	270.7	1	90.0	0.0	2	550.0	285.0
Chill-coma recovery [sec]	1-1	10	566.0	120.6	8	468.1	11.1	13	521.5	116.6	17	455.3	73.0
	2-2	69	473.0	13.3	61	429.2	17.2	56	360.6	14.3	50	365.4	15.8
	1-2	79	550.8	50.0	94	515.5	31.9	71	454.4	35.5	59	409.7	28.7
	2-3	15	556.7	61.5	10	404.0	31.8	22	451.1	72.4	16	515.6	99.6
	1-3	7	614.3	52.2	5	419.0	35.9	5	352.0	27.8	6	568.3	167.8
	1-4	3	426.7	39.3	6	474.2	36.0	4	586.3	208.8	0		

Publication list

Chapters 5, 6.1, 6.2, 7.1 and 7.2 have been published or are under consideration for publication in international peer-reviewed journals as follows:

Chapter 5 – The mechanistic basis of the temperature-size-rule

Karl, I. and Fischer, K. (2008). Why get big in the cold? Towards a solution of a life-history puzzle. Oecologia 155: 215-225.

Chapter 6 – Altitudinal patterns in traits potentially related to thermal performance

Chapter 6.1

Karl, I., Janowitz, S.A. and Fischer, K. (2008). Altitudinal life-history variation and thermal adaptation in the copper butterfly *Lycaena tityrus*. Oikos 117: 778-788.

Chapter 6.2

Karl, I., Sørensen, J.G., Loeschcke, V. and Fischer, K HSP70 expression in the Copper butterfly *Lycaena tityrus* depends on altitude and temperature. In revision.

Chapter 7 – The genetic background of altitudinal variation in life-history and temperature stress resistance traits

Chapter 7.1

Karl, I., Schmitt, T. and Fischer, K. Genetic differentiation between alpine and lowland populations of a butterfly is caused by variation at the PGI locus. Submitted.

Chapter 7.2

Karl, I., Schmitt, T. and Fischer, K. (in press). PGI genotype affects life history traits and cold stress resistance in a Copper butterfly. Functional Ecology doi: doi:10.1111/j.1365-2435.2008.01438.x.

Record of contributions to this thesis

All experiments (including experimental design, execution and analyses) and the survey of relevant literature were conducted by myself or under my direct supervision – if not stated otherwise (see below). Chapters 1 to 4 of this thesis, providing a general introduction, the synopsis and the summary, as well as all published articles summarised in Chapters 5, 6 and 7 were written by myself.

All subchapters of Chapters 5, 6 and 7 have been published, are accepted or submitted for publication in international peer-reviewed journals with the following <u>co-authors:</u>

Prof. Dr. Klaus Fischer: He is the supervisor of my thesis and co-author of all publications. He contributed support and supervision in all stages of the projects, discussion of experimental designs, analyses and results and critical comments on the first drafts of the chapters.

Chapter 6.1 Susann Janowitz carried out *experiment 2* (analyses of flight performance and morphological traits) under my direct supervision (as a student research project).

Chapter 6.2 This work was done in cooperation with **Dr. Jesper G. Sørensen** and **Prof. Dr. Volker Loeschcke**. They supervised me during my stay in Aarhus, Denmark and instructed me how to measure the expression of heat-shock proteins.

Chapter 7.1 and 7.2 Analysis of allozymes was done in cooperation with **Dr. Thomas Schmitt**, University of Trier**.**

Experiments presented in Chapters 5, 6 and 7 were supported by the following <u>student assistants and colleagues</u>:
Chapter 5 Experiments were conducted and analysed by myself with the help of Jana Perlick, who aided with feeding larvae, and PD Dr. M.W. Lorenz, who helped with protein analyses.

Chapter 6.1 Experiments were conducted and analysed by myself, assistance for feeding the larvae was provided by S.S. Bauerfeind and C. Pflicke. The experimental part investigating flight duration and morphological traits in *experiment 2* was done by Susann Janowitz as a student research project (see above).

Chapter 6.2 Experimental assistance in butterfly rearing was provided by I. Thamke.

Chapter 7.1 Experiments were conducted and analysed by myself, assistance was povided by S.S. Bauerfeind, J. Hager, S.A. Janowitz, M. Weibart, and I. Zeilstra, all of whom helped collecting butterflies for allozyme analyses.

Chapter 7.2 Experiments were conducted and analysed by myself.

Isabell Karl

Bayreuth, June 2008

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Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

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