Thermal adaptation in insects:
Effects of inbreeding, environment and selection

Inauguraldissertation
zur
Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer.nat.)
an der Mathematisch-Naturwissenschaftlichen Fakultät
der
Ernst-Moritz-Arndt-Universität Greifswald

vorgelegt von
Anneke Dierks
geboren am 27.03.1976
in Neunkirchen am Brand

Greifswald, Mai 2012

Dekan: Prof. Dr. Klaus Fesser

1. Gutachter: Prof. Dr. Klaus Fischer
2. Gutachter: Prof. Dr. Klaus H. Hoffmann

Tag der Promotion: 12.10.2012

Contents

1. Introduction

1. Introduction

Environmental stress and the need for (thermal) adaptation

Biotic and abiotic conditions are not constant over time, so the long-term persistence of any given species or population depends on its ability to respond to environmental change (Bijlsma and Loeschcke 2005, 2012; Malcolm 2011). Among the associated environmental stressors challenging a species` survival, temperature can be considered a particularly important one (e.g. Hoffmann et al. 2003; Sørensen et al. 2005; Dahlhoff and Rank 2007). The importance of an individual's temperature stress resistance will further increase in the future due to global warming, causing a raise in mean temperatures but also in temperature extremes, which may comprise the largest anthropogenic challenge ever placed on natural systems (Sala et al. 2000; Thomas et al. 2004; Diffenbaugh et al. 2005, 2007; Deutsch et al. 2008).

Phenotypic plasticity and/or genetic adaptation

According mechanisms to adjust phenotypic values to environmental conditions, including behavioural, physiological and molecular ones, are indeed generally found (Hoffmann et al. 2003; Sørensen et al. 2003; Chown and Terblanche 2007). Conceptually, they can be categorized into two classes: longer-term genetic adaptation or phenotypic plasticity (Bradshaw 1965; Pigliucci 2001). Phenotypic plasticity allows a quick response to different environmental conditions, as a genotype can produce different phenotypes (Travis 1994; West-Eberhard 2003), but may be alternatively a non-adaptive biochemical or physiological interaction of an organism with its environment (Bradshaw 1965; Pigliucci 2001). Genetic adaptation in contrast needs longer periods of time. Here, allele frequencies change as a result of the selection pressure exerted by the environment (e.g. Bijlsma and Loeschcke 2005; David et al. 2005; Sørensen et al. 2005). However, long-term exposure to a constant environment may lead to fixation of alleles being favorable in that environment, while the same alleles may be disadvantageous in novel environments (Via and Hawthorne 2002). Thus, genetic variation might be reduced after a long period in this constant environment

preventing adaption to novel environments (Barrett and Bell 2006). Although phenotypic plasticity allows for maximal flexibility, plastic responses seem to involve non-trivial costs, as it is not generally favored by selection (DeWitt et al. 1998; Relyea 2002; Pigliucci 2005). In contrast, it has been repeatedly suggested that genetic adaptation may come at reduced costs, thus being favoured in stable environments (DeWitt et al. 1998; Relyea 2002; Aubret and Shine 2010). This notion, however, has been challenged by some recent findings (Meyer and Di Giulio 2003; Bourguet et al. 2004). Therefore, the relative importance of genetic adaptation versus phenotypic plasticity remains a matter of controversial discussion (e.g. Ayrinhac et al. 2004, Samietz et al. 2005).

Constraints to adaptation

Inbreeding and genetic variation

Inbreeding, the mating between closely related individuals, exerts direct detrimental effects on e.g. survival rate, reproduction and development in naturally outbreeding species (Darwin 1876; Charlesworth and Charlesworth 1987; Frankham et al. 2002; Frankham 2005). These detrimental effects, known as inbreeding depression, are caused by increased levels of homozygosity, implying either an increased expression of deleterious recessive alleles (partial dominance hypothesis) or an increased homozygosity at loci with heterozygote advantage (overdominance hypothesis; Charlesworth and Charlesworth 1999; Pedersen et al. 2008; Vermeulen et al. 2008; Charlesworth and Willis 2009). Because of the expected increase in deleterious alleles, inbred individuals are predicted to be more sensitive to environmental stress than outbred individuals (Lynch and Walsh 1998; Armbruster and Reed 2005; Fox and Reed 2010; Fox et al. 2011). Indeed, experimental studies found often an increase in inbreeding depression in more stressful environments, although there are exceptions and even contrary findings as well (Bijlsma et al. 1999; Dahlgaard and Hoffmann 2000; Keller and Waller 2002; Armbruster and Reed 2005; Martin and Lenormand 2006; Waller et al. 2008; Fox and Reed 2010; Fox et al. 2011). Therefore, it is important to consider

‘genetic’ and environmental stress together as both may interactively decrease population fitness (Kristensen et al. 2003).

Beside the direct detrimental effects and enhanced sensitivity to environmental stress, inbreeding is thought to hamper the evolutionary potential, because sufficient genetic variation is supposed to be of crucial importance for adaptation to new environmental conditions (Falconer and Mackay 1996; Roff 1997; Bijlsma and Loeschke 2005; Bouzat 2010), as the presence of beneficial variants within a population seem to play a major role (Blows and Hoffmann 2005; Orr and Unckless 2008; Bijlsma and Loeschcke 2012). A reduction in effective population size may reduce genetic variation through inbreeding or genetic drift and thus diminish the evolutionary potential (Bijlsma et al. 1997; Frankham et al. 2002, Frankham 2005; Willi et al. 2006; Keyghobadi 2007; Vandergast et al. 2007; Mikkelsen et al. 2010; Bijlsma and Loeschcke 2012).

Adding further complexity, survival rates of inbred populations are difficult to predict due to ‘purging’, i.e. selection against deleterious recessive alleles (Templeton and Read 1984; Keller et al. 1994; Hedrick and Kalinowski 2000; Crnokrak and Barrett 2002). Nevertheless, the effectiveness of purging is still under debate. Beside the beneficial influence, effects of purging often turned out to be small or even contrary (e.g. Saccheri et al. 1996; Meffert and Regan 2006; Fox et al. 2008; Mikkelsen et al. 2010). This may arise from e.g. variation in the rate of inbreeding, stochastic effects associated with bottlenecks, the specific genetic background, the environment in which inbreeding occurs, immigration, mutation, and population size (Hedrick and Kalinowski 2000; Wang 2000; Leberg and Firmin 2008; Mikkelsen et al. 2010). Thus, purging may have limited success in variable environments when applied to small populations (Jamieson et al. 2003; Boakes et al. 2007).

Genetic trade-offs

Perhaps the most obvious limit to genetic adaptation are negative genetic correlations (caused by antagonistic pleiotropy), in which a beneficial genetic change in one trait is linked to a detrimental change in another (Stearns 1989; Roff 2002). Over recent decades our

knowledge on genetic trade-offs has much increased, especially due to the use of artificial selection experiments, representing the most straight-forward approach (e.g. Bell and Koufopanou 1986; Brakefield 2003; Czesak and Fox 2003; Bubliy and Loeschcke 2005; Bertoli et al. 2010 Fischer et al. 2006; Bauerfeind and Fischer 2007). For instance, artificial selection in *Drosophila melanogaster* revealed a strong trade-off between larval development time and adult weight (Nunney 1996), and between cold tolerance and starvation resistance (Hoffmann et al. 2005). In general, there is agreement that such negative genetic correlations among traits related to fitness have the potential to maintain genetic variation within populations, and more importantly to bias or constrain evolutionary change (Cheverud 1984; Roff 2002). While such genetic trade-offs have been quite frequently measured among different traits (see above), hardly any data is available on potential trade-offs across developmental stages (but e.g. Tucic 1979; Cheverud et al. 1983; Loeschcke and Krebs 1996). Throughout ontogeny, though, selection at any age is expected to result in correlated responses in all other life-history stages. Such genetic influences may on principle yield (1) consistent effects throughout development, (2) divergent patterns across developmental stages, or (3) phenotypic effects in one or two consecutive ages only (Cheverud et al. 1983).

Study organisms: The butterfly *Bicyclus anynana* and the fly *Protophormia terranovae*

All studies except one used the butterfly *Bicyclus anynana* (Butler, 1879) (Lepidoptera Nymphalidae) as model organism. For studying the effects of photoperiod, the temperate fly *Protophormia terranovae* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae) has been used.

B. anynana is a tropical, fruit-feeding butterfly distributed from South Africa to Ethiopia (Larsen 1991). It exhibits two seasonal morphs as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen et al. 2004). Reproduction is confined to the warmer, wet season, when oviposition plants are available and where 2-3 generations occur. During the colder dry season reproduction pauses and butterflies do not start to mate before the first rains announce the beginning of the next wet season (Brakefield 1997; Windig et al. 1994). A

laboratory stock population was established at Greifswald University, Germany, in 2007. The origins of this population lead back to a stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from 80 gravid females caught at a single locality in Malawi. Since then, several hundred individuals are reared in each generation to avoid inbreeding and to maintain high levels of heterozygosity (Van't Hof et al. 2005; Bauerfeind and Fischer 2007). For this study butterflies from the Greifswald stock population were used.

P. terranovae is a widespread temperate-zone fly with a holarctic distribution (Byrd and Castner 2001). It is a multivoltine species developing up to six generations per year in Central Europe (Gautier and Schumann 1973). Life-cycles last between ca. 9 (35°C) and ca. 38 (15°C) days (Grassberger and Reiter 2002). *P. terranovae* is considered to be the most cold-tolerant of all calliphorid species. It can withstand extreme temperatures and can be found as close as 550 miles from the North Pole (Byrd and Castner 2001). Adults feed on nectar, preferring flowers of Apiaceae, larvae feed on various sources of organic, protein-rich matter such as carcasses (Conn 2006). A stock population at Greifswald University, which exists for at least 200 generations, originated from wild-caught individuals caught in the vicinity of Greifswald. In order to avoid detrimental effects of inbreeding and laboratory adaptation, several 100 individuals are reared each generation and several wild-caught individuals are added once a year. Stock flies are bred at 20-25°C under ambient day-light conditions. For oviposition, flies are continuously offered fish heads. Larvae are fed with a mixture of bran (ca. 75%), pork blood and sawdust (the latter to maintain appropriate levels of humidity) ad libitum. Adults are fed with highly concentrated sugar solution and milk.

Objective and key elements of this thesis

Using insects as model organisms, this study focuses on thermal adaptation through plastic and/or genetic adjustments in ectotherms. The ability to withstand (thermal) stress is considered to be crucial for an individual`s fitness and a species survival, especially in times of global warming and the associated changes in the frequency and severity of thermal

stress (Wilmer et al. 2000; Bijlsma and Loeschcke 2000; Chown and Terblanche 2007; Marshal and Sinclair 2010). But given that temperature has always been fluctuating and temperature extremes occur more or less in all ecosystems, organisms needed to develop effective mechanisms to ensure survival under such stressful conditions. In contrast to the effects of ambient temperature, other environmental factors potentially affecting temperature stress resistance have recently received less attention (Andersen et al. 2010). Under these circumstances, chapter 5.1 and chapter 5.2 of this thesis investigate phenotypic adjustments in temperature stress resistance following different temperature- and other environmental manipulations (e.g. food limitation, photoperiod, time of day) in the butterfly *B. anynana* and the fly *P. terranovae*, addressing the following question:

To what extent is stress resistance plastic and reversible and is there an influence of other environmental factors on temperature stress resistance?

Chapter 5.1 and Chapter 5.2

Additionally, many species are confronted with a human induced loss and fragmentation of natural habitats reducing population sizes. Therefore, genetic diversity might be reduced through inbreeding and/or genetic drift (Bijlsma et al. 1997; Frankham et al. 2002) and thus hamper a species ability to cope with changing conditions. This might increase the extinction risk of small populations (Frankham 1995; Bijlsma et al. 2000; Swindell and Bouzat 2006; Bouzat 2010). While the detrimental impact of inbreeding has often been documented, (e.g., Darwin 1876; Crnokrak and Roff 1999; Armbruster et al. 2000; Carr and Eubanks 2002) its consequences for the ability to cope with temperature stress are still poorly understood. Whereas chapter 6.1 is concerned with investigating the effect of inbreeding on acute stress tolerance in comparison to the effect of inbreeding on life history traits, chapter 6.2 is engaged in exploring the effect of inbreeding on evolutionary potential (here the ability to increase cold resistance). Specifically, I addressed the following questions:

Does inbreeding increase the thermal sensitivity to environmental stress?

Chapter 6.1

Is response to selection on cold tolerance constrained by inbreeding?

Chapter 6.2

Another, well-known limit to genetic adaptation are negative genetic correlations (caused by antagonistic pleiotropy). While our knowledge on genetic trade-offs has much increased, especially due to the use of artificial selection experiments, only a few studies have targeted genetic correlations across developmental stages (but e.g. Tucic 1979; Cheverud et al. 1983; Loeschcke and Krebs 1996). Against this background, the last chapter investigates genetic links between the expressions of a specific phenotype at different time points in the ontogeny:

Does selection on increased cold tolerance in the adult stage confer resistance throughout development?

Chapter 6.3

2. Synopsis

2. Synopsis

2.1 Plastic responses: Environmental effects on temperature stress resistance

2.1.1 Environmental effects on temperature stress resistance

In a series of experiments we here investigated phenotypic adjustment in temperature stress resistance following environmental manipulations in the butterfly *Bicyclus anynana*. Therefore, organisms were acclimated to different temperatures and/or put under different hardening temperatures. Resistance was tested using either cold- or heat shocks. Additionally, the influence of other factors (larval/ adult food stress, age, time of day) on thermal resistance was tested.

Chill coma recovery time is frequently used as a proxy for cold resistance. Testing different assay conditions of inducing a chill coma (with a temperature ranging between +1°C and -8°C and exposure times between 15 min and 19 h) clarified that the acclamatory response is largely independent from the method used. Therefore, the use of rather artificial assay conditions may not be that critical for measuring plastic responses to different thermal environments. Additionally, our data show that chill coma recovery time is closely related to the survival rate after cold exposure (Rako and Hoffmann 2006). In nature, quick plastic responses to colder temperature might be related to longer activities in the evening or earlier activities in the morning, elongating times of mating, foraging and reproduction.

Cooler compared to warmer acclimation temperatures generally increased cold but decreased heat stress resistance and vice versa. Thus, there has been an adaptive response to changing conditions (Rako and Hoffmann 2006; Karl et al. 2008; Rajamohan and Sinclair 2009). Acclamatory response to cold stress in *B. anynana* is fairly fast: The temperature experienced the last 24 hours had the dominating effect while previous temperatures experienced seem to have only a subtle, modulating effect. Similar to cold stress resistance, acclamatory response in heat stress resistance can be expected to occur

within 24 hours. Here, cold exposure (19 h, 1°C) can interfere with acclimation to warmer temperatures, but does not cause a diminishing performance in general.

In contrast, short time hardening responses revealed more complex patterns. Cold stress resistance turned out to be highest at intermediate hardening temperatures. While 20°C had a positive effect on cold tolerance, cooler (although at least 13°C sometimes occurring in the field) and warmer temperatures on the contrary negatively affected cold resistance, turning out to be stressful for this tropical butterfly and partly reflecting results obtained in *Drosophila* (30). 20°C in contrast is well within the range of temperatures frequently experienced by the butterflies in the field (reflecting the daily highs during the cooler dry season (Windig et al. 1994; Brakefield 1997).

Only ‘hardening’ at fairly high temperature (39°C) showed a positive effect on heat tolerance compared to butterflies ‘hardened’ at 20°C while other ‘hardening’ temperatures within the range did not. Therefore, there seem to be different mechanisms between hardening response and (longer-term) acclimation response (Macdonald et al. 2004; Sinclair and Roberts 2005; Rako and Hoffmann 2006). The hardening response to heat stress appears to be a sort of emergency mechanism, being activated under acute heat stress only (Sejerkilde et al. 2003; Jensen et al. 2007; Rajamohan and Sinclair 2009). On the other hand, exposure time of one hour or recovery time may have been too short (Kellett et al. 2005) to induce a measurable response. Further, the results indicate that different mechanisms seem to underlie heat and cold hardening, as the same hardening temperatures (20°C, 27°C and 34°C) produced a clear response in cold stress resistance.

In contrast to expectations, cold resistance slightly increased during the first eight days of adult life. While this result is in contrast with results on heat shock survival in various insects (Sørensen and Loeschcke 2002; Pappas et al. 2007) and the result obtained in chapter 2.3.2., the few data available for effects of age on chill-coma recovery time are contradictory and show no consistent pattern (Bowler and Terblanche 2008; Lalouette et al. 2010).

Adult food stress had a negative effect on heat but not on cold stress resistance. Additionally, larval feeding treatment showed interactive effects with adult feeding for heat but not for cold stress resistance, indicating that nitrogenous larval resources may set an upper limit to performance under heat stress. Nevertheless, best performance showed those butterflies that had neither suffered as larvae nor as adults. The difference between heat and cold resistance suggests that mechanisms increasing heat resistance may be more costly than those increasing cold resistance and are strikingly different (Sørensen et al. 2003; Sinclair and Roberts 2005; Overgaard et al. 2005 2008, Bowler and Terblanche 2008; Andersen et al. 2010). While the basic mechanism underlying heat stress resistance seems to be the heat shock response, cold stress resistance seems to involve several mechanisms including cryoprotectants, antifreeze proteins, glycerol, heat-shock proteins and changes in membrane fluidity and composition (Sinclair and Roberts 2005).

Light cycle had marginal effects on temperature stress resistance only. While heat stress resistance was not affected by light cycle, cold stress resistance varied significantly across time of day. As cold resistance during day-time and especially in the afternoon was higher compared to the night, this might suggest an adaptive pattern assuring higher levels of activity despite unfavorably cool weather conditions (although butterflies seem to rely in the first place on their cryptic coloration here, thus avoiding unnecessary flight (Brakefield and Reitsma 1991; Brakefield 1997.

In summary, this study clearly found evidence for environmentally induced variation in temperature stress resistance thus supporting the beneficial acclimation hypothesis, which has been repeatedly challenged over recent years (Wilson and Franklin 2002; Woods and Harrison 2002). Plasticity is thus an effective tool to greatly modulate temperature stress resistance within very short periods of time, thus increasing survival probability under temperature stress (Sejerkilde et al. 2003; Ayrinhac et al. 2004, Loeschcke and Hoffmann 2007; Rajamohan and Sinclair 2008). However, resistance traits are not only affected by ambient temperature, but also by, e.g., food availability and age, making their measurement challenging. The latter effects are largely underexplored and deserve more future attention.

Owing to their magnitude, plastic responses in thermal tolerance should be incorporated into models trying to forecast effects of global change on extant biodiversity.

Although the methods used were highly artificial, we think they have been close enough to resemble natural conditions: All acclimation temperatures used are within the range of temperatures experienced by *B. anynana* in its natural environment, which is also true for the majority of 'hardening' temperatures (Brakefield and Reitsma 1991; Brakefield 1997). Even the heat shock temperature of 45°C can be sometimes reached by solar radiation close to the ground. One exception is the temperatures used to induce a chill coma, but we could also demonstrate that the patterns obtained are largely independent from the assay conditions.

Nevertheless, as plastic responses may thus modify phenotypes to environmental needs including thermal challenges, the potential costs associated with plastic responses or more plastic genotypes remains a recurrent and still largely unresolved issue in evolutionary biology (Auld et al. 2010). Additionally, we suggest investigating factors like limited resource availability should be further included in future research, as they may influence plastic response and might go hand in hand with climate change due to the impact of man. Therefore, another environmental factor which caught our interest is photoperiod, for which some studies suggest an important role in mediating thermal resistance (e.g. Mahoney and Hutchinson 1969; Ansart et al. 2001; Goldman 2001; Udaka et al. 2008) and has become the topic of our next study.

2.1.2 Effects of photoperiod on temperature stress resistance in a fly

Here, we again have been engaged exploring phenotypic adjustments in temperature stress resistance, focusing on the effects of photoperiod (and temperature) on thermal resistance (and life history traits). Light conditions provide a constant measure indicating the advancement of seasons. Thus shorter day lengths, indicating cooler seasons, should promote more cold-resistant phenotypes (Kimura et al. 1994; Hori and Kimura 1998) and

vice versa. However, data on photoperiodic effects on heat resistance are still scarce (but e.g. Sørensen and Loeschcke 2002) and we are not aware of any study that has investigated the effects of different photoperiods exclusively in the adult stage in any insect.

Photoperiod affected development time and body size, the significance of which is currently unclear. Variation in development times were partly contradictory (reduced larval time accompanied with higher growth rates versus prolonged pupal time at shorter day length) and thus overall negligible. Variation in body size across photoperiods and temperatures showed patterns according to the 'temperature size rule' (see also Atkinson 1994; Angilletta et al. 2004; Karl and Fischer 2008) meaning pupal- as well as adult mass were higher at the shorter day length and at the lower temperature and vice versa.

Our study shows that variation in temperature stress resistance can be triggered by photoperiod, with shorter day lengths inducing more cold- and longer day lengths more heat-tolerant phenotypes. Such plastic changes can be induced during development but also (at least for cold tolerance) in the adult stage. In line with this interpretation it was recently shown that seasonal variation in heat and cold tolerance in a slug corresponds with changes in ambient temperatures, and is caused by both temperature and photoperiodic cues (Udaka et al. 2008). We also found plastic changes to temperature happen much faster than changes to photoperiod. Typically, plastic responses to temperature in stress resistance traits can be induced within hours (rapid hardening response; e.g. Hoffmann et al. 2003; Loeschcke and Hoffmann 2007; Overgaard and Sørensen 2008; Rajamohan and Sinclair 2009; Fischer et al. 2010). The fact that responses to photoperiod take longer was not unexpected, as photoperiod unlike temperature does not show very rapid and sometimes unpredictable changes, but alters exclusively in a gradual manner. Seasonal adjustment in contrast to buffering detrimental effects of sudden heat or cold waves simply does not require particularly fast responses. We suggest that this pattern represents adaptive seasonal plasticity. The mechanisms promoting variation in thermal resistance do not seem to be directly linked to the processes causing reproductive diapause as plastic changes were not related to different developmental pathways (reproductive activity or reproductive diapause).

Photoperiodic information seems to be used here as an indicator of the advancement of seasons very similar to life cycle decisions such as diapause induction (Denlinger 1991; Danks 1994, 2005; Kimura et al. 1994; Hori and Kimura 1998; Schmidt et al. 2005, 2008), suggesting adaptive phenotypic plasticity. As mentioned in the previous study, the adaptive value could be related to higher levels of activity at suboptimal temperatures, providing more time for essential behaviors such as foraging, mate location and reproduction (Sørensen and Loeschcke 2002). Moreover, both traits are closely related to survival rates following temperature stress (Rako and Hoffmann 2006; Fischer et al. 2010). Beside the influence of different environmental factors on thermal resistance, reduced genetic diversity might be another factor playing an important role in a species ability to cope with changing conditions. In the next chapter we therefore explored the influence of reduced genetic diversity on thermal resistance using different levels of inbreeding.

2.2 Effects of inbreeding on life history traits and temperature stress resistance

As global warming, loss and fragmentation of natural habitats will jointly challenge a species ability to survive, we considered genetic constraints and environmental stress (inbreeding; temperature shocks) together as both may interactively reduce population fitness (Bijlsma et al. 1997; Kristensen et al. 2003; Chown and Terblanche 2007) To understand better the degree to which inbreeding causes negative effects on temperature stress resistance, we compared the effects of inbreeding on temperature stress resistance and life-history traits. In contrast to other studies (using in general 4–5 full-sib matings; see also Dahlgaard et al 1995; Pedersen et al. 2005), we decided to use low levels of inbreeding (1 and 2 full-sib matings) as this ought to be more ecologically relevant (Mikkelsen et al. 2010)

We measured a total of seven traits (5 life-history traits, 2 temperature stress resistance traits) and found five to be negatively affected by inbreeding: Egg hatching success, larval time, larval growth rate, pupal mass and cold stress resistance. The results for life-history traits largely resemble those obtained in previous studies on various organisms like flies,

crickets, beetles or butterflies (Roff 1998; Haikola 2001; Fox and Scheibly 2006; Mikkelsen et al. 2010). Especially the strong effect of inbreeding on egg hatching success demonstrates a large genetic load carried by *B. anynana*, a result also obtained in a previous experiment by Saccheri 1996.

Interestingly, only cold- but not heat stress resistance was affected by inbreeding. However, the few studies (using *Drosophila* as model organism) concerned with observing variation in stress tolerance in relation to inbreeding did come to similar results: no effect of inbreeding on heat tolerance, but on cold tolerance (Dahlgaard and Loeschcke 1997; Mikkelsen et al. 2010). Measuring the magnitude of inbreeding depression resulted in a reduction of performance ranging between 8 and 41% in life history traits (8% for pupal mass, 8% for larval time, 11% for growth rate, and 41% for egg hatching rate) and 12% for cold tolerance. Therefore we conclude that stress tolerance may not be generally reduced by inbreeding, at least when using environmental conditions which are beneficial prior to testing (Dahlgaard and Loeschcke 1997) and when inbreeding levels are low (2 full-sib matings). But note that despite the rather low levels of inbreeding we gained clear responses in cold tolerance and life history-traits. Consequently, regarding the results on cold tolerance, inbreeding may negatively affect an organism's ability to react to changing environmental conditions and thereby enhancing a small population`s extinction risk.

2.3 Genetic adaptation/Response to selection on cold tolerance

2.3.1 Response to selection and effects of inbreeding on selection

Sufficient standing genetic variation is supposed to be of crucial importance for adaptation to new environmental conditions (Falconer and Mackay 1996; Roff 1997; Bijlsma and Loeschke 2005; Bouzat 2010). But modern land-use practices often result in small and isolated populations, increasing the risk of extinction through reduced genetic diversity as a consequence of inbreeding or drift. Such genetic erosion may also interfere with a population's evolutionary potential.

To explore constraints on evolution, we applied artificial selection to chill-coma recovery time, starting from three levels of inbreeding (outbred control, one or two full-sib matings) and selected 10 generations for increased cold tolerance. Our study showed a clear response to selection on cold stress resistance. Inbred lines showed a weaker response to selection, indicating reduced evolutionary potential and thereby constraints on evolution. The reduced response to selection could on principal be caused by either additive or non-additive (dominance, epistasis) genetic effects. Following the neutral quantitative genetic theory, inbreeding is supposed to decrease the additive genetic variance of quantitative traits (and thereby their evolutionary potential) proportional to the inbreeding coefficient (Falconer and Mackay 1996; Frankham et al. 2002; Roff and Emerson 2006; Van Heerwaarden et al. 2008). On the other hand non-additive effects might also be involved. Here, additive genetic variance may actually increase rather than decrease through inbreeding (e.g. Bryant et al. 1986; Meffert 1995; Wade et al. 1996; Saccheri et al. 2001; Van Heerwarden et al. 2008; Bouzat 2010). While additive effects may have caused the overall weaker response to selection in the inbred lines, inbreeding level 2, which showed a stronger response to selection than inbreeding level 1, may have partially benefitted from non-additive effects, releasing novel additive variance. This is particularly likely for life-history traits closely related to fitness, as is the case here, because they are more likely to have a non-additive genetic architecture than e.g. morphological traits (Roff and Emerson 2006; Van Bushkirk and Willi 2006; Willi et al. 2006). Alternatively, the differences between inbreeding level 1 and 2 in our study could be caused by chance effects. Several studies reported large variation across inbred lines despite identical inbreeding coefficients (e.g. Fowler and Whitlock 1999; Reed et al. 2002; Kristensen et al. 2003; Wright et al. 2008), with some inbred lines even outperforming outbred controls. Such lineage effects probably result from the number of founding individuals carrying deleterious recessive alleles (Fowler and Whitlock 1999; Reed et al. 2002; Wright et al. 2008), and are an ubiquitous feature in studies on inbreeding depression (Armbruster and Reed 2005).

Correlated responses to selection in 10 different life history and stress resistance traits (egg number, egg hatching, larval time, pupal time, pupal mass, growth rate, heat tolerance, survival after heat exposure, longevity, longevity in cold-stressed animals) were essentially absent. In line with our findings, studies on *Drosophila* also yielded mainly negative or inconclusive results regarding correlated responses (e.g. Watson and Hoffmann 1996; Anderson et al. 2005; Mori and Kimura 2008; Bertoli et al. 2010). In summary, cold adaptation obviously operates at least largely independent from other traits including heat tolerance.

Inbreeding depression was still measurable in some traits after the course of selection (Table 1). Traits more closely related to fitness showed a clear fitness rebound, suggesting a trait-specific impact of purging (e.g. Frankham et al. 2001; Pedersen et al. 2005; Mikkelsen et al. 2010). Such effects should be most pronounced in traits closely related to fitness, which may apply here for instance to egg hatching success (Ehiobu et al. 1989; Pedersen et al. 2005). The prolonged negative effects of inbreeding on fecundity suggests, in line with Saccheri et al. (1996), that inbreeding depression is far less severe for fecundity than for fertility.

Table 1: Effects of inbreeding on various traits before (i.e. immediately after full-sib-matings) and after the selection experiment (i.e. 11-12 generations later). Complete (no effects of inbreeding measurable anymore after selection) and partial (effects still significant, though effect size has decreased) fitness rebounds are also indicated. '-': negatively affected; '0': not affected, '+': positively affected (though note the very small effect size; see text); '*': Not measured prior to selection. T: time.

Trait	**Before**	**After**	**Rebound**
Egg hatching success	**-**	**0**	**Yes**
Fecundity	*****	**-**	
Larval development T	**-**	**0**	**Yes**
Pupal development T	**0**	**+**	
Larval growth rate	**-**	**-**	**Partly**
Pupal mass	**-**	**-**	**Partly**
Longevity	*****	**0**	
Heat survival	*****	**-**	
Heat knowck-down T	**0**	**0**	
Chill-coma recovery T	**-**	**0**	**Yes**

We believe that our results have large implications for the survival of small populations in fragmented landscapes, and therefore for many species inhabiting cultivated landscapes. Small populations are evidently prone to a loss of genetic diversity through drift and/or inbreeding, while being at the same time under pressure through human-induced deterioration of their habitats. Importantly, we here experimentally demonstrate that increased levels of inbreeding indeed reduce evolutionary potential, and therefore the ability to cope with environmental change. In this context, our findings on long-lasting detrimental effects of relatively mild and putatively ecologically relevant levels of inbreeding seem also important, as this will further increase the risk of extinction.

2.3.2 Genetic correlations across development stages

Perhaps the most obvious limit to genetic adaptation are negative genetic correlations (caused by antagonistic pleiotropy), in which a beneficial genetic change in one trait is linked to a detrimental change in another (Stearns 1989; Roff 2002). While such genetic trade-offs have been quite frequently measured among different traits, hardly any data is available on potential trade-offs across developmental stages (but e.g. Tucic 1979; Cheverud et al. 1983; Loeschcke and Krebs 1996). Against this background, we here investigated genetic links between the expression of a specific phenotype at different time points in the ontogeny of the butterfly *Bicyclus anynana*. We used the lines from 2.3.1, selected for decreased chill-coma recovery time and according controls, which had originally been set up from three levels of inbreeding (outbred control, one or two full-sib matings). Four generations after selection procedure had been terminated, a significant response to selection was still found in one day-old butterflies (the age at which selection took place). Seven day-old adults showed a very similar though weaker response, with 61% longer chill coma recovery times compared to one day-old butterflies. As individual performance is expected to decrease with age because of senescence, i.e. a decline in physiological functions (Descamps et al. 2008), this effect was expected. Nevertheless, previous studies on effects of age on chill coma recovery times though yielded contradictory results, thus challenging the notion of a linear correlation between age and cold tolerance (see Fischer et al. 2010; Lalouette et al. 2010).

Cold resistance did not increase in either egg, larval or pupal stage in the selection lines. Note that we have necessarily measured different traits, namely cold stress survival during egg, larval, and pupal development, and chill-coma recovery time in the adult stage. However, both traits are clearly related, for which there is direct evidence in *B. anynana* (Fischer et al. 2010). While positive effects were thus lacking throughout, increased cold tolerance in the adult stage was even associated with reduced cold stress survival for eggs, with according tendencies being found in young larvae (except in the I2 treatment), and furthermore in all other developmental stages investigated (though not in all groups). We considered several explanations leading to these results: (1) the involvement of different

mechanisms in cold stress survival versus chill-coma recovery time, although both traits are related (Anderson et al. 2005; Hoffmann et al. 2002; Fischer et al. 2010); (2) the involvement of different mechanisms facilitating cold tolerance in the adult stage and during development (note that Gilchrist et al. 1997 revealed clear effects of thermal experimental evolution in the adult but not in the egg stage); (3) the presence of resource-allocation trade-offs with an enhanced investment into adult cold tolerance leaving less resources available for offspring provisioning. Distinguishing between these possibilities will be an important task for future research. However, for the time being we favor a resource-based explanation: A wealth of studies indicates that allocation trade-offs, with energy-demanding functions competing for limited energy resources, play an important role in life-history evolution (Stearns 1989, 1992; Roff 2002, 2007; Roff and Fairbairn 2007). Consequently, resources allocated to increased cold tolerance in the adult stage may not be available anymore for offspring provisioning, which may in turn diminish subsequent offspring performance. Such effects should be most pronounced in early developmental stages, which is indeed the case here.

Our results thus highlight that increased performance in a specific trait may not only be traded off against other energy-demanding functions, but also against performance throughout ontogeny, which may substantially affect optimal responses to selection pressures (Marshall and Sinclair 2011; Stoks and De Block 2011).

3. References

3. References

Andersen LH, Kristensen TN, Loeschcke V, Toft S, Mayntz D (2010) Protein and carbohydrate composition of larval food affects tolerance to thermal stress and desiccation in adult *Drosophila melanogaster*. J Insect Physiol 56:336-340

Anderson AR, Hoffmann AA, McKechnie SW (2005) Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: physiology and life-history traits. Genet Res 85:15-22

Angilletta MJ Jr, Steury TD, Sears MW (2004) Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. Integr Comp Biol 44:498-509

Ansart A, Vernon P, Daguzan J (2001) Photoperiod is the main cue that triggers supercooling ability in the land snail, *Helix aspersa* (Gastropoda: Helicidae). Cryobiology 42:266-273

Armbruster P, Hutchinson RA, Linvell T (2000) Equivalent inbreeding depression under laboratory and field conditions in a treehole- breeding mosquito. Proc R Soc B 267:1939-1945

Atkinson D (1994) Temperature and organism size – a biological law for ectotherms. Adv Ecol Res 25:1-58

Aubret F, Shine R (2010) Fitness costs may explain the post-colonisation erosion of phenotypic plasticity. J Exp Biol 213:735-739

Auld JR, Agrawal AA, Relyea RA (2010) Re-evaluating the costs and limits of adaptive phenotypic plasticity. Proc R Soc Lond B 277: 503-511

Ayrinhac A, Debat V, Gibert P, Kister AG, Legout H, Moreteau B, Vergilino B, David JR (2004) Cold adaptation in geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more important than genetic variability. Funct Ecol 18:700-706

Bauerfeind SS, Fischer K (2007) Maternal body size as an evolutionary constraint on egg size in a butterfly. Evolution 61:2374-2385

Barrett RDH, Bell G (2006). The dynamics of diversification in evolving *Pseudomonas* populations. Evolution 60:484-490

Bell G, Koufopanou V (1986) The cost of reproduction. Oxford University Press, Oxford.

Bertoli CI, Scannapieco AC, Sambucetti P, Norry FM (2010) Direct and correlated responses to chill-coma recovery selection in *Drosophila buzzatii*. Entomol Exp Appl 134:154-159

Bijlsma R, Loeschcke V (2000) Environmental stress, adaptation and evolution: an overview. J Evol Biol 18: 744-749

Bijlsma R, Loeschcke V (2005) Environmental stress, adaptation and evolution: an overview. J Evol Biol 18:744-749

Bijlsma R, Loeschcke V (2012) Genetic erosion impedes adaptive responses to stressful environments. Evol Appl 5:117-129

Bijlsma R, Bundgaard J, Boerema AC, Van Putten WF (1997) Genetic and environmental stress, and the persistence of populations. In: Bijlsma RR and Loeschcke V (eds) Environmental stress: adaptation and evolution. Birkhäuser, Basel, pp 105-146

Bijlsma R, Bundgaard J, Van Putten WF (1999) Environmental dependence of inbreeding depression and purging in Drosophila melanogaster. J Evol Biol 12:1125-1137

Blows MW, Hoffmann AA (2005) A reassessment of genetic limits to evolutionary limits. Ecology 86:1371-1384

Boakes EH, Wang J, Amos W (2007) An investigation of inbreeding depression and purging in captive pedigreed populations. Heredity 98:172-182

Bourguet D, Guillemaud T, Chevillon C, Raymond M (2004) Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. Evolution 58:128-135

Bouzat JL (2010) Conservation genetics of population bottlenecks: the role of chance, selection, and history. Conserv Genet 11:463-478

Bowler K, Terblanche JS (2008) Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? Biol Rev 83: 339-355

Bradshaw AD (1965) Evolutionary significance of phenotypic plasticity in plants. Adv Genet 13:115-155

Brakefield PM (1997) Phenotypic plasticity and fluctuating asymmetry as responses to environmental stress in the butterfly *Bicyclus anynana*. In: Bijlsma RR and Loeschcke V (eds) Environmental stress: adaptation and evolution. Birkhäuser, Basel, pp 105-146

Brakefield PM (2003) Artificial selection and the development of ecologically relevant phenotypes. Ecology 84:1661-1671

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

Bryant EHS, McCommas A, Combs LM (1986) The effect of an experimental bottleneck upon quantitative genetic variation in the housefly. Genetics 114:191-121

Bubliy OA, Loeschcke V (2005) Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. J Evol Biol 18:789-803

Byrd J, Castner J (2001) Forensic entomology: the utility of arthropods in legal investigations. CRC Press, New York

Carr DE, Eubanks MD (2002) Inbreeding alters resistance to insect herbivory and host plant quality in *Mimulus guttatus* (Scrophulariaceae). Evolution 56:22-30

Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. Annu Rev Ecol Syst 18:237-268

Charlesworth B, Charlesworth D (1999) The genetic basis of inbreeding depression. Genet Res 74:329-340

Charlesworth D, Willis JH (2009) The genetics of inbreeding depression. Genetics 10:783–796

Cheverud JM (1984) Quantitative genetics and developmental constraints on evolution by selection. J Theor Biol 110:155-171

Cheverud JM, Rutledge JJ, Atchley WR (1983) Quantitative genetics of development - genetic correlations among age-specific trait values and the evolution of ontogeny. Evolution 37:895-905

Chown SL, Terblanche JS (2007) Phyiological diversity in insects: ecological and evolutionary contexts. Adv Insect Physiol 33:50-152

Conn M (2006) Handbook of models for human aging. Elsevier Academic Press, London

Crnokrak P, Roff DA (1999) Inbreeding depression in the wild. Heredity 83:260-270

Crnokrak P, Barrett S (2002) Purging the genetic load: a review of the experimental evidence. Evolution 56:2347-2358

Czesak ME, Fox CW (2003) Evolutionary ecology of egg size and number in a seed beetle: genetic trade-off differs between environments. Evolution 57:1121-1132

Dahlgaard J, Loeschcke V (1997) Effects of inbreeding in three life stages of *Drosophila buzzatii* after embryos were exposed to a high temperature stress. Heredity 78:410-416

Dahlgaard J, Hoffmann AA (2000) Stress resistance and environmental dependency of inbreeding depression in *Drosophila melanogaster*. Conserv Biol 14:1187-1192

Dahlgaard J, Krebs RA, Loeschcke V (1995) Heat-shock tolerance and inbreeding in Drosophila buzzatii. Heredity 74:157-163

Dahlhoff EP, Rank NE (2007) The role of stress proteins in responses of a montane willow leaf beetle to environmental temperature variation. J Biosci 32:477-488

Danks HV (1994) Insect life cycle polymorphism. Kluwer Academic Publisher, Dordrecht

Danks HV (2005) Key themes in the study of seasonal adaptations in insects. 1. Patterns of cold hardiness. Appl Entomol Zool 40:199-211

Darwin CR (1876) The effects of cross and self fertilisation in the vegetable kingdom. John Murray, London

David JR, Araripe LO, Chakir M, Legout H, Lemos B, Petavy G, Rohmer C, Joly D, Moreteau B (2005) Male sterility at extreme temperatures: a significant but neglected phenomenon for understanding *Drosophila* climatic adaptations. J Evol Biol 18:838-846

Denlinger DL (1991) Relationship between coldhardiness and diapause. In: Lee RE and Denlinger DL (eds) Insects at low temperature. Chapman and Hall, New York, pp 174-198

Deutsch CA, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, Haak DC, Martin PR (2008) Impacts of climate warming on terrestrial ectotherms across latitude. Proc Natl Acad Sci U S A 105:6668-6672

Descamps S, Boutin S, Berteaux D, Gaillard JM (2008) Age-specific variation in survival, reproductive success and offspring quality in red squirrels: evidence of senescence. Oikos 117:1406-1416

DeWitt TJ, Sih A, Wilson DS (1998) Costs and limits of phenotypic plasticity. Trends Ecol Evol 13:77-81

Diffenbaugh NS, Pal JS, Trapp RJ, Giorgi F (2005) Fine-scale processes regulate the response of extreme events to global climate change. Proc Natl Acad Sci US A 102:15774-15778

Diffenbaugh NS, Pal JS, Giorgi F, Gao X (2007) Heat stress intensification in the Mediterranean climate change hotspot. Geophysical Research Letters 34:L11706

Ehiobu NG, Goddard ME, Taylor JF (1989) Effect of rate of inbreeding on inbreeding depression in *Drosophila melanogaster*. Theor Appl Genet 77:123-127

Emerson KJ, Uyemura AM, McDaniel KL, Schmidt PS, Bradshaw WE, Holzapfel CM (2009) Environmental control of ovarian dormancy in natural populations of *Drosophila melanogaster*. J Comp Physiol A 195:825-829

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics. Longman, Essex

Fischer K, Fiedler K (2001) Resource-based territoriality in the butterfly *Lycaena hippothoe* and environmentally induced behavioural shifts. Anim Behav 61:723-732

Fischer K, Bot ANM, Brakefield PM, Zwaan BJ (2006) Do mothers producing large offspring have to sacrifice fecundity? J Evol Biol 19:380-391

Fischer K, Dierks A, Franke K, Geister TL, Liszka M, Winter S, Pflicke C (2010) Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. PLOS One: e15284

Fowler K, Whitlock MC (1999) The distribution of phenotypic variance with inbreeding. Evolution 53:1143-1156

Fox CW, Scheibly KL (2006) Variation in inbreeding depression among populations of the seed beetle, Stator limbatus. Entomol Exp Appl 121:137-144.

Fox CW, Reed DH (2010) Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. Evolution 65:246-258

Fox CW, Scheibly KL, Reed DH (2008) Experimental evolution of the genetic load and its implications for the genetic basis of inbreeding depression. Evolution 62:2236-2249

Fox CW, Stillwell RC, Wallin WG, Curtis CL, Reed DH (2011) Inbreeding-environment interactions for fitness: complex relationships between inbreeding depression and temperature stress in a seed-feeding beetle. Evol Ecol 25:25-43

Frankham R (1995) Inbreeding and extinction: a threshold effect. Conserv Biol 9:792-799

Frankham R (2005) Genetics and extinction. Biol Conserv 126:131-140

Frankham R, Gilligan DM, Morris D, Briscoe DA (2001) Inbreeding and extinction: effects of purging. Conserv Genet 2:279-284

Frankham R, Ballou JD, Briscoe DA (2002) Introduction to conservation genetics. Cambridge University Press, Cambridge

Gautier A, Schumann H (1973) Puparia of the subarctic or black blowfly *Protophormia terraenovae* (Robineau-Desvoidy, 1830) in a skull of a late eemian bison at Zemst, Brabant (Belgium). Palaeogeogr, Palaeoclimatol, Palaeoecol 14:119-125

Geister TL, Fischer K (2007) Testing the beneficial acclimation hypothesis: cool males – hot love? Behav Ecol 18:658-664

Gilchrist GW, Huey RB, Partridge L (1997) Thermal sensitivity of *Drosophila melanogaster:* evolutionary responses of adults and eggs to laboratory natural selection at different temperatures. Physiol Zoo 70:403-414

Goldman BD (2001) Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283-301

Gotthard K, Nylin S, Wiklund C (1999) Seasonal plasticity in two satyrine butterflies: state-dependent decision making in relation to daylength. Oikos 84:453-462

Grassberger M, Reiter C (2002) Effect of temperature on development of the forensically important holarctic blow fly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae). Forensic Sci Int 128:177-182

Haikola S (2001) Inbreeding depression and the maintenance of genetic load in *Melitaea cinxia* metapopulations. Conserv Genet 2:325-335

Hedrick PW, Kalinowski ST (2000) Inbreeding depression in conservation biology. Annu Rev Ecol Syst 31:139-162

Hoffmann AA, Hallas RJ, Dean JA, Schiffer M (2003) Low potential for climatic stress adaptation in a rainforest *Drosophila* species. Science 301:100-102

Hoffmann AA, Anderson A, Hallas R (2002) Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. Ecol Lett 5:614-618

Hoffmann AA, Sørensen JG, Loeschcke V (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J Therm Biol 28: 175-216. 6.

Hori Y, Kimura MT (1998) Relationship between cold stupor and cold tolerance in *Drosophila* (Diptera: Drosophilidae). Environ Entomol 27:1297-1302

Jamieson IG, M. Roy S, Lettinek M (2003) Sex specific consequences of recent inbreeding in an ancestrally inbred population of New Zealand Takahe. Conserv Biol 17:708-716

Jensen D, Overgaard J, Sørensen JG (2007) The influence of developmental stage on cold shock resistance and ability to cold harden in *Drosophila melanogaster*. J Insect Physiol 53: 179-186

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

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

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. Trends Ecol Evol 17:230-341

Keller LF, Arcese P, Smith JNM, Hochachka WM, Stearns SC (1994) Selection against inbred song sparrows during a natural population bottleneck. Nature 372:356-357

Kellett M, Hoffmann AA, McKechnie SW (2005) Hardening capacity in the *Drosophila melanogaster* species group is constrained by basal thermotolerance. Funct Ecol 19:853-858

Keyghobadi N (2007) The genetic implications of habitat fragmentation for animals. Can J Zool. 85:1049-1064

Kimura MT, Ohtsu T, Yoshida T, Awasaki T, Lin LJ (1994) Climatic adaptations and distributions in the *Drosophila takahashii* species subgroup (Diptera: Drosophilidae). J Nat History 28:401-409

Kristensen TN, Dahlgaard J, Loeschcke V (2003) Effects of inbreeding and environmental stress on fitness—using *Drosophila buzzatii* as a model organism. Conserv Genet 4:453-465

Lalouette L, Vernon P, Amat H, Renault D (2010) Ageing and thermal performance in the sub-Antarctic wingless fly Anatalanta aptera (Diptera: Shaeoceridae): older is better. Biol Lett 6:346-349

Larsen TB (1991) The butterflies of Kenya. Oxford University Press, Oxford

Leberg PL, Firmin BD (2008) Role of inbreeding depression and purging in captive breeding and restoration programmes. Mol Ecol 17:334-343

Loeschcke V, Krebs RA (1996) Selection for heat-shock resistance in larval and adult *Drosophila buzzatii*: comparing direct and indirect responses on viability and development. Evolution 50: 2354-2359

Loeschcke V, Hoffmann AA (2007) Consequences of heat hardening on a field fitness component in *Drosophila* depend on environmental temperature. Am Nat 169:175-183

Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates, Sunderland

Lyytinen A, Brakefield PM, Lindstro¨m L, Mappes JA (2004) Does predation maintain eyespot plasticity in Bicyclus anynana? Proc R Soc B 271:279-283

Macdonald SS, Rako L, Batterham P, Hoffmann AA (2004) Dissecting chill coma recovery as a measure of cold resistance: evidence for a biphasic response in *Drosophila melanogaster*. J Insect Physiol 50: 695-700

Mahoney JJ, Hutchinson VH (1969) Photoperiod acclimation and 24-hour variations in the critical thermal maxima of a tropical and a temperate frog. Oecologia 2:143-161

Marshall KE, Sinclair BJ (2010) Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster.* Proc R Soc *B* 277: 963-969

Martin G, Lenormand T (2006) The fitness effect of mutations across environments: a survey in light of fitness landscape models. Evolution 60:2413-2427

Malcolm JW (2011) Smaller gene networks permit longer persistence in fast-changing environments. PLoS One 6:e14747.

Meffert LM (1995) Bottleneck effects on genetic variance for courtship repertoire. Genetics 139:365-374

Meffert LM, Regan JL (2006) Reversed selection responses in small populations of the housefly (*Musca domestica* L.). Genetica 127:1-9

Meyer JN, Di Giuliuo RT (2003) Heritable adaptation and fitness costs in killifish (*Fundulus beteroclitus*) inhabiting a polluted estuary. Ecol. Appl. 13:490-503

Mikkelsen K, Loeschke V, Kristensen TN (2010) Trait specific consequences of fast and slow inbreeding: lessons from captive populations of Drosophila melanogaster. Conserv Genet11:497-488

Mori N, Kimura MT (2008)Selection for rapid and slow recovery from chill- and heat-coma in *Drosophila melanogaster*. Biol J Linn Soc:95:72-80

Nunney L (1996) The response to selection for fast larval development in *Drosophila melanogaster* and its effect on adult weight: an example of a fitness trade-off. Evolution 50:1193-1204

Orr HA, Unckless RL (2008) Population extinction and the genetics of adaptation. Am Nat 172:160-169

Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrupp M (2005) Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. J Insect Physiol 51:1173-1182

Overgaard J, Tomcala A, Sørensen JG, Holmstrup M, Krogh PH, et al. (2008) Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly *Drosophila melanogaster*. J Insect Physiol 54:619-629

Pappas C, Hyde D, Bowler K, Loeschcke V, Sørensen JG (2007) Post-eclosion decline in 'knock-down' thermal resistance and reduced effect of heat hardening in *Drosophila melanogaster*. Comp Biochem Physiol A 146:355-359

Pedersen KS, Kristensen TN, Loeschcke V (2005) Effects of inbreeding and rate of inbreeding in *Drosophila melanogaster* – Hsp70 expression and fitness. J Evol Biol 18:756-762

Pigliucci M (2001) Phenotypic plasticity: beyond nature and nurture. John Hopkins University Press, Baltimore, Maryland

Pigliucci M (2005) Evolution of phenotypic plasticity: where are we going now? Trends Ecol Evol 20: 481-486

Rajamohan A, Sinclair BJ (2008) Short-term hardening effects on survival of acute and chronic cold exposure by *Drosophila melanogaster* larvae. J Insect Physiol 54:708-718

Rajamohan A, Sinclair BJ (2009) Hardening trumps acclimation in improving cold tolerance of Drosophila melanogaster larvae. Physiol Entomol 34:217-223

Rako L, Hoffmann AA (2006) Complexity of the cold acclimation response in *Drosophila melanogaster*. J Insect Physiol 52:94-104

Reed DH, Briscoe DA. Frankham R (2002) Inbreeding and extinction: the effect of environmental stress and lineage. Conserv. Genet.3:301-307

Relyea RA (2002) Costs of phenotypic plasticity. Am Nat 159:272-282

Roff DA (1997) Evolutionary quantitative genetics. Chapman & Hall, New York

Roff DA (1998) Effects of inbreeding on morphological and life history traits of the sand cricket, *Gryllus firmus*. Heredity 81:28-37

Roff DA (2007) Contributions of genomics to life-history theory. Genetics 8:116-125

Roff DA, Emerson K (2006) Epistasis and dominance: evidence for differential effects in life-history versus morphological traits. Evolution 60:1981-1990

Roff DA, Fairbairn DJ (2007) The evolution of trade-offs: where are we? J Evol Biol 20:433-447

Saccheri IJ, Brakefield PM, Nichols RA (1996) Severe inbreeding depression and rapid fitness rebound in the butterfly *Bicyclus anynana* (Satyridae). Evolution 50:2000-2013

Saccheri IJ, Nichols RA, Brakefield PM (2001) Effects of bottlenecks on quantitative genetic variation in the butterfly *Bicyclus anynana*. Genet Res:77:167-181

Sala OE, Chapin FS, Armesto JJ, Berlow E, Bloomfield J, et al. (2000) Biodiversity: global biodiversity scenarios for the year 2100. Science 287:1770–1774

Samietz J, Salser MA, Dingle H (2005) Altitudinal variation in behavioural thermoregulation: local adaptation vs. plasticity in California grasshoppers. J Evol Biol 18: 1087-1096

Schmidt PS, Matzkin L, Ippolito M, Eanes WF (2005) Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. Evolution 59:1721-1732

Schmidt PS, Zhu C-T, Das J, Mariska B, Li Y, Eanes WF (2008) An amino acid polymorphism in the couch potato gene forms the basis for climatic adaptation in *Drosophila melanogaster*. Proc Natl Acad Sci USA 105: 16207-16211Sejerkilde M, Sørensen JG, Loeschcke V (2003) Effects of cold- and heat hardening on thermal resistance in *Drosophila melanogaster*. J Insect Physiol 49:719-726

Sinclair BJ, Roberts SP (2005) Acclimation, shock and hardening in the cold. J Therm Biol 30: 557-562

Sørensen JG, Loeschcke V (2002) Natural adaptation to environmental stress via physiological clock-regulation of stress resistance in *Drosophila*. Ecol Lett 5:16-19

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6:1025-1037

Sørensen JG, Norry FM, Scannapieco AC, Loeschcke V (2005) Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. J Evol Biol 18:829-837

Stearns SC (1989) Trade-offs in life-history evolution. Funct Ecol 3:259-268

Stearns SC (1992) The evolution of life histories. Oxford University Press, New York

Stoks R, De Block M (2011) Rapid growth reduces cold resistance: evidence from latitudinal variation in growth rate, cold resistance and stress proteins. PLoS ONE 6: e16935

Swindell WR, Bouzat JL (2006) Ancestral inbreeding reduces the magnitude of inbreeding depression in *Drosophila melanogaster*. Evolution 60:762-767

Templeton AR, Read B (1984) Factors eliminating inbreeding depression in a captive herd of Speke's gazelle (*Gazella spekei*). Zool Biol 3:177-199

Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, Collingham YC, Erasmus BFN, de Siqueira MF, Grainger A, Hannah L, Hughes L, Huntley B, van Jaarsveld AS, Midgley GF, Miles L, Ortega-Huerta MA, Peterson AT, Phillips OL, Williams SE (2004) Extinction risk from climate change. Nature 427:145-148

Travis J (1994) Evaluating the adaptive role of morphological plasticity. Ecological Morphology (eds P.C. Wainwright and S.M. Reilly), pp. 99-122. University of Chicago Press, Chicago.

Tucic N (1979) Genetic capacity for adaptation to cold resistance at different developmental stages of *Drosophila melanogaster*. Evolution 33:350-358

Udaka H, Goto SG, Numata H (2008) Effects of photoperiod and acclimation temperature on heat and cold tolerance in the terrestrial slug, *Lehmannia valentiana* (Pulmonata: Limacidae). Appl Entomol Zool 43:547-551

Van Bushkirk J, Willi Y (2006) The change in quantitative genetic variation with inbreeding. Evolution 60:2428-2434

Vandergast AG, Bohonak AJ, Weissman DB, Fisher RN (2007) Understanding the genetic effects of recent habitat fragmentation in the context of evolutionary history:

phylogeography and landscape genetics of a southern California endemic Jerusalem cricket (Orthoptera: Stenopelmatidae: Stenopelmatus). Mol. Ecol. 16:977-992

Van Heerwaarden B, Willi Y, Kristensen TN, Hoffmann A (2008) Population bottlenecks increase additive genetic variance but do not break a selection limit in rain forest *Drosophila*. Genetics 179:2135-2146

Van't Hof AE, Zwaan BJ, Saccheri IJ, Daly D, Bot ANM, Brakefield PM (2005) Characterization of 28 microsatellite loci for the butterfly *Bicyclus anynana*. Mol Ecol Notes 5:169-172

Vermeulen CJ, Bijlsma R, Loeschcke V (2008) QTL mapping of inbreeding-related cold sensitivity and conditional lethality in *Drosophila melanogaster*. J Evol Biol 21:1236-1244

Via S, Hawthorne DJ (2002) The genetic architecture of ecological specialization: correlated gene effects on host use and habitat choice in pea aphids. Am Nat 159: S76-S88

Wade MJ, Shuster SM, Stevens L (1996) Inbreeding: its effect on response to selection for pupal weight and the heritable variance in fitness in the flour beetle, *Tribolium castaneum*. Evolution 50:723-733

Waller DM, Dole J, Bersch AJ (2008) Effects of stress and phenotypicvariation on inbreeding depression in *Brassica rapa*. Evolution 62:917-931

Wang J (2000) Effects of population structures and selection strategies on purging of inbreeding depression due to deleterious mutations. Genet Res 76:75-86

Watson MJO, Hoffmann AA (1996) Acclimation, cross-generation effects, and the response to selection for increased cold resistance in *Drosophila*. Evolution 50:1182-1192

West-Eberhard MJ (2003) Developmental plasticity and evolution. Oxford University Press, New York.

Willi Y, Bushkirk JV, Hoffmann AA (2006) Limits to the adaptive potential of small populations. Annu Rev Ecol Evol Syst 37:433-458

Willmer P, Stone G, Johnston IA (2000) Environmental physiology of animals. Blackwell, Oxford UK

Windig JJ, Brakefield PM, Reitsma N, Wilson JGM (1994) Seasonal polyphenism in the wild: survey of wing patterns in five species of *Bicyclus* butterflies in Malawi. Ecol Entomol 19:285-298

Wilson RS, Franklin CE (2002) Testing the beneficial acclimation hypothesis. Trends Ecol Evol 17:66-70

Wright LI, Tregenza T, Hosken DJ (2008) Inbreeding, inbreeding depression and extinction. Conserv Genet 9:833-843

Woods HA, Harrison JF (2002) Interpreting rejections of the beneficial acclimation hypothesis: when is physiological plasticity adaptive? Evolution 56:1863-1866

4. Publication list

4. Publication list

Chapter 5.1: Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*

- Fischer, K., **Dierks, A.**, Franke, K., Geister, T.L., Liszka, M., Winter, S. & C. Pflicke (2010): Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. PLoS One 5: e15284.

Contributions:

1. Rearing of butterflies for experiment 3
2. Preparing and supervising of experiment 3, which was carried out as a student research project
3. Analyzing data of experiment 3
4. Drafting parts of the manuscript
5. Editing the manuscript

Chapter 5.2: Phenotypic plasticity in temperature stress resistance is triggered by photoperiod in a fly

- Fischer, K., Liniek, S., Bauer, M., Baumann, B., Richter, S. & **A. Dierks** (2011): Adaptive phenotypic plasticity in temperature stress resistance is triggered by photoperiod in a fly. Evolutionary Ecology. DOI 10.1007/s10682-011-9547-x

Contributions:

1. Establishing the methods for flies in pilot experiments
2. Rearing of flies
3. Preparing and supervising the experiments which were carried out as student research projects or diploma thesis
4. Drafting parts of the manuscript
5. Editing the manuscript

Chapter 6.1: Effects of inbreeding on life-history and thermal performance in the tropical butterfly *Bicyclus anynana*

- **Dierks, A**., Hoffmann, B., Bauerfeind, S.S. & K. Fischer (2012): Effects of inbreeding on life history and thermal performance in the tropical butterfly *Bicyclus anynana*. Population Ecology 54: 83-90.

Contributions:

1. Experimental Design
2. Rearing of butterflies
3. Establishing the full-sib breeding design
4. Data acquisition of life history traits
5. Conducting cold- and heat tolerance assays
6. Statistical analysis
7. Writing and editing the manuscript

Chapter 6.2: Response to selection on cold tolerance is constrained by inbreeding

- **Dierks, A**., Baumann, B. & K. Fischer (2012): Response to selection on cold tolerance is constrained by inbreeding. Evolution. DOI: 10.1111/j.1558-5646.2012.01604.x.

Contributions:

1. Experimental Design
2. Rearing and preparing of selection lines
3. Conducting selection procedure
4. Conducting cold tolerance assays
5. Statistical analysis
6. Writing and editing the manuscript

Chapter 6.3: Does selection on increased cold tolerance in the adult stage confer resistance throughout development?

- **Dierks, A**., Kölzow, N., Franke, K. & K. Fischer (in press): Does selection on increased cold tolerance in the adult stage confer resistance throughout development? Journal of Evolutionary Biology

Contributions:

1. Experimental Design
2. Establishing the methods for eggs, larvae and pupae in pilot experiments
3. Rearing and preparing of butterflies for the experiments
4. Data acquisition of survival rates
5. Conducting cold tolerance assays
6. Statistical analysis
7. Writing and editing the manuscript

Prof. Klaus Fischer Anneke Dierks

5. Environmental effects on temperature stress resistance

5.1 Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*

Klaus Fischer, Anneke Dierks, Kristin Franke, Thorin L. Geister, Magdalena Liszka, Sarah Winter, Claudia Pflicke

Published as:

Fischer, K., Dierks, A., Franke, K., Geister, T.L., Liszka. M., Winter, S. and Pflicke, C. (2010). Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. PLoS ONE 5(12): e15284. doi:10.1371/journal.pone.0015284

Environmental Effects on Temperature Stress Resistance in the Tropical Butterfly *Bicyclus Anynana*

Klaus Fischer[1]*, Anneke Dierks[1], Kristin Franke[1], Thorin L. Geister[2], Magdalena Liszka[2], Sarah Winter[1], Claudia Pflicke[2]

1 Zoological Institute and Museum, University of Greifswald, Greifswald, Germany, **2** Department of Animal Ecology I, University of Bayreuth, Bayreuth, Germany

Abstract

Background: The ability to withstand thermal stress is considered to be of crucial importance for individual fitness and species' survival. Thus, organisms need to employ effective mechanisms to ensure survival under stressful thermal conditions, among which phenotypic plasticity is considered a particularly quick and effective one.

Methodology/Principal Findings: In a series of experiments we here investigate phenotypic adjustment in temperature stress resistance following environmental manipulations in the butterfly *Bicyclus anynana*. Cooler compared to warmer acclimation temperatures generally increased cold but decreased heat stress resistance and vice versa. In contrast, short-time hardening responses revealed more complex patterns, with, e.g., cold stress resistance being highest at intermediate hardening temperatures. Adult food stress had a negative effect on heat but not on cold stress resistance. Additionally, larval feeding treatment showed interactive effects with adult feeding for heat but not for cold stress resistance, indicating that nitrogenous larval resources may set an upper limit to performance under heat stress. In contrast to expectations, cold resistance slightly increased during the first eight days of adult life. Light cycle had marginal effects on temperature stress resistance only, with cold resistance tending to be higher during daytime and thus active periods.

Conclusions/Significance: Our results highlight that temperature-induced plasticity provides an effective tool to quickly and strongly modulate temperature stress resistance, and that such responses are readily reversible. However, resistance traits are not only affected by ambient temperature, but also by, e.g., food availability and age, making their measurement challenging. The latter effects are largely underexplored and deserve more future attention. Owing to their magnitude, plastic responses in thermal tolerance should be incorporated into models trying to forecast effects of global change on extant biodiversity.

Citation: Fischer K, Dierks A, Franke K, Geister TL, Liszka M, et al. (2010) Environmental Effects on Temperature Stress Resistance in the Tropical Butterfly *Bicyclus Anynana*. PLoS ONE 5(12): e15284. doi:10.1371/journal.pone.0015284

Editor: Brian Gratwicke, Smithsonian's National Zoological Park, United States of America

Received August 30, 2010; **Accepted** November 4, 2010; **Published** December 20, 2010

Funding: This work was funded by the German Research Council (DFG grants no. Fi 846/1-2, 1–3 and 1–4 to KFi). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: klaus.fischer@uni-greifswald.de

Introduction

The ability to withstand environmental stress is of crucial importance for any species' longer-term survival, and the associated stressors are consequently deemed among the strongest forces of natural selection [1–3]. Among the large number of stressors temperature is considered a particularly important one, because variable thermal environments are common and may pose substantial challenges for individual survival and reproduction [4–8]. The importance of an individual's temperature stress resistance will further increase in the future due to global warming, causing a raise in mean temperatures but also in temperature extremes, which may comprise the largest anthropogenic challenge ever placed on natural systems [9–13].

Given that variation in temperature is all pervasive and that temperature extremes may occur in most ecosystems more or less frequently, organisms need to employ effective mechanisms to ensure survival under such stressful conditions. According mechanisms to adjust phenotypic values to environmental conditions, including behavioural, physiological and molecular ones, are indeed generally found [5,14–15]. Conceptually, they can be categorized into two classes: longer-term genetic adaptation (e.g. through changes in allele frequencies) and phenotypic plasticity [16–17]. Phenotypic plasticity, on which we will focus here, comprises environmental effects on phenotypic expression, being either an adaptive strategy to cope with short-term environmental variation, or alternatively a non-adaptive biochemical or physiological interaction of an organism with its environment [16–17].

Plastic responses to temperature variation can offer quick and effective means to cope with thermal stress, including, amongst others, rapid hardening and acclimation. Rapid hardening refers to an increased performance under temperature extremes after a brief (typically 1–2 hours) pre-exposure to less extreme temperatures, which has been described in several insect species and some other arthropods, e.g. [18–21]. Acclimation, in turn, is defined as a facultative response to changes in a single environmental variable, typically in the adult stage [1,22]. The difference to hardening is that acclimation typically involves longer periods of time, typically several days. Both mechanisms have been repeatedly found to

affect temperature stress resistance in *Drosophila* and some other organisms, e.g. [5,15,23–29], though still fairly little is known especially for the effects of hardening and acclimation on chill-coma recovery time [30]. Consequently, we mainly focus on cold stress resistance here.

To investigate environmental effects on temperature stress resistance, we here use two well-established proxies: chill-coma recovery time (i.e. the time needed to regain mobility following cold exposure) and heat knock-down time (i.e. the time until being knocked down under heat stress). Both indices are considered reliable proxies of climatic cold and heat adaptation, respectively, e.g. [7,27,31–32]. We have chosen the tropical butterfly *Bicyclus anynana* as model organism for this study for the following reasons: (1) the specific population used here inhabits a seasonal environment with an adverse (cool) dry season and a beneficial (warm) wet season, thus promoting phenotypic plasticity [33], (2) there is a solid knowledge on plastic responses in some life-history traits already, e.g. [25,34–35], and (3) because of its tropical origin [36]. The latter seems important as recent studies suggested that tropical ectotherms, living currently close to their (upper) critical thermal limits already, may be particularly vulnerable to global warming [13,37], and may further show very limited evolutionary potential to respond to future climate change [38–39].

In a series of experiments we here address the following research questions: 1) Does temperature stress resistance in *B. anynana* respond to acclimation temperature, how long does it take for an acclimation response to occur, and to what extent is this response reversible? 2) Does stress resistance further respond to short time exposure to different temperatures ('hardening')? 3) What is the effect of extreme cold stress on the acclimation response? 4) Does cold stress resistance and the ability to acclimate to a novel environment diminish with age? 5) Does the acclimation response in cold stress resistance depend on the specific assay conditions used? 6) Is chill-coma recovery time related to other proxies for fitness such as survival rate? 7) Does larval and adult food stress interfere with the ability to withstand thermal stress? 8) Does temperature stress resistance show variation in relation to daily light cycle?

Assuming that phenotypic changes in temperature stress resistance are adaptive, we predict that warm acclimation and hardening temperatures will increase heat stress resistance, while cool acclimation and hardening temperatures will increase cold stress resistance, e.g. [24–26,28–29]. However, how long such responses need to take effect, whether they are readily reversible and to what extent they depend on prior thermal experience has thus far received little attention, while these issues may be of great ecological importance in environments showing strong temperature fluctuations. Another largely open question is whether the ability to acclimate to a novel environment depends on age. If such plastic responses come at any meaningful cost (as is suggested by theory [4,40–41]), older individuals with less resources available should show a decreasing performance with increasing age, e.g. [42–43]. Note in this context that butterflies, as other holometabolous insects, typically loose mass as they age, indicating resource depletion [44–46]. If temperature stress resistance was indeed subject to resource-allocation trade-offs, food stress is also predicted to negatively impact on thermal performance, as is the case for many other traits, but is essentially unknown for temperature resistance traits [47]. Further, resistance traits may show variation in relation to time of day. It might be expected that animals are more cold resistant in the morning where temperature is typically low, thus allowing earlier activity, but more heat resistant in the early afternoon where temperature may reach stressfully high values. Low-altitude populations of *Drosophila buzzatti*, for instance, show diel shifts in high-temperature resistance, which is controlled by a circadian rhythm in order to synchronize highest resistance with peak activity ([48]; see also [49]). Finally, two of the questions addressed above (5 & 6) concentrate more on methodological issues. When using chill-coma recovery time as a proxy for cold stress resistance, one might argue that the conditions used to induce a chill coma may include highly artificial settings. We therefore decided to use a range of different temperatures and exposure times in order to explore its effects on the patterns found. Further, the adaptive significance of a shorter recovery time is not always straightforward, although it correlates with differences in the thermal niche occupied [50] as well as geographical variation in cold stress resistance, e.g. [31,51–52]. Therefore, we test whether cold-acclimated animals showing shorter recovery times also show increased survival rates following cold exposure.

Materials and Methods

Study organism

Bicyclus anynana is a tropical, fruit-feeding butterfly ranging from Southern Africa to Ethiopia [36]. It exhibits striking phenotypic plasticity with two seasonal morphs, which functions as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and predation [53–54]. Reproduction in this butterfly is essentially confined to the warmer wet season when oviposition plants are abundantly available, and where 2–3 generations occur. During the colder dry season reproduction ceases and butterflies do not mate before the first rains at the beginning of the next wet season [53,55]. Laboratory stock populations were established at Bayreuth University, Germany, in 2003, and at Greifswald University, Germany, in 2008, both from several hundred eggs derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from 80 gravid females caught at a single locality in Malawi. In each generation several hundred individuals are reared maintaining high levels of heterozygosity at neutral loci [56]. For this study butterflies from either the Bayreuth or the Greifswald stock population were used.

Experimental design

For all experiments outlined below, *B. anynana* eggs were collected from several hundred stock females and reared at either 20°C or 27°C (depending on experiment and for purely logistic reasons), high relative humidity (70±10%), and a photoperiod of L12:D12. The temperatures chosen reflect the daily highs in the butterfly's natural environment during the dry and wet season, respectively [53]. Larvae were reared in population cages and fed on young maize (*Zea mays*) plants ad libitum (except for *experiment 8*; see below). Pupae were collected daily and transferred to cylindrical hanging cages, which were checked daily for eclosed butterflies. On their eclosion day, butterflies were randomly allocated to different adult treatment groups as outlined below, except for *experiment 9*, where individuals were allocated to treatments as young larvae already (see below). Thus, except for *experiment 9*, larvae were always reared in a common environment. Unless otherwise stated (cf. *experiment 8*), adult butterflies were fed with moist banana ad libitum.

For measuring chill-coma recovery time, 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 to the cold, usually using 19 h at 1°C (for exceptions see below). This method proved to be successful in an earlier study [25]. After cold exposure, trays were transferred to an environ-

mental cabinet with a constant temperature of 20°C. Recovery time was defined as the time elapsed between taking the tray out of the cold until a butterfly was able to stand on its legs. Butterflies were observed for a maximum of 60 min, and this maximum value was used for all animals that had not yet recovered (excluding those few animals from subsequent analyses would not change any of the results presented here qualitatively). To determine heat knock-down time, butterflies were placed in small, sealed glass vials (40 ml), which were submerged in a water bath or transferred to a climate cabinet (Sanyo MIR-553), both set at a constant temperature of 45°C (again in a randomized block design). Note that heating rates may differ between the water bath and the climate cabinet. This, however, does not confound any result shown, as always the same method (either water bath or climate cabinet) was used within one experiment. 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. Throughout, there was no re-use of any butterflies, i.e. each butterfly was tested only once. In total nine different experiments were carried out. Differences in experimental designs stem partly from follow-up experiments and thus previous results, partly from logistic reasons (i.e. the butterfly numbers available).

Effects of acclimation and hardening temperature on cold stress resistance. To investigate effects of acclimation and hardening temperature on cold stress resistance, five different experiments were carried out as detailed below (for an overview of all experiments see Table 1). In *experiment 1*, butterflies were randomly divided among four treatment groups in order to assay effects of acclimation temperature and the reversibility of the acclimation response: exposure for 3 days to 20°C, exposure for 3 days to 27°C, exposure for 3 days to 20°C followed by 3 days at 27°C, and exposure for 3 days to 27°C followed by 3 days at 20°C. Consequently, butterflies were tested on day 3 or 6 of adult life. We predicted that cold-acclimated individuals show shorter recovery times than warm-acclimated ones and that this plastic response is reversible.

In *experiment 2*, butterflies from two acclimation groups (3 days at 20°C or 27°C) were compared for acclamatory responses using different (largely arbitrarily chosen) methods to induce a chill coma, namely 19 h at 1°C, 50 min at −3.5°C, 90 min at −3.5°C, 45 min at −5°C, and 15 min at −8°C. This experiment was designed to investigate whether the acclimation response depends on assay conditions. After having measured chill-coma recovery time, butterflies were transferred to a climate cabinet set at an intermediate temperature of 23.5°C, and survival was recorded 24 h later. We here tested the hypothesis that cold-acclimated butterflies show a higher cold tolerance than warm-acclimated ones regardless of assay conditions.

To further investigate how quickly butterflies with different thermal histories are able to respond to acclimation temperature, eight treatment groups were used in *experiment 3*. Butterflies were acclimated on three consecutive days to different combinations of 20°C and 27°C prior to testing: 20-20-20°C (i.e. acclimated for 3 days to 20°C; treatment 1), 27-20-20°C (acclimated for 1 day to 27°C, followed by 2 days at 20°C; treatment 2), 20-27-20°C (3), 27-27-20°C (4), 20-20-27°C (5), 27-20-27°C (6), 20-27-27°C (7), 27-27-27°C (8). Afterwards, chill-coma recovery time was measured after exposing the butterflies for 19 h to 1°C. We hypothesized that the final day prior to testing has the largest impact on cold resistance, while earlier thermal experience may have some subtle, modulating impact.

Table 1. Overview over all experiments carried out.

Experiment	Groups	Factors	Treatments	Dependent variables
1	4	Acclimation treatment	3 d at 20°C, 3 d at 27°C, 3 d at 20°C followed by 3 d at 27°C or 3 d at 27°C followed by 3 d at 20°C	CCRT after 19 h at 1°C
2	2	Acclimation temperature	3 d at 20°C or 27°C	CCRT after 19 h at 1°C, 50 min at −3.5°C, 90 min at −3.5°C, 45 min at −5°C or 15 min at −8°C; survival after 24 h
3	8	Acclimation treatment	Acclimation on three consecutive days to: 20-20-20°C, 27-20-20°C, 20-27-20°C, 27-27-20°C, 20-20-27°C, 27-20-27°C, 20-27-27°C or 27-27-27°C	CCRT after 19 h at 1°C
4	16	Acclimation treatment & Age	Four acclimation groups acclimated to 20°C, 27°C, 27°C followed by 20°C or 20°C followed by 27°C; each tested after 2, 4, 6 and 8 d	CCRT after 19 h at 1°C
5	5	Hardening temperature	1 h at 6°C, 13°C, 20°C, 27°C or 34°C	CCRT after 19 h at 1°C or after 4 min at −20°C
6a	9	Acclimation & Hardening temperature	Factorial design with 3 acclimation (2d) and 3 hardening temperatures (1 h, with 20°C, 27°C, 34°C each)	HKDT at 45°C
6b	3	Hardening temperature	1 h at 20°C, 27°C or 34°C	HKDT at 45°C
6c	2	Hardening temperature	1 h at 20°C or 39°C	HKDT at 45°C
7a	4	Acclimation treatment	6 d at 20°C, 6 d at 27°C, 6 d at 20°C with cold stress on day 3, 6 d at 27°C with cold stress on day 3	HKDT at 45°C
7b	2	Acclimation temperature	24 h at 20°C or 27°C	HKDT at 45°C
8	4	Larval & Adult food stress	Factorial design with 2 larval and 2 adult feeding treatments (stress versus control each)	CCRT after 19 h at 1°C or HKDT at 45°C
9	6	Time of day	Tests at 6 different times of day	CCRT after 19 h at 1°C, CCRT after 20 min to −5°C or HKDT at 45°C

Given are number of experiment, number of treatment groups (excluding sex), factors (also excluding sex), a short description of the treatments involved, and the dependent variables and how they were measured. For details see Experimental design. d: day; CCRT: chill-coma recovery time; h: hour; min: minute; HKDT: heat knock-down time.

doi:10.1371/journal.pone.0015284.t001

Experiment 4 addressed whether the ability to acclimate to a novel environment diminishes with age. Therefore, butterflies from four treatment groups were tested at days 2, 4, 6, and 8 of adult life. Two control treatment groups were exposed permanently to 20°C and 27°C, respectively. In the other two treatment groups, butterflies were first exposed to the one, but for the last two days prior to testing to the alternative acclimation temperature (treatments 27-20°C and 20-27°C, respectively). Note that in the transfer treatments the 'day 2' group did consequently not experience an acclimation temperature change, as butterflies were exposed to the 'second' temperature on their eclosion day already. For the other groups, butterflies were exposed for 2 days to 20°C and 2 days to 27°C (day 4 group), for 4 days to 20°C and 2 days to 27°C (day 6 group), and for 6 days to 20°C and 2 days to 27°C (day 8 group, for the 20-27°C treatment) and vice versa (for the 27-20°C treatment). For all individuals used here, pupal mass was measured on day 2 after pupation. After weighing, pupae were kept singly in translucent plastic cups (125 ml) until adult eclosion, after which all butterflies were individually marked for future reference. We here tested the prediction that the ability to acclimate to a novel environment diminishes with increasing with age.

Experiment 5 investigated the response to different 'hardening' temperatures. Butterflies maintained at 27°C were exposed for one hour to 6°C, 13°C, 20°C, 27°C or 34°C. Thereafter, butterflies were either immediately exposed for 19 h to 1°C, or back-transferred to 27°C for one hour for recovery. The latter group was thereafter exposed for 4 min to −20°C (in order to quickly induce a chill coma) prior to measuring chill-coma recovery time. This treatment was included as it appeared questionable whether hardening effects (1 hour exposure) would be measurable after a long-term exposure (19 h) to the cold. Note that, of course, butterflies will not equilibrate to −20°C within 4 minutes and that the temperatures experienced by the butterflies are unknown. However, this matter should be irrelevant for comparing different hardening groups, as conditions were identical across treatment groups and the specific method used to induce a chill coma seems not crucial for such comparisons (see results of experiment 2). We predicted that colder hardening temperatures increase but warmer hardening temperatures decrease cold resistance.

Effects of acclimation and hardening temperature on heat stress resistance. Two experiments focussed on temperature effects on heat stress resistance. *Experiment 6* used a full-factorial design with three acclimation and three 'hardening' temperatures. Butterflies were acclimated to 20°C, 27°C or 34°C for two days, after which they were divided among the same three temperatures for 1 hour (short time acclimation, here referred to as 'hardening'). After another two hours at the respective acclimation temperature for recovery, butterflies were tested for heat knock-down time. As no effect of hardening temperature was found using the above design (see below), the experiment was repeated using only one acclimation temperature (27°C, for logistic reasons), from which butterflies were once again exposed for one hour to 20°C, 27°C or 34°C, but tested immediately thereafter (i.e. without a recovery period). Additionally, butterflies (only females, for logistic reasons) acclimated to 27°C were exposed for one hour to 20°C or a more extreme temperature of 39°C, and tested immediately thereafter. We hypothesized that warmer acclimation and hardening temperatures increase heat tolerance and vice versa.

In *experiment 7* the effect of a longer-term cold exposure on the acclimation response was investigated using four treatment groups. Butterflies were acclimated for six days to either 20°C or 27°C. Per acclimation temperature, half of the individuals was exposed for 19 h to 1°C on day 3 of adult life, and afterwards back-transferred to their original acclimation temperature. The other half remained at the respective acclimation temperature throughout (control). We additionally tested whether one day is sufficient to induce a significant acclimation response, by dividing butterflies (only females, again for logistic reasons) reared and maintained at 27°C among 20°C and 27°C for 24 h on day 2 of adult life, prior to investigating heat stress resistance. We here test the prediction that severe cold stress will decrease subsequent heat stress resistance.

Effects of larval and adult food limitation on temperature stress resistance. In *experiment 8* a full-factorial design was used to investigate the effects of larval and adult food limitation on temperature stress resistance. Larvae were reared at 27°C and either fed ad libitum (larval control) or starved two-times for 24 h by removing any food from the rearing cages, with one day of food access in-between. To proof that the starvation treatment was successful in imposing stress, pupal mass was measured for all individuals on day 2 after pupation. Upon adult eclosion, butterflies were once again allocated to either a control (fed with banana ad libitum) or a starvation treatment (having access to water only). Temperature stress tolerance was assessed on day 3 of adult life. The experiment consisted of two parts, one addressing effects on chill-coma recovery (A), the other on heat knock-down time (B). Both larval and adult food stress were expected to decrease temperature stress resistance.

Effects of light cycle on temperature stress resistance. In *experiment 9* we investigated effects of the time of day on temperature stress resistance. Therefore, we used six climate cabinets (Sanyo MLR-351H) set at 27°C, 70% relative humidity, and a photoperiod of L12:D12. Thus, conditions were the same throughout except for the onset of the light phase, starting at 10 a.m., 2 p.m., 6 p.m., 10 p.m., 2 a.m. or 6 a.m. All individuals were allocated to treatments as young larvae, i.e. ca. 20 days prior to testing temperature stress resistance. This design enabled us to test individuals from all treatments at the same time using a randomized block design. However, the test time equalled different time points with respect to the onset of the light phase from the butterflies' perspective. The experiment consisted of three parts, investigating chill-coma recovery time after exposure for 19 h to 1°C (A), chill-coma recovery time after exposure for 20 min to −5°C (B), or heat knock-down time at 45°C (C). During chilling animals were kept in the dark. Part B was included as it seemed questionable whether effects of photoperiod would be measurable after 19 h in a common environment, after which recovery time was eventually assayed. Butterflies were tested on day 2 (A, C) or 4 (B) of adult life. For part A butterflies were transferred to the cold at 3 p.m., allowing to measure recovery time at 10 a.m. the following day, while for parts B and C exposure to extreme temperatures started at 10 a.m. throughout. We hypothesized that butterflies are more cold-tolerant early in the morning, but more heat-tolerant in the early afternoon.

Data analyses

Effects of treatment (which may consist of up to two full factors; e.g. acclimation and hardening temperature) and sex on stress resistance traits were analysed using full-factorial AN(C)OVAs, including all factors as fixed effects. Pupal mass was added as covariate in *experiments 4* and *8*. In order to meet ANOVA requirements, data were transformed as appropriate. To standardize between blocks, all stress resistance data were adjusted to block means prior to analysis. Pair-wise comparisons were performed employing Tukey's HSD. Survival data in *experiment 2* were analyzed with nominal logistic regressions on binary data (dead or alive). When comparing two groups only, the t-test

(*experiment 6*) or the Mann-Whithney U test (*experiment 7*, as the requirements for parametric testing were not met) was used. All statistical analyses were done using JMP version 4.02 (SAS Institute, 2000) or Statistica 6.1 (StatSoft, 2003). Throughout, all means are given ±1 SE.

Results

Effects of acclimation and hardening on cold stress resistance

Experiment 1. Chill-coma recovery time varied significantly across acclimation groups ($F_{3,117} = 11.7$, $p<0.001$), but not between sexes ($F_{1,117} = 2.9$, $p = 0.090$; treatment by sex interaction: $F_{3,117} = 0.2$, $p = 0.909$). Three-day old butterflies exposed to 20°C showed the shortest recovery time, followed by the group exposed first to 27°C but afterwards to 20°C, while both groups exposed to 27°C before testing showed considerably longer recovery times and were statistically indistinguishable (20°C<27-20°C<27°C = 20-27°C; Tukey HSD after ANOVA; Fig. 1).

Experiment 2. Using five different methods with a temperature range between +1°C and -8°C and exposure times between 15 min and 19 h yielded a consistent pattern of shorter recovery times for animals acclimated to 20°C compared to 27°C (Table 2A). Consequently, the general pattern of such acclamatory responses is largely independent of the method used to induce a chill coma. Moreover, another proxy for cold stress resistance, mortality rate measured 24 h after cold exposure, invariably revealed a better performance for 20°C- compared to 27°C-acclimated butterflies (n.s. in one case; Table 2B).

Experiment 3. In the next experiment we further corroborated that *B. anynana* readily responds to different acclimation temperatures, that this acclimation response is largely reversible, and that the last 24 h prior to testing have the largest impact on stress resistance. Chill-coma recovery time differed significantly across acclimation groups ($F_{7,566} = 19.4$, $p<0.0001$) and sexes ($F_{1,566} = 4.9$, $p = 0.0280$; interaction: $F_{7,566} = 1.0$, $p = 0.3994$). The groups exposed to 20°C during the last 24 h prior to testing showed much reduced recovery times compared to those exposed to 27°C, and females showed shorter recovery times than males (1916±50 sec versus 2045±49 sec; Fig. 2). Note that the most extreme values coincide with permanent exposure to 20°C and 27°C, respectively, while the treatments 27-27-20°C and 20-20-27°C produced fairly intermediate phenotypes.

Experiment 4. Following up on the above experiments, the acclimation response was additionally examined in relation to age. Chill-coma recovery time was significantly affected by acclimation treatment, age and the covariate pupal mass (Table 3; Fig. 3). Individuals permanently exposed to 20°C showed the shortest recovery times followed by the group exposed to 20°C during the last two days before testing (27-20°C), while the groups permanently or during the last two days before testing exposed to 27°C needed longer recovery times and were statistically indistinguishable (20°C<27-20°C<20-27°C = 27°C; Tukey HSD after ANOVA, combined for age groups). The age effect indicates that recovery times slightly decreased with increasing age. The non-significant interaction indicates that, within the age classes investigated here, the acclimation response was not negatively affected by age, although recovery times tended to increase at days 6 and 8 for the 27-20°C group (cf. Fig. 3).

Experiment 5. The response to different hardening temperatures depended on methodology. Exposing butterflies for 19 h to 1°C for measuring chill-coma recovery time yielded no significant effect of hardening temperature (hardening temperature: $F_{4,227} = 0.49$, $p = 0.7473$; sex: $F_{1,227} = 0.81$, $p = 0.3701$; interaction: $F_{2,227} = 1.33$, $p = 0.2622$). Using a four minute exposure to −20°C to induce a chill coma, in contrast, showed that both high and low hardening temperatures significantly increased recovery times (6°C = 34°C≥13°C = 27°C (control) ≥20°C; Tukey HSD after ANOVA; hardening temperature: $F_{4,229} = 3.08$, $p = 0.0169$; sex: $F_{1,229} = 1.19$, $p = 0.2763$; interaction: $F_{2,229} = 0.54$, $p = 0.7068$; Fig. 4).

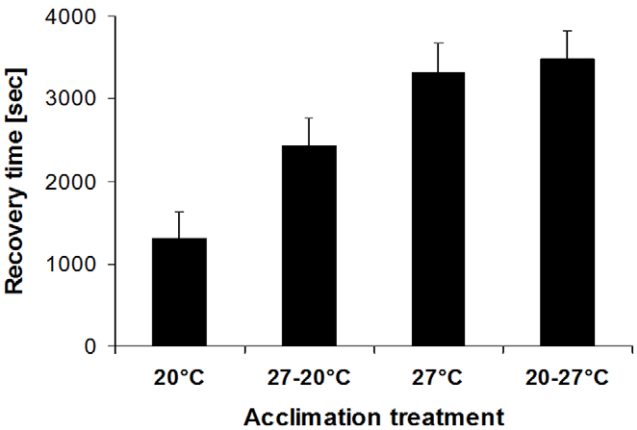

Figure 1. Chill-coma recovery time of *Bicyclus anynana* in relation to acclimation treatment. Butterflies reared in a common environment were acclimated for 3 days to 20°C (20°C), 3 days to 27°C followed by 3 days to 20°C (27-20°C), 3 days to 27°C (27°C), or for 3 days to 20°C followed by 3 days to 27°C (20-27°C). We predicted that cold-acclimated individuals show shorter recovery times than warm-acclimated ones and that this plastic response is reversible. Chill-coma recovery time varied significantly across acclimation groups ($F_{3,117} = 11.7$, $p<0.001$; 20°C<27-20°C<27°C = 20-27°C; Tukey HSD after ANOVA), with predictions being largely met. Given are means +1 SE. Sample sizes range between 28 and 34 per group.
doi:10.1371/journal.pone.0015284.g001

Table 2. Chill-coma recovery time and mortality rates in *Bicyclus anynana*.

(A) Treatment	20°C [sec]	27°C [sec]	*P*
19 h at 1°C	1547±114	2427±114	**<0.0001**
50 min at −3.5°C	1350±49	1496±49	**0.0402**
90 min at −3.5°C	1312±45	1789±45	**<0.0001**
45 min at −5°C	1198±55	1352±55	**0.0354**
15 min at −8°C	530±55	686±52	**0.0145**
(B) Treatment	**20°C [%]**	**27°C [%]**	***P***
19 h at 1°C	15.3	43.5	**0.0128**
50 min at −3.5°C	5.0	25.0	**0.0105**
90 min at −3.5°C	35.0	55.0	0.0696
45 min at −5°C	27.5	50.0	**0.0342**
15 min at −8°C	2.5	20.0	**0.0053**

Chill-coma recovery time (**A**, means ±1 SE) and mortality 24 h after cold exposure (**B**; in %) for 20°C- and 27°C-acclimated *Bicyclus anynana* butterflies across five induction treatments. Significant p-values, as tested by ANOVAs **(A)** and nominal logistic regressions **(B)**, are given in bold. Sexes differed in one out of ten analyses only, with females (47.5%) showing higher mortality rates than males (10.5%) when exposed for 19 h to 1°C (results not shown). Sample sizes are 39 or 40 throughout, except for recovery times in the final treatment, where sample size is only 18 and 20, respectively.
doi:10.1371/journal.pone.0015284.t002

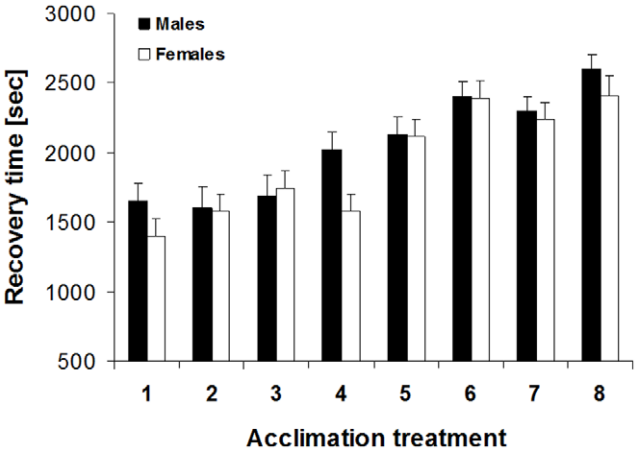

Figure 2. Chill-coma recovery time of *Bicyclus anynana* in relation to sex and acclimation treatment. Butterflies reared in a common environment were acclimated on three consecutive days to different combinations of 20°C and 27°C prior to testing: 20-20-20°C (acclimated for 3 days to 20°C; treatment 1), 27-20-20°C (acclimated for 1 day to 27°C and then for 2 days to 20°C; treatment 2), 20-27-20°C (3), 27-27-20°C (4), 20-20-27°C (5), 27-20-27°C (6), 20-27-27°C (7), 27-27-27°C (8). We hypothesized that the final day prior to testing has the largest impact on cold resistance, while earlier thermal experience may have some subtle, modulating impact. Chill-coma recovery time differed significantly across acclimation groups ($F_{7,566} = 19.4$, $p < 0.0001$) and sexes ($F_{1,566} = 4.9$, $p = 0.0280$), with the thermal environment experienced during the last day prior to testing having the largest impact ($1 = 2 = 3 = 4 < 5 < 6 = 7 = 8$; Tukey HSD after ANOVA). Given are means +1 SE. Sample sizes range between 31 and 46 per group.
doi:10.1371/journal.pone.0015284.g002

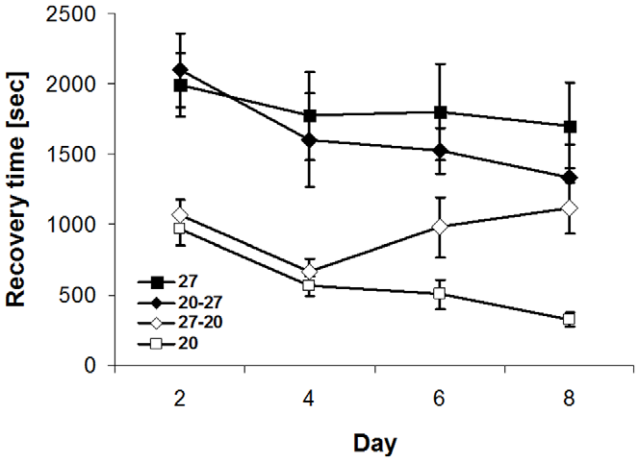

Figure 3. Chill-coma recovery time of *Bicyclus anynana* in relation to acclimation treatment and age. Butterflies reared in a common environment were acclimated for up to 8 days to 20°C, 27°C, 20°C and afterwards 27°C or to 27°C and afterwards 20°C. Butterflies of the 'transfer groups' were, depending on age at testing, exposed for 0–6 days to the first, but always for 2 days to the second temperature. Note that accordingly day 2 butterflies did not experience a temperature change. We tested the prediction that the ability to acclimate to a novel environment diminishes with increasing with age, which was not supported by empirical data (interaction between treatment and age not significant; $F_{1,298} = 0.7$, $p = 0.70$). Given are means +1 SE. Sample sizes range between 12 and 26 per group.
doi:10.1371/journal.pone.0015284.g003

Effects of acclimation and hardening on heat stress resistance

Experiment 6. Heat knock-down time (measured two hours after hardening) was significantly affected by acclimation temperature (34°C>27°C>20°C; Tukey HSD after ANOVA), but not by hardening temperature and sex (Table 4). A significant acclimation by hardening temperature interaction indicates that the differences among acclimation temperatures were rather consistent throughout, except that the individuals acclimated to 27°C and hardened at 34°C showed very similar knock-down times compared to the individuals acclimated and hardened at 34°C (Fig. 5). Testing butterflies acclimated to 27°C immediately after hardening (for 1 hour at 20°C, 27°C or 34°C) once again did not yield a significant effect of hardening temperature on heat knock-down time (hardening temperature: $F_{2,84} = 0.94$, $p = 0.3934$; sex: $F_{1,84} = 0.30$, $p = 0.5879$; interaction: $F_{2,84} = 1.27$, $p = 0.2871$). However, using a more extreme hardening temperature of 39°C revealed a significantly longer knock-down time compared to animals exposed to 20°C (39°C: 561±19; 20°C: 456±19 sec; $t_{46} = 4.0$, $p < 0.0001$, $n = 48$).

Table 3. ANCOVA results for the effects of pupal mass (covariate), acclimation treatment and age on chill-coma recovery time. Significant p-values are given in bold.

	DF	MQ	*F*	*P*
Pupal mass	1	3882912	4.8	**0.0293**
Acclimation treatment	3	26150191	32.3	**<0.0001**
Age	3	3312293	4.1	**0.0072**
Accl. treatment x age	9	576528	0.7	0.6977
Error	298	809584		

doi:10.1371/journal.pone.0015284.t003

Experiment 7. A clear response to acclimation temperature can be expected within one day already. Butterflies reared and maintained at 27°C, being divided among 20 and 27°C on day two of adult life for 24 h, differed significantly in heat knock-down

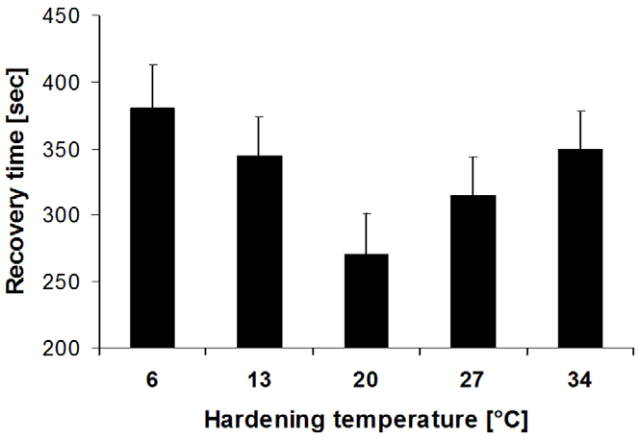

Figure 4. Chill-coma recovery time of *Bicyclus anynana* in relation to hardening temperature. We predicted that colder hardening temperatures increase but warmer hardening temperatures decrease cold resistance. While hardening temperature indeed affected recovery time ($F_{4,229} = 3.08$, $p = 0.0169$), both high and low hardening temperatures increased recovery times (6°C = 34°C ≥ 13°C = 27°C (control) ≥ 20°C; Tukey HSD after ANOVA). Given are means +1 SE. Sample sizes range between 47 and 48 per group.
doi:10.1371/journal.pone.0015284.g004

Table 4. ANOVA results for the effects of acclimation temperature, hardening temperature and sex on heat knock-down time.

	DF	MQ	*F*	*p*
Acclimation T	2	0.888	62.6	**<0.0001**
Hardening T	2	0.032	2.2	0.1080
Sex	1	0.021	1.5	0.2202
Accl. T x Hard. T	4	0.034	2.4	**0.0478**
Accl. T x Sex	2	0.002	0.2	0.8582
Hard. T x Sex	2	0.033	2.3	0.0988
Accl. T x Hard. T x Sex	4	0.017	1.2	0.3202
Error	321	0.014		

Significant p-values are given in bold. T: temperature.
doi:10.1371/journal.pone.0015284.t004

time with 27°C-acclimated individuals (528±87 sec, n = 20) being much more heat resistant than 20°C-acclimated ones (209±15 sec, n = 20; Mann-Whitney U-test: Z = −3.81, p = 0.0001). However, longer exposure to extreme temperatures may impair the acclimation response. When being subjected for 19 h to 1°C on day three of adult life, butterflies did not regain increased heat stress resistance after a recovery period of another three days at different temperatures: While the 27°C control group showed a significantly increased heat knock-down time, the 27°C group exposed to the cold did not differ significantly from both groups acclimated to 20°C (Tukey HSD after ANOVA; acclimation group: $F_{3,157} = 11.2$, $p<0.001$; sex: $F_{1,157} = 0.1$, $p = 0.7146$; interaction: $F_{3,157} = 0.4$, $p = 0.7458$; Fig. 6).

Effects of larval and adult food limitation on temperature stress resistance

Experiment 8. Food stress during larval development significantly reduced pupal mass ($F_{1,364} = 24.2$, $p<0.001$), and

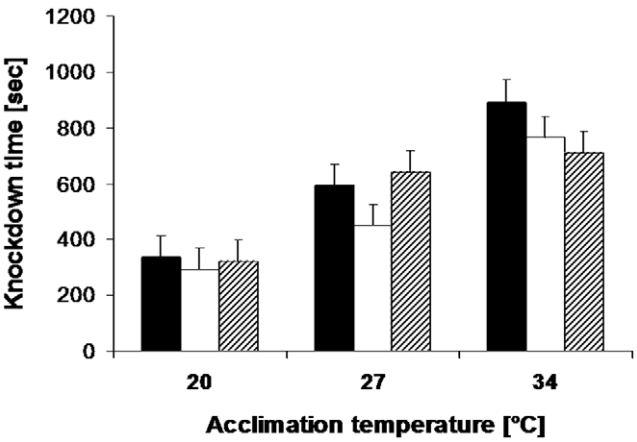

Figure 5. Heat knock-down time of *Bicyclus anynana* in relation to acclimation and hardening temperature. We hypothesized that warmer acclimation and hardening temperatures increase heat tolerance and vice versa. While this prediction was met for acclimation temperature ($F_{2,321} = 62.6$, $p<0.0001$; 34°C>27°C>20°C; Tukey HSD after ANOVA), the effect of hardening temperature was not significant ($F_{2,321} = 2.2$, $p = 0.11$). Black bars: hardened at 20°C; open bars: hardened at 27°C; hatched bars: hardened at 34°C. Given are means +1 SE. Sample sizes range between 37 and 38 per group.
doi:10.1371/journal.pone.0015284.g005

females were significantly heavier than males ($F_{1,364} = 59.0$, $p<0.001$). As indicated by a significant food stress by sex interaction ($F_{1,364} = 6.9$, $p = 0.009$), females were more strongly affected by food stress than males (food stressed males: 136.8±3.7 mg; control males: 143.4±3.0 mg; food stressed females: 151.4±2.4 mg; control females: 173.1±2.2 mg). While chill-coma recovery time was not affected by either factor, heat knock-down time differed significantly across adult feeding treatments and sexes (Table 5). Butterflies deprived of adult food showed reduced knock-down times compared to controls (326±35 sec versus 371±36 sec), and females were more heat resistant than males (406±34 sec versus 326±31 sec; Fig. 7). A significant interaction between larval and adult feeding treatment indicates that lack of adult food greatly reduced heat stress resistance compared to controls in animals fed as larvae ad libitum (303±43 sec versus 431±47 sec), while it tended to increase heat stress resistance in animals having experienced larval food stress (352±47 sec versus 313±45 sec). The latter partly results from the fact that throughout lack of adult food reduced heat knock-down time, except for the males having experienced larval food stress (significant three-way interaction; Fig. 7).

Note that, regarding the larval by adult feeding interaction, there was a very similar tendency also for chill-coma recovery time ($p = 0.058$; Table 5A), with a negative effect of adult food stress (prolonged recovery times) in animals having experienced no larval food stress (4477±300 sec versus 3534±254 sec), while cold stress resistance was very similar across adult feeding treatments in animals having experienced larval food stress (3745±405 sec versus 3772±355 sec).

Effects of light cycle on temperature stress resistance

Experiment 9. Independent of the method used to induce a chill coma, light cycle had a marginally significant effect on cold stress resistance (Table 6). In the experiment using 19 h at 1°C to induce a chill coma, recovery times tended to be shortest between 10 a.m. and 10 p.m. (Fig. 8A), though note that a post-hoc

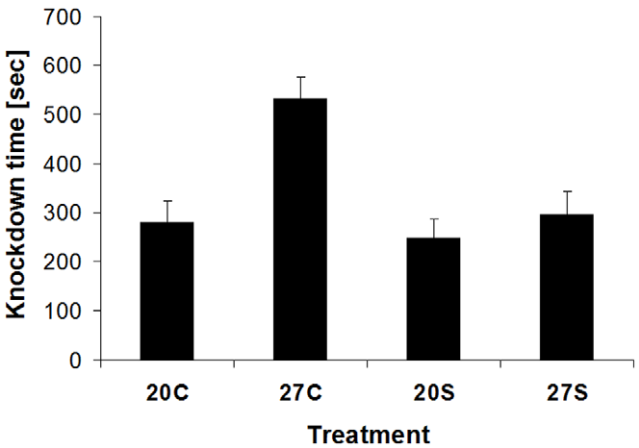

Figure 6. Heat knock-down time of *Bicyclus anynana* in relation to acclimation treatment. Butterflies were acclimated for 6 days to either 20 or 27°C, with half of each acclimation group being subjected for 19 h to 1°C after 3 days. We here test the prediction that severe cold stress will decrease subsequent heat stress resistance, which was confirmed by empirical data ($F_{3,157} = 11.2$, $p<0.001$; 27C>27S = 20C = 20S; Tukey HSD after ANOVA). 20C: 20°C control; 20S: 20°C cold-stressed; 27C: 27°C control; 27S: 27°C cold-stressed. Given are means +1 SE. Sample sizes range between 34 and 49 per group.
doi:10.1371/journal.pone.0015284.g006

Table 5. ANCOVA results for the effects of pupal mass (covariate), larval feeding treatment, adult feeding treatment and sex on chill-coma recovery time (A) and heat knock-down time (B).

(A)	DF	MQ	*F*	*P*
Pupal mass	1	1953.6	1.02	0.3142
Larval starvation	1	166.6	0.09	0.7684
Adult starvation	1	1440.2	0.75	0.3872
Sex	1	668.3	0.35	0.5556
Larv. starv. x adult starv.	1	6986.4	3.65	0.0583
Larv. starv. x sex	1	133.2	0.07	0.7924
Adult starv. x sex	1	589.0	0.31	0.5800
Larv. starv. x adult starv. x sex	1	467.7	0.25	0.6219
Error	123	1912.8		
(B)	**DF**	**MQ**	***F***	***P***
Pupal mass	1	0.1	0.01	0.9150
Larval starvation	1	13.1	2.22	0.1376
Adult starvation	1	28.7	4.87	**0.0284**
Sex	1	29.2	4.95	**0.0271**
Larv. starv. x adult starv.	1	57.1	9.71	**0.0021**
Larv. starv. x sex	1	2.9	0.49	0.4838
Adult starv. x sex	1	<0.1	<0.01	0.9561
Larv. starv. x adult starv. x sex	1	32.8	5.57	**0.0191**
Error	227	5.9		

Significant p-values are given in bold.
doi:10.1371/journal.pone.0015284.t005

comparison (Tukey HSD) did not locate any significant differences among groups. The experiment using 20 min at −5°C indicated that chill-coma recovery time was shortest in the afternoon and evening (Fig. 8B), with butterflies tested at 7 p.m. having a significantly shorter recovery time than those tested at 11 a.m. (Tukey HSD after ANOVA; all other pair-wise comparisons n.s.). Further, here females (924±29 sec) needed significantly shorter times to recover than males (1030±32 sec). Heat stress resistance, in contrast, was neither affected by light cycle or sex (Table 6).

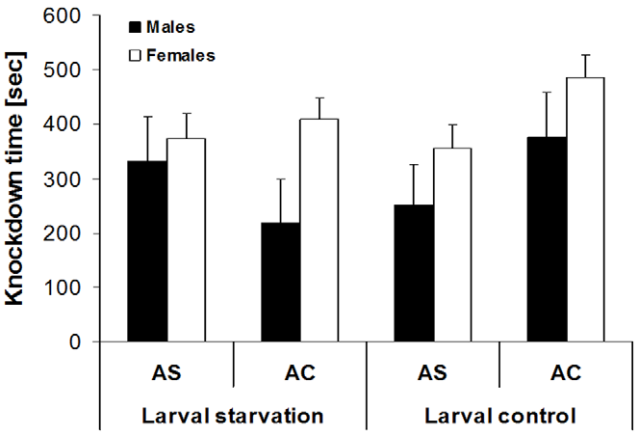

Figure 7. Heat knock-down time of *Bicyclus anynana* in relation to sex, larval and adult feeding treatment. Both larval and adult food stress were expected to decrease temperature stress resistance. Indeed, adult food stress significantly reduced heat tolerance ($F_{1,227}=4.9$, $p=0.03$), while larval food stress did not ($F_{1,227}=2.2$, $p=0.14$; though note the significant interaction with adult starvation, cf. Table 5). AS: adult starvation, AC: adult control. Given are means +1 SE. Sample sizes range between 12 and 50 per group.
doi:10.1371/journal.pone.0015284.g007

Discussion

Temperature effects on cold stress resistance

Throughout, cool-acclimated butterflies showed a shorter chill-coma recovery time compared to warm-acclimated ones, meaning that the former are more resistant to cold stress than the latter and thus indicating an adaptive response to temperature variation (see below; cf. e.g. [27,29–30]). Such increased performance under stressful temperatures may on the other hand induce non-trivial costs in other traits [4,30,57]. The relatively intermediate phenotype of the group first exposed to 27°C and subsequently to 20°C in *experiment 1* (Fig. 1) suggests some carry-over effects of previous thermal experience, which were still measurable after a three-day acclimation period at another temperature. However, a similar tendency was not obvious in the other transfer group, being transferred from 20°C to 27°C, thus questioning the generality of such effects. An alternative explanation would be that the poorer performance of the 27-20°C compared to the 20°C group results from the higher age in the former group (6 versus 3 days). Independent of the lack of consistency in the other transfer group (see above), e*xperiment 4* renders this possibility unlikely, as recovery times decreased rather than increased with age in three out of four groups. Also, a fundamental difference between transfers from warm to cool versus cool to warm is unsupported by additional data (see below, *experiment 3*, but *experiment 4*; cf. [25]).

Experiment 2 clearly demonstrates that such acclamatory responses are largely independent of the method used to induce a chill coma (see also [30,52,58]). We therefore argue that the specific assay conditions used may not be that critical for measuring plastic responses to different thermal environments.

Table 6. ANOVA results for the effects of light cycle and sex on chill-coma recovery time after 19 h at 1°C (A) or after 20 min at −5°C (B), and on heat knock-down time at 45°C (C).

(A)	DF	MQ	*F*	*p*
Light cycle	5	504.4	2.3	**0.0448**
Sex	1	341.0	1.6	0.2136
Light cycle x sex	5	410.4	1.9	0.0990
Error	360	219.6		
(B)	**DF**	**MQ**	***F***	***p***
Light cycle	5	95.1	2.3	**0.0463**
Sex	1	244.6	5.9	**0.0160**
Light cycle x sex	5	44.5	1.1	0.3782
Error	317	41.7		
(C)	**DF**	**MQ**	***F***	***p***
Light cycle	5	1518.0	1.1	0.3895
Sex	1	443.0	0.3	0.5808
Light cycle x sex	5	782.4	0.5	0.7462
Error	420	1449.8		

Significant p-values are given in bold.
doi:10.1371/journal.pone.0015284.t006

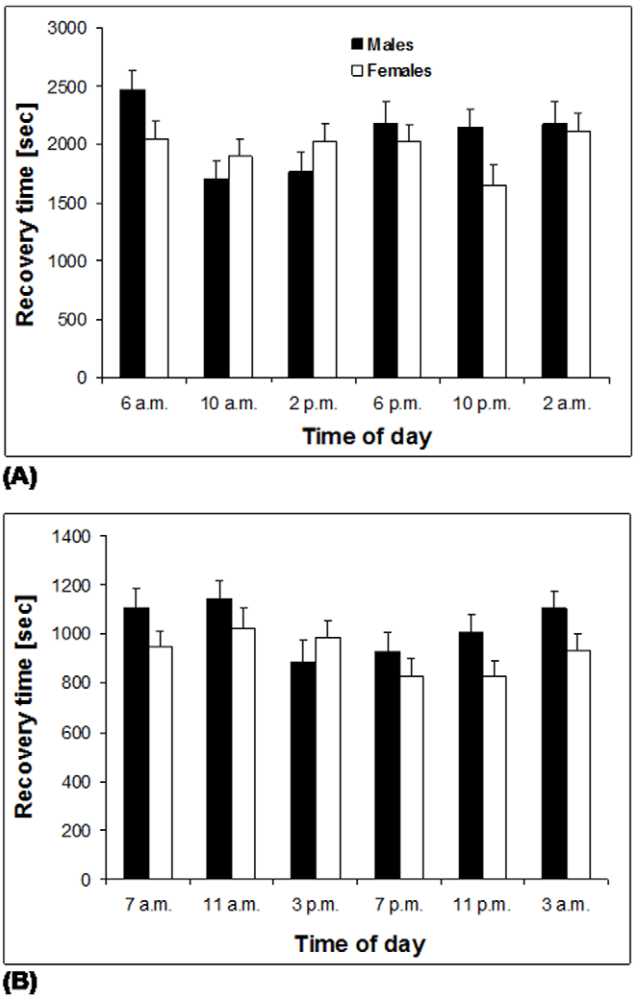

Figure 8. Chill-coma recovery time of *Bicyclus anynana* in relation to time of day and sex. We hypothesized that butterflies are more cold-tolerant early in the morning, but more heat-tolerant in the early afternoon. **(A)** Measured after 19 h at 1°C; **(B)** measured after 20 min at −5°C. In both cases light cycle had a marginally significant effect ($F_{5,360} = 2.3$, $p = 0.045$ and $F_{5,317} = 2.3$, $p = 0.046$), with cold stress resistance tending to be higher during daytime. The photo phase lasted for 12 h from 6 a.m. to 6 p.m. Given are means +1 SE. Sample sizes range between 22 and 41 per group for **(A)** and between 19 and 35 in **(B)**.
doi:10.1371/journal.pone.0015284.g008

The adaptive value of shorter recovery times after cold exposure could be related to an earlier activity in the mornings following relatively cool nights, a longer activity in the evenings and/or generally higher levels of activity at suboptimal temperatures. Consequently, more time would be available for essential behaviours such as foraging, mate location and reproduction. Note in this context that flight performance is strongly related to ambient temperature in butterflies and other ectotherms [59,60]. Moreover, our data clearly demonstrate that chill-coma recovery time, frequently used as a proxy for cold stress resistance, is closely related to the survival rate after cold exposure [30].

Given that *experiment 1* did not yield conclusive results on the effects of previous thermal experience, this issue was once again addressed in *experiment 3*, using a more sophisticated design. The respective results show that the acclimation response is fairly fast, because the temperature experienced during the last 24 hours prior to testing had a dominating effect on thermal performance (Fig. 2). Although post-hoc comparisons revealed little significant variation except from the clear distinction between the above mentioned groups (i.e. between the animals having experienced 20°C versus 27°C during the last 24 hours prior to testing), the data suggest that the previous thermal environment does have some subtle, modulating effects on cold stress resistance, with effect size increasing with increasing exposure time to an alternative temperature. Results from *experiment 4* corroborate the above data, in showing that the temperature experienced directly before testing has the largest effect on stress tolerance, and that a previous temperature change does modulate the acclimation response to some extent (though significantly only in the 27-20°C group). Further, visual inspection of figure 3 does suggest that the ability to acclimate to another temperature does diminish with longer exposure to a given temperature and thereby age, though this tendency is statistically not supported.

In contrast to expectations, recovery times slightly decreased rather than increased with age (see also [61]). Thus, at least over the age classes tested here, age did not negatively affect recovery times, but rather tended to increase performance. This as well as the lack of interactive effects might be related to the fact that exclusively relatively young butterflies were tested here (note that *B. anynana* may reach substantially longer life spans in the laboratory; [25,62]). However, we consider this time span as of particular ecological importance, as butterflies (apart from diapausing individuals) typically have fairly short life spans in the field [63]. In any case our data rule out that there is a simple linear decrease in performance with age, which contrasts with results on heat shock survival showing in various insects a strong decline during the early adult period [42,48]. The few data available for effects of age on chill-coma recovery time to date revealed contradictory evidence and thus no consistent pattern [43,61].

In addition to the acclimation temperatures discussed above, short-time exposure to different ('hardening') temperatures also affected cold stress tolerance (*experiment 5*). However, the response depended on the methodology used to induce a chill coma, as exposure for 19 hours to 1°C did not reveal any significant effect. We assume that the exposure time to 1°C was simply too long in order to yield a measurable effect of a 1 hour exposure to different hardening temperatures. This notion is supported by our additional experiment using a four minute exposure to −20°C following hardening. Here, recovery time was shortest after 'hardening' at 20°C, a temperature which is well within the range of temperatures frequently experienced by the butterflies in the field (reflecting the daily highs during the cooler dry season [53,55]). Interestingly though, cooler as well as warmer temperatures increased recovery times. Thus, higher temperatures negatively affected cold stress resistance as expected, but the same was true for lower temperatures of 6°C and 13°C, with at least the latter certainly occurring occasionally in the field. These data clearly suggest that the latter temperatures are already stressful for this tropical butterfly, thus diminishing rather than improving subsequent performance [22,25]. Partly similar results regarding negative effects of hardening on chill-coma recovery time were obtained in *Drosophila* [30].

Temperature effects on heat stress resistance

As with cold stress resistance, acclimation temperature induced a significant effect on heat stress resistance, with heat knock-down time increasing substantially with increasing acclimation temperature: butterflies acclimated to 34°C resisted heat stress roughly twice as long compared to butterflies acclimated to 20°C [27,64]. These data once again clearly indicate adaptive phenotypic plasticity, in particular since heat coma is typically followed soon

by lethal stress levels. Using short-time exposure to the same three temperatures (20°C, 27°C, 34°C; 'hardening'), in contrast, did not yield a significant effect, independent of whether butterflies were left to recover for two hours or tested immediately after hardening. These findings suggest that the hardening temperatures used were not extreme enough to induce a rapid hardening response, although the same temperatures were effective in inducing an acclimation response. This notion is supported by an additional set of data comparing hardening temperatures of 20°C and 39°C, where a significant effect of one hour exposure showed a significantly better performance of the latter under heat stress (but e.g. [20] for *Drosophila*). Thus, the mechanisms underlying the hardening response may differ fundamentally from those underlying the (longer-term) acclimation response [30,58,65]. The hardening response to heat stress appears to be a sort of emergency mechanism, being activated under acute heat stress only [19,29,66]. Alternatively, the exposure time of one hour may have been too short to induce a measurable response when using less extreme temperatures, or the time for recovery may have been too short [20]. Further, our results suggest that heat and cold hardening may also differ with regard to underlying mechanisms [29,65], as the same hardening temperatures (20°C, 27°C, 34°C) elucidated a clear response in cold stress resistance (see above), but not in heat stress resistance.

Similar to cold stress resistance, variation in heat stress resistance can be expected to occur within 24 hours spent at different temperatures. Nevertheless, exposure for many (19) hours to 1°C clearly reduced subsequent heat stress resistance, although butterflies spent three full days at different acclimation temperatures following the cold stress. Consequently, an extreme (cold) stress event yields longer-lasting effects on subsequent heat stress resistance, lasting well beyond periods typically inducing a clear acclimation response. Interestingly, the negative effect was restricted to the group acclimated to 27°C, while there was no obvious response to extreme cold stress across both groups acclimated to 20°C. Thus, cold exposure interfered with acclimation to warmer temperatures, rather than causing a generally diminished performance.

Food access and temperature stress resistance

Our larval treatment was successful in imposing food stress, as evidenced by a significant reduction in pupal mass by ca. 10%. Similarly, adult food deprivation is known to reduce body mass in *B. anynana* [46]. Despite the clear evidence for a reduction in the amount of resources available to butterflies, cold stress resistance was not affected by larval or adult feeding treatment. Heat stress resistance, in contrast, was negatively affected by adult food stress, but not (directly) affected by larval food stress (cf. [47] for effects of different larval feeding treatments in *Drosophila*). The latter indicates that the resources carried over from the larval stage may not be that crucial for temperature stress resistance (note in this context the non-significant effect of the covariate pupal mass in both analyses). However, this view is challenged by the interactive effect between larval and adult food stress for heat stress resistance. This significant interaction suggests that only the butterflies having experienced neither larval nor adult food stress showed a better performance under heat stress compared to the other three groups. In other words, butterflies having experienced larval food stress could not take any advantage from having access to adult food (Fig. 7; cf. [67] for reproductive traits). These findings suggest that putatively nitrogenous larval resources, lack of which cannot be compensated for in the adult stage, do actually play a crucial role for stress resistance, setting an upper limit to performance under heat stress [47,67]. Why adult food access actually reduced heat stress resistance in males having experienced larval food stress, thus showing the opposite pattern compared to all other treatment groups, is difficult to explain and might reflect a chance effect of allocation to treatments.

The discrepancy between heat and cold stress resistance, with the former not being affected by any type of food stress, suggests that the mechanisms involved in heat resistance (e.g. the heat shock response [14]) might be more costly than those involved in cold stress resistance [65,68–69]. However, regarding the above larval by adult feeding treatment interaction for heat stress resistance, there was an analogous statistical trend for cold stress resistance ($p = 0.058$). Here, adult food stress tended to have a negative impact on cold resistance only if individuals had experienced no larval food stress, while all other groups performed comparably well. Thus, these data may suggest that individuals having been challenged during the larval stage were better prepared to handle adult food stress compared to control individuals [35,70–71]. In any case patterns obtained for heat versus cold stress resistance were strikingly different, which again suggests divergent underlying mechanisms [43,47]. While the basic mechanism underlying heat stress resistance seems to be the heat shock response, cold stress resistance seems to involve several mechanisms including cryoprotectants, antifreeze proteins, glycerol, heat-shock proteins and changes in membrane fluidity and composition [65].

Light cycle and temperature stress resistance

While heat stress resistance was not affected by light cycle, cold stress resistance did vary significantly across time of day, independently of the method used to induce a chill coma. However, effect size was small and significance marginal only, which may suggest that the patterns found are biologically irrelevant. On the other hand it is striking that two independent experiments using different approaches yielded qualitatively fairly similar patterns. Overall, it appeared that cold stress resistance was slightly higher during daytime, especially in the afternoon/evening, as compared to night time. Such a pattern could on principle be adaptive, as it might enable higher levels of activity during spells of unfavourably cool weather or in the cool dry season (although butterflies seem to rely in the first place on their cryptic coloration here, thus avoiding unnecessary flight [33,53]).

Sex differences and effects of pupal mass

In some experiments sex differences could not be investigated due to the exclusive use of females for logistic reasons. However, in a total of 22 statistical analyses sex was included as factor, yielding in 18 cases a non-significant result. In the remaining cases females showed twice a higher cold stress resistance than males, once a higher heat stress resistance, while survival rates after cold exposure were once lower in females than in males. These results indicate that, overall, sexes seem to be equally stress resistant in *B. anynana* [23,27,66].

In two experiments the effect of body size on temperature stress resistance was investigated, by including pupal mass as covariate in the statistical models. While two analyses on heat and cold stress resistance, respectively, revealed no significant effect of pupal mass, it did affect cold stress resistance in *experiment 4*. However, this effect was marginal, and a subsequent Pearson correlation between pupal mass and chill-coma recovery time was non-significant (data not shown). Thus, body size is clearly of subordinate importance for temperature stress resistance in *B. anynana*, challenging the common notion of a positive association between stress resistance and body size.

Conclusions

Having overall found clear evidence for environmentally-induced variation in temperature stress resistance, one crucial question remains: Did our experimental designs resemble natural conditions closely enough to extrapolate from our results to field conditions? Our answer is a tentative 'yes'. The acclimation temperatures used are definitively within the range of temperatures experienced by *B. anynana* in its natural environment, which is also true for the majority of 'hardening' temperatures [33,53]. This is the main reason why we decided to use relatively mild 'hardening' temperatures throughout. Further, the temperature used to assess heat stress resistance (45°C) will be regularly reached during high solar radiation, at least close to the ground. More critically seem to be the assays on cold stress resistance, as the temperatures used to induce a chill coma were necessarily very low, probably largely without the range of temperatures usually experienced by the butterflies in their natural environment. However, our results also document that the patterns obtained are largely independent of the specific assay conditions used. Further, both heat knock-down and chill-coma recovery time seem to be closely related to fitness, as both correlate with survival rates. We therefore argue that both should be considered convenient proxies of temperature adaptation, even if the experimental conditions chosen do not perfectly resemble natural conditions. Overall, the measurement of acclimation responses seems much less susceptible to assay conditions as compared to critical thermal limits [72]. Nevertheless, we need more studies examining the impact and consequences of using more natural versus more artificial settings.

Our results suggest that temperature-induced plasticity in stress resistance is a striking example of adaptive phenotypic plasticity, thus supporting the beneficial acclimation hypothesis, which has been repeatedly challenged over recent years [22,73]. Plasticity is thus an effective tool to greatly modulate temperature stress resistance within very short periods of time, thus increasing survival probability under temperature stress [19,31,57,74]. This is likely to be true for the vast majority of organisms, while in some specific cases species may exhibit reduced levels of plasticity, thus limiting quick responses to changing thermal environments [27,38–39,75]. Such species may be exceptionally vulnerable to the impact of global change [75]. Except from extreme events, such plastic responses are further largely and fairly quickly reversible, enabling adequate responses to fluctuating thermal conditions. As plastic responses may thus fine-tune phenotypes to environmental needs including thermal challenges, the potential costs associated with plastic responses or more plastic genotypes remains a recurrent and still largely unresolved issue in evolutionary biology [41]. In any case the species' ability to respond plastically needs to be incorporated into models trying to forecast effects of global change on extant biodiversity [75–76]. Further, we suggest that other factors at least potentially affecting plastic responses, such as limited resource availability which may well go hand in hand with climate change due to the ubiquitous impact of man on natural systems, should not be neglected. Especially investigating interactive effects between food availability and temperature challenges should prove to be a fruitful and valuable area for future research. Facing the increasing temperatures at the global scale, investigating genetic but also plastic responses to temperature will be at the forefront of evolutionary and ecological research for some time to come.

Acknowledgments

We thank Marise Giertz, Gudrun Schneider and Alexander Sell for help with experiment 3, and Stephanie Bauerfeind for valuable comments on an earlier version of this paper.

Author Contributions

Conceived and designed the experiments: KFischer TLG. Performed the experiments: AD KFranke ML SW CP. Analyzed the data: KFischer AD KFranke TLG ML SW CP. Wrote the paper: KFischer.

References

1. Willmer P, Stone G, Johnston IA (2000) Environmental Physiology of Animals. Oxford: Blackwell.
2. Bijlsma R, Loeschcke V (2000) Environmental stress, adaptation and evolution: an overview. J Evol Biol 18: 744–749.
3. Marshall KE, Sinclair BJ (2010) Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*. Proc R Soc Lond B 277: 963–969.
4. Clarke A (2003) Costs and consequences of evolutionary temperature adaptation. Trends Ecol Evol 18: 573–581.
5. Hoffmann AA, Sørensen JG, Loeschcke V (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J Therm Biol 28: 175–216.
6. Sinclair BJ, Vernon P, Klok CJ, Chown SL (2003) Insects at low temperatures: an ecological perspective. Trends Ecol Evol 18: 257–262.
7. Sørensen JG, Norry FM, Scannapieco AC, Loeschcke V (2005) Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. J Evol Biol 18: 829–837.
8. Dahlhoff EP, Rank NE (2007) The role of stress proteins in responses of a montane willow leaf beetle to environmental temperature variation. J Biosci 32: 477–488.
9. Diffenbaugh NS, Pal JS, Trapp RJ, Giorgi F (2005) Fine-scale processes regulate the response of extreme events to global climate change. Proc Natl Acad Sci U S A 102: 15774–15778.
10. Diffenbaugh NS, Pal JS, Giorgi F, Gao X (2007) Heat stress intensification in the Mediterranean climate change hotspot. Geophysical Research Letters 34: L11706.
11. Sala OE, Chapin FS, Armesto JJ, Berlow E, Bloomfield J, et al. (2000) Biodiversity: global biodiversity scenarios for the year 2100. Science 287: 1770–1774.
12. Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, et al. (2004) Extinction risk from climate change. Nature 427: 145–148.
13. Deutsch CA, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, et al. (2008) Impacts of climate warming on terrestrial ectotherms across latitude. Proc Natl Acad Sci U S A 105: 6668–6672.
14. Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6: 1025–1037.
15. Chown SL, Terblanche JS (2007) Phyiological diversity in insects: ecological and evolutionary contexts. Adv Insect Physiol 33: 50–152.
16. Bradshaw AD (1965) Evolutionary significance of phenotypic plasticity in plants. Adv Genet 13: 115–155.
17. Pigliucci M (2001) Phenotypic plasticity: beyond nature and nurture. Baltimore: John Hopkins University Press.
18. Lee RE, Chen C-P, Denlinger DL (1987) A rapid cold-hardening process in insects. Science 238: 1415–1417.
19. Sejerkilde M, Sørensen JG, Loeschcke V (2003) Effects of cold- and heat hardening on thermal resistance in *Drosophila melanogaster*. J Insect Physiol 49: 719–726.
20. Kellett M, Hoffmann AA, McKechnie SW (2005) Hardening capacity in the *Drosophila melanogaster* species group is constrained by basal thermotolerance. Funct Ecol 19: 853–858.
21. Marais E, Terblanche JS, Chown SL (2009) Life stage-related differences in hardening and acclimation of thermal tolerance traits in the kelp fly, *Paratora dreuxi* (Diptera, Helcomyzidae). J Insect Physiol 55: 336–343.
22. Wilson RS, Franklin CE (2002) Testing the beneficial acclimation hypothesis. Trends Ecol Evol 17: 66–70.
23. Zeilstra I, Fischer K (2003) Cold tolerance in relation to developmental and adult temperature in a butterfly. Physiol Entomol 30: 92–95.
24. Deere JA, Sinclair BJ, Marshall DJ, Chown SL (2006) Phynotypic plasticity of thermal tolerances in five oribatid mite species from sub-Antarctic Marion Island. J Insect Physiol 52: 693–700.
25. Geister TL, Fischer K (2007) Testing the beneficial acclimation hypothesis: cool males – hot love? Behav Ecol 18: 658–664.

26. Terblanche JS, Marais E, Chown SL (2007) Stage-related variation in rapid cold hardening as a test of the environmental predictability hypothesis. J Insect Physiol 53: 455–462.
27. Karl I, Janowitz SA, Fischer K (2008) Altitudinal life-history variation and thermal adaptation in the Copper butterfly *Lycaena tityrus*. Oikos 117: 778–788.
28. Bahrndorff S, Loeschcke V, Pertoldi C, Beier C, Holmstrup M (2009) The rapid cold hardening response of *Collembola* is influenced by thermal variability of the habitat. Funct Ecol 23: 340–347.
29. Rajamohan A, Sinclair BJ (2009) Hardening trumps acclimation in improving cold tolerance of *Drosophila melanogaster* larvae. Physiol Entomol 34: 217–223.
30. Rako L, Hoffmann AA (2006) Complexity of the cold acclimation response in *Drosophila melanogaster*. J Insect Physiol 52: 94–104.
31. Ayrinhac A, Debat V, Gibert P, Kister A-G, Legout H, et al. (2004) Cold adaptation in geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more important than genetic variability. Funct Ecol 18: 700–706.
32. Castañeda L, Lardies MA, Bozinovic F (2005) Interpopulational variation in recovery time from chill coma along a geographic gradient: a study in the common woodlouse, *Porcellio laevis*. J Insect Physiol 51: 1346–1351.
33. Brakefield PM, Reitsma N (1991) Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. Ecol Entomol 16: 291–303.
34. Fischer K, Brakefield PM, Zwaan BJ (2003) Plasticity in butterfly egg size: why larger offspring at lower temperatures? Ecology 84: 3138–3147.
35. Bauerfeind SS, Perlick JEC, Fischer K (2009) Disentangling environmental effects on adult life span in a butterfly across the metamorphic boundary. Exp Geront 44: 805–811.
36. Larsen TB (1991) The Butterflies of Kenya. Oxford: University Press.
37. Tewksbury JJ, Huey RB, Deutsch CA (2008) Ecology: Putting the heat on tropical animals. Science 320: 1296–1297.
38. Kellermann VM, Van Heerwaarden B, Sgro CM, Hoffmann AA (2009) Fundamental evolutionary limits in ecological traits drive *Drosophila* species distributions. Science 325: 1244–1246.
39. Kellermann VM, Van Heerwaarden B, Hoffmann AA, Sgro CM (2006) Very low additive genetic variance and evolutionary potential in multiple populations of two rainforest *Drosophila* species. Evolution 60: 1104–1108.
40. Hoffmann AA, Parsons PA (1991) : Evolutionary genetics and environmental stress. Oxford: University Press.
41. Auld JR, Agrawal AA, Relyea RA (2010) Re-evaluating the costs and limits of adaptive phenotypic plasticity. Proc R Soc Lond B 277: 503–511.
42. Pappas C, Hyde D, Bowler K, Loeschcke V, Sørensen JG (2007) Post-eclosion decline in 'knock-down' thermal resistance and reduced effect of heat hardening in *Drosophila melanogaster*. Comp Biochem Physiol A 146: 355–359.
43. Bowler K, Terblanche JS (2008) Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? Biol Rev 83: 339–355.
44. Karlsson B (1994) Feeding habits and change of body composition with age in three nymphalid butterflies species. Oikos 69: 224–230.
45. Stjernholm F, Karlsson B (2006) Reproductive expenditure affects utilization of thoracic and abdominal resources in male *Pieris napi* butterflies. Funct Ecol 20: 442–448.
46. Fischer K, Perlick J, Galetz T (2008) Residual reproductive value and male mating success: older males do better. Proc R Soc Lond B 275: 1517–1524.
47. Andersen LH, Kristensen TN, Loeschcke V, Toft S, Mayntz D (2010) Protein and carbohydrate composition of larval food affects tolerance to thermal stress and desiccation in adult *Drosophila melanogaster*. J Insect Physiol 56: 336–340.
48. Sørensen JG, Loeschcke V (2002) Natural adaptation to environmental stress via physiological clock-regulation of stress resistance in *Drosophila*. Ecol Lett 5: 16–19.
49. McMillan DM, Fearnley SL, Rank NE, Dahlhoff EP (2005) Natural temperature variation affects larval survival, development and HSP70 expression in a leaf beetle. Funct Ecol 19: 844–852.
50. Gibert P, Huey RB (2001) Chill-coma temperature in *Drosophila*: effects of developmental temperature, latitude, and phylogeny. Physiol Biochem Zool 74: 429–434.
51. Gibert P, Moreteau B, Petavy G, Karan D, David JR (2001) Chill-coma tolerance, a major climatic adaptation among *Drosophila* species. Evolution 55: 1063–1068.
52. David J, Gibert P, Moreteau B, Gilchrist GW, Huey RB (2003) The fly that came in from the cold: geographic variation of recovery time from low-temperature exposure in *Drosophila subobscura*. Funct Ecol 17: 425–430.
53. Brakefield PM (1997) Phenotypic plasticity and fluctuating asymmetry as responses to environmental stress in the butterfly *Bicyclus anynana*. In: Bijlsma RR, Loeschcke V, eds. Environmental Stress: Adaptation and Evolution. Basel: Birkhäuser. pp 105–146.
54. Lyytinen A, Brakefield PM, Lindstrom L, Mappes JA (2004) Does predation maintain eyespot plasticity in *Bicyclus anynana*? Proc R Soc Lond B 271: 279–283.
55. Windig JJ, Brakefield PM, Reitsma N, Wilson JGM (1994) Seasonal polyphenism in the wild: survey of wing patterns in five species of *Bicyclus* butterflies in Malawi. Ecol Entomol 19: 285–298.
56. Van't Hof AE, Zwaan BJ, Saccheri IJ, Daly D, Bot ANM, et al. (2005) Characterization of 28 microsatellite loci for the butterfly *Bicyclus anynana*. Mol Ecol Notes 5: 169–172.
57. Loeschcke V, Hoffmann AA (2007) Consequences of heat hardening on a field fitness component in *Drosophila* depend on environmental temperature. Am Nat 169: 175–183.
58. Macdonald SS, Rako L, Batterham P, Hoffmann AA (2004) Dissecting chill coma recovery as a measure of cold resistance: evidence for a biphasic response in *Drosophila melanogaster*. J Insect Physiol 50: 695–700.
59. Merckx T, Karlsson B, Van Dyck H (2006) Sex- and landscape-related differences in flight ability under suboptimal temperatures in a woodland butterfly. Funct Ecol 20: 436–441.
60. Karl I, Hoffmann KH, Fischer K (2010) Food stress sensitivity and flight performance across phosphoglucose isomerase enzyme genotypes in a Copper butterfly. Popul Ecol 52: 307–315.
61. Lalouette L, Vernon P, Amat H, Renault D (2010) Ageing and thermal performance in the sub-Antarctic wingless fly *Anatalanta aptera* (Diptera: Shaeoceridae): older is better. Biol Lett 6: 346–349.
62. Pijpe J, Brakefield PM, Zwaan BJ (2008) Increased life span in a polyphenic butterfly artificially selected for starvation resistance. Am Nat 171: 81–90.
63. Fischer K, Fiedler K (2001) Resource-based territoriality in the butterfly *Lycaena hippothoe* and environmentally induced behavioural shifts. Anim Behav 61: 723–732.
64. Loeschcke V, Sørensen JG (2005) Acclimation, heat shock and hardening - a response from evolutionary biology. J Therm Biol 30: 255–257.
65. Sinclair BJ, Roberts SP (2005) Acclimation, shock and hardening in the cold. J Therm Biol 30: 557–562.
66. Jensen D, Overgaard J, Sørensen JG (2007) The influence of developmental stage on cold shock resistance and ability to cold harden in *Drosophila melanogaster*. J Insect Physiol 53: 179–186.
67. Bauerfeind SS, Fischer K (2005) Effects of food stress and density in different life stages on reproduction in a butterfly. Oikos 111: 514–524.
68. Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrupp M (2005) Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. J Insect Physiol 51: 1173–1182.
69. Overgaard J, Tomcala A, Sørensen JG, Holmstrup M, Krogh PH, et al. (2008) Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly *Drosophila melanogaster*. J Insect Physiol 54: 619–629.
70. Karl I, Lorenz MW, Fischer K (2007) Energetics of reproduction: consequences of divergent selection on egg size for egg composition and reproductive effort in a butterfly. Biol J Linn Soc 91: 403–418.
71. Karl I, Fischer K (2009) Altitudinal and environmental variation in lifespan in the Copper butterfly *Lycaena tityrus*. Funct Ecol 23: 1132–1138.
72. Chown SL, Jumbam KR, Sørensen JG, Terblanche JS (2009) Phenotypic variance, plasticity and heriability estimates of critical thermal limits depend on methodological context. Funct Ecol 23: 133–140.
73. Woods HA, Harrison JF (2002) Interpreting rejections of the beneficial acclimation hypothesis: when is physiological plasticity adaptive? Evolution 56: 1863–1866.
74. Rajamohan A, Sinclair BJ (2008) Short-term hardening effects on survival of acute and chronic cold exposure by *Drosophila melanogaster* larvae. J Insect Physiol 54: 708–718.
75. Karl I, Sørensen JG, Loeschcke V, Fischer K (2009) HSP70 expression in the Copper butterfly *Lycaena tityrus* across altitudes and temperatures. J Evol Biol 22: 172–178.
76. Calosi P, Bilton DT, Spicer JI (2008) Thermal tolerance, acclimatory capacity and vulnerability to global climate change. Biol Lett 4: 99–102.

5.2 Phenotypic plasticity in temperature stress resistance is triggered by photoperiod in a fly

Klaus Fischer, Susann Liniek, Michael Bauer, Birgit Baumann, Stefan Richter and Anneke Dierks

Published as:

Fischer, K., Liniek, S., Bauer, M., Baumann, B., Richter, S. and Dierks, A. (2011). Phenotypic plasticity in temperature stress resistance is triggered by photoperiod in a fly. Evolutionary Ecology. DOI 10.1007/s10682-011-9547-x

Evol Ecol
DOI 10.1007/s10682-011-9547-x

ORIGINAL PAPER

Phenotypic plasticity in temperature stress resistance is triggered by photoperiod in a fly

Klaus Fischer · Susann Liniek · Michael Bauer · Birgit Baumann · Stefan Richter · Anneke Dierks

Received: 14 July 2011 / Accepted: 13 December 2011

Abstract Temperature variation poses a substantial challenge for individual survival and reproductive success, warranting effective means to counter negative effects of temperature extremes. Phenotypic plasticity offers a particularly powerful mechanism to cope with short-time temperature fluctuations. While temperature-induced plasticity in thermal tolerance has been widely explored, effects of other environmental factors have received much less attention. Using a full-factorial design we here show that variation in temperature stress resistance can be triggered by photoperiod (and temperature) in the fly *Protophormia terraenovae*, with shorter day lengths inducing more cold- and longer day lengths more heat-tolerant phenotypes. Such plastic changes were not related to different developmental pathways (reproductive activity or reproductive diapause), and can be induced during development but also in the adult stage (at least for cold tolerance). We suggest that short-term, photoperiod-mediated changes in insect thermal tolerance represent a mechanism of adaptive seasonal plasticity. Photoperiod further affected development time and body size, the significance of which is currently unclear.

Keywords Cold tolerance · Heat tolerance · Life-history plasticity · *Protophormia terraenovae* · Seasonal plasticity · Thermal stress

Introduction

The longer-term survival of any species and population depends on the ability to adjust phenotypic values to environmental conditions. In particular, effective means to deal with environmental stress are needed to buffer detrimental effects on fitness (Overgaard and Sørensen 2008). From the various stressors affecting population viability and individual fitness in nature, temperature stress is considered a particular important one

K. Fischer (✉) · S. Liniek · M. Bauer · B. Baumann · S. Richter · A. Dierks
Zoological Institute and Museum, University of Greifswald, Johann-Sebastian-Bach-Str. 11/12, 17489 Greifswald, Germany
e-mail: klaus.fischer@uni-greifswald.de

Published online: 22 December 2011

(e.g. Hoffmann et al. 2003; Sørensen et al. 2005; Dahlhoff and Rank 2007). Consequently, and because of increasing concerns about the potential effects of globally rising temperatures on biodiversity, factors affecting temperature stress resistance have gained increasing attention over the last decades (Thomas et al. 2004; Deutsch et al. 2008; Kellermann et al. 2009; Tewksbury et al. 2008).

Adaptations to thermal stress may result from longer-term genetic adaptation, phenotypic plasticity or both (Sørensen et al. 2003; Chown and Terblanche 2007; Karl et al. 2008; Fischer and Karl 2010). Phenotypic plasticity is a particularly effective way to cope with short-time temperature fluctuations (Nylin and Gotthard 1998; Pigliucci 2001). Over the last years, plastic responses in temperature stress resistance to ambient temperature have been extensively studied in ectotherms. As a general result, cooler developmental, adult acclimation and/or hardening temperatures were found to increase cold but decrease heat tolerance and vice versa, though not necessarily in every study (e.g. Sejerkilde et al. 2003; Geister and Fischer 2007; Terblanche et al. 2007; Bahrndorff et al. 2009; Rajamohan and Sinclair 2009).

In contrast to the effects of ambient temperature, other environmental factors potentially affecting temperature stress resistance have recently received less attention (Andersen et al. 2010). One such factor is photoperiod, for which some studies suggest an important role in mediating thermal resistance. In the land snail *Helix aspersa*, for instance, photoperiod triggers supercooling ability (Ansart et al. 2001), and in a terrestrial slug seasonal changes in heat and cold tolerance are promoted by acclimation to photoperiod (Udaka et al. 2008). In rodents, photoperiodic acclimation results in changes in thermoregulatory mechanisms (Haim et al. 1998; Goldman 2001), and in amphibians in adjustments of the critical maximum temperature (e.g. Brattstrom and Lawrence 1962; Mahoney and Hutchinson 1969). These and other studies (e.g. Hoar 1956; Ballinger et al. 1969; Woiwode and Adelman 1992) suggest that light conditions, providing a highly reliable cue indicating the advancement of seasons, modulate thermal resistance. In general, shorter day lengths indicative of cooler seasons promoted more cold-resistant phenotypes and vice versa.

In insects, decreasing day length indicating approaching winter conditions typically induces diapause, and many studies have shown that diapausing insects show increased cold resistance (e.g. increased freeze tolerance), allowing them to overwinter successfully (Denlinger 1991; Danks 2005; Tsumuki and Hirai 2007; Bale and Hayward 2010). Such variation is typically associated with different developmental pathways. For instance, different species of *Drosophila* were found to be more cold-resistant when reared under short-day conditions inducing reproductive diapause (Kimura et al. 1994; Hori and Kimura 1998). In contrast, data on photoperiodic effects on heat resistance are scarce (but e.g. Sørensen and Loeschcke 2002), and we are not aware of any study that has investigated the effects of different photoperiods exclusively in the adult stage in any insect.

Against the above background we here investigate the effects of photoperiod (and temperature) on cold and heat stress resistance in the fly *Protophormia terraenovae*. We explore whether photoperiodic effects on thermal tolerance can be exclusively induced during development, or also within relatively short periods of time in the adult stage. As adults of *P. terraenovae* may enter a reproductive diapause, we additionally investigate the effects of reproductive status on stress resistance. Specifically we hypothesize that flies should be more cold-resistant when experiencing short photoperiods, indicating an early or late time point in the season and thus risk of cold stress (cf. Lanciani et al. 1990, 1992). Long photoperiods, in contrast, indicating summer conditions and risk of heat stress, should induce more heat-tolerant phenotypes.

Materials and methods

Study organism

Protophormia terraenovae (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae) is a widespread temperate-zone fly with a Holarctic distribution (Byrd and Castner 2001). It is a multivoltine species with up to six generations per year in Central Europe (Gautier and Schumann 1973). The life cycle lasts between ca. 9 (35°C) and ca. 38 (15°C) days (Grassberger and Reiter 2002). The fly is particularly common in cool regions and can be found as close as 550 miles from the North Pole. It can withstand extreme temperatures and is considered the most cold-tolerant of all calliphorid species (Byrd and Castner 2001). While adults feed on nectar preferring flowers of Apiaceae, larvae feed on various sources of organic, protein-rich matter such as carcasses (Conn 2006). The flies used here originated from a laboratory stock population kept at Greifswald University for at least 200 generations. Flies were originally collected in the vicinity of Greifswald, northeast Germany. Per generation several 100 individuals are reared. Additionally several wild-caught individuals are added once a year to the stock in order to avoid detrimental effects of inbreeding and laboratory adaptation. Stock flies are bred at 20–25°C under ambient day–light conditions. For egg-laying, flies are continuously offered fish heads. Subsequently, larvae are fed with a mixture of bran (ca. 75%), pork blood and sawdust (the latter to maintain appropriate levels of humidity) ad libitum. Adults are fed with highly concentrated sugar solution and milk.

Experimental design

Four different experiments were carried out as detailed below. All eggs used for these experiments were collected within a single photo phase from the stock population. Throughout, four treatment groups involving a lower or a higher temperature and a short or a long day length were used: constant temperature of 20°C and photoperiod of L:D 12:12 (treatment 1); 20°C and L:D 18:6 (2); 27°C and L:D 12:12 (3); 27°C and L:D 18:6 (4). While the long day length indicates summer conditions, the short one indicates spring/autumn conditions. During experiments flies were reared and maintained in climate cabinets (Sanyo MLR-315H) set at 60% relative humidity. Climatic conditions within cabinets were continuously monitored by data loggers. Throughout all experiments, larvae were fed with the above food mixture, and adults with a highly concentrated sucrose solution ad libitum.

For *experiment 1*, ca. 300 eggs were collected for each of the above treatments. In order to synchronize adult eclosion, eggs for both 20°C treatments (1 and 2) were collected 11 days earlier than the eggs for the 27°C treatments (3 and 4). The eggs were transferred to translucent plastic pots (1 l) filled with food and covered with gauze. The pots were checked daily for pupae, which were removed, weighed and afterwards kept individually in small, translucent pots (125 ml) covered with gauze. For all individuals larval development time (from egg laying to pupation, thus including egg development), larval growth rate (ln pupal mass/larval time), pupal development time (from pupation to adult eclosion), pupal and adult mass (to the nearest 0.1 mg; Sartorius LE225D) were recorded. For taking adult mass, flies were chilled after temperature stress resistance had been measured (see below). Note that all flies spent their complete life cycle (i.e. from egg stage through to adult life) at the respective treatments.

To assess temperature stress resistance we used chill-coma recovery time (i.e. the time needed to regain mobility following cold exposure) and heat knock-down time (i.e. the time until being knocked down under heat stress). Both indices are considered reliable proxies of climatic cold and heat adaptation, respectively (e.g. Ayrinhac et al. 2004; Castañeda et al. 2005; Sørensen et al. 2005; Karl et al. 2008). To score chill-coma recovery time, 1–3 day-old flies from all four treatment groups, being kept individually in translucent plastic cups (125 ml), were arranged on a tray in a randomized block design (32 flies per block). Chill coma was induced by exposing the flies for 20 h to −5°C in a climate cabinet (Sanyo MIR-553). Preliminary studies showed that shorter exposure times caused very quick recovery, while longer ones induced substantial mortality (data not shown). Afterwards, the trays were transferred to a room with a constant temperature of 24.3 ± 0.7°C to score recovery time, which was defined as the time elapsed between taking the tray out of the climate cabinet until a fly was able to stand on its legs. Measurements were terminated after a maximum of 60 min, and all animals needing longer to recover (<5% throughout) were removed from subsequent analyses (as it remained unclear whether they needed longer times to recover or whether they had died).

Heat knock-down time was scored in a climate cabinet at 45°C (Sanyo MIR-553). Two day-old flies were placed individually in translucent plastic cups (125 ml) in a block design. Per block, 16 flies were placed on a laboratory shaker located within the climate cabinet. The shaker was used to facilitate easier detection of the flies' physical knock down, i.e. to avoid that flies would hold on to the cup after having entered heat coma. The time until a fly was knocked down was recorded through the glass front door of the climate cabinet. All flies were frozen after having measured temperature stress resistance at −20°C for later sex determination by the distance between the eyes (which is much smaller in males compared to females; Conn 2006).

Experiments 2 and *3* investigated effects of adult acclimation. Therefore, all individuals were raised in a common environment at intermediate conditions, i.e. at 23.5°C and L:D 15:9. On their eclosion day, adult flies were collected from the rearing pots and randomly divided among the four treatment groups outlined above, thus including changes to both alternative temperatures and photoperiods. Flies were placed individually in small, translucent pots (125 ml) covered with gauze. Heat and cold stress resistance were scored as detailed above after an acclimation period of 4 (*experiment 2*) or 10 days (*experiment 3*).

Experiment 4. As the above conditions may result in differential proportions of reproductively active/diapausing individuals (Numata and Shiga 1995), we dissected females from an additional set of animals to check for fully developed ovaries indicative of reproductive activity. We dissected females which had been reared and kept at treatments 1–4, and additionally at 23.5°C/L:D 15:9, on day ten of adult life. Additionally, we dissected females reared at 23.5°C and L:D 15:9, being divided among treatments 1–4 on their eclosion day, on day 13 of adult life. In the latter 4 groups we also tested for an impact of reproductive status on chill-coma recovery and heat knock-down time. Dissections were restricted to females as reproductive status cannot be easily investigated in males.

Statistical analyses

The effects of photoperiod, temperature and sex on life-history traits (larval and pupal development time, larval growth rate, pupal and adult mass) and stress resistance traits were analyzed using three-way AN(C)OVAs, with temperature, photoperiod, and sex as fixed factors. Body mass was added as covariate as appropriate. In the analysis of

chill-coma recovery time in *experiment 1*, age was included as an additional factor, because fly age differed between 1 and 3 days at testing (note that in all other experiments flies were of exactly the same age while being tested for stress resistance). Data on development times were log-transformed prior to analysis to meet ANOVA requirements.

To test for the effects of reproductive status on stress resistance traits, we used two different approaches. First, we used 3-way ANOVAs with temperature, photoperiod, and reproductive status (reproductively active or reproductive diapause) as fixed factors. Note that sex was not included here as reproductive status could be measured in females only (see above). Due to the low number of reproductively active females in certain treatment groups, this approach is a bit problematic due to highly imbalanced sample sizes. Therefore, we additionally analyzed the effects of reproductive status within treatment groups (i.e. separately for each temperature by photoperiod combination), using standard non-parametric U tests.

Throughout, minimum adequate models were constructed by sequentially removing non-significant interaction terms (if $P > 0.10$). Correlations between two factors were computed using Pearson correlations. Differences in the proportions of reproductively active/diapausing females were explored using contingency tables. All statistical tests were performed using STATISTICA 6.1 (StatSoft Inc. 2003). Throughout, all means are given $\pm$ 1 SE. As significance threshold we used $P < 0.05$ throughout.

Results

Life-history traits and developmental plasticity (experiment 1)

All life-history traits measured were affected by both photoperiod and temperature while none was affected by sex (Table 1). The shorter compared to the longer day length caused a reduction in larval development time (8.2 $\pm$ 0.2 vs. 8.5 $\pm$ 0.3 days) accompanied by higher growth rates (6.5 $\pm$ 0.1 vs. 6.0 $\pm$ 0.2 mg/day), an increase in pupal development time (7.6 $\pm$ 0.3 vs. 7.3 $\pm$ 0.2 days), and an increase in pupal (50.3 $\pm$ 0.6 vs. 46.2 $\pm$ 0.6 mg) as well as adult mass (27.7 $\pm$ 0.3 vs. 26.0 $\pm$ 0.3 mg, $n = 94$ throughout). The cooler compared to the higher temperature induced longer larval (10.8 $\pm$ 0.1 vs. 5.9 $\pm$ 0.1 days) and pupal (9.9 $\pm$ 0.1 vs. 5.1 $\pm$ 0.1 days) development times associated with lower larval growth rates (4.9 $\pm$ 0.1 vs. 7.6 $\pm$ 0.1 mg/day), and a higher pupal (51.6 $\pm$ 0.1 vs. 45.0 $\pm$ 0.1 mg) as well as adult mass (27.7 $\pm$ 0.1 vs. 26.0 $\pm$ 0.1 mg; sample size is 93 or 95 throughout). Significant temperature by photoperiod interactions were present for larval and pupal time, larval growth rate and pupal mass, indicating that for all those traits differences across photoperiods were more pronounced at 20°C than at 27°C (Table 2). The significant temperature by sex interaction for adult mass, finally, reflects that males were slightly lighter than females at 20°C, but slightly heavier than females at 27°C.

Reproductive status (experiment 4)

The proportions of reproductively active females, as measured in an additional set of individuals, differed significantly across rearing environments ($\chi^2_4 = 162$, $P < 0.0001$). It was close to 100% in the treatments involving higher rearing temperatures (27°C/18 h: 98%; 27°C/12 h: 96%; 23.5°C/15 h: 100%), but was much lower in the treatments involving lower rearing temperatures (20°C/18 h: 60%; 20°C/12 h: 6%; $n = 50$

Table 1 ANOVA results for the effects of photoperiod, temperature and sex on larval time, larval growth rate, pupal time, pupal and adult mass in *Protophormia terraenovae*

	df	MS	*F*	*P*
Larval time				
Photoperiod	1	1.8	4.2	**0.0423**
Temperature	1	1,068.0	2,495.8	**<0.0001**
Sex	1	<0.1	<0.1	0.8300
Temperature × photoperiod	1	6.2	14.4	**0.0002**
Error	183	0.4		
Larval growth rate				
Photoperiod	1	1.1×10^{-5}	21.3	**<0.0001**
Temperature	1	34.1×10^{-5}	680.9	**<0.0001**
Sex	1	$<0.1 \times 10^{-5}$	0.1	0.7486
Temperature × photoperiod	1	0.3×10^{-5}	6.7	**0.0105**
Error	183	0.1×10^{-5}		
Pupal time				
Photoperiod	1	5.1	22.8	**<0.0001**
Temperature	1	1,068.9	4,793.4	**<0.0001**
Sex	1	<0.1	<0.1	0.9609
Temperature × photoperiod	1	1.6	7.1	**0.0086**
Error	183	0.2		
Pupal mass				
Photoperiod	1	8.3×10^{-4}	32.7	**<0.0001**
Temperature	1	19.6×10^{-4}	77.4	**<0.0001**
Sex	1	0.2×10^{-4}	0.9	0.3375
Temperature × photoperiod	1	1.2×10^{-4}	4.8	**0.0302**
Error	183	0.3×10^{-4}		
Adult mass				
Photoperiod	1	15.4×10^{-5}	19.0	**<0.0001**
Temperature	1	12.0×10^{-5}	14.8	**0.0002**
Sex	1	0.3×10^{-5}	0.4	0.5217
Temperature × sex	1	3.3×10^{-5}	4.0	**0.0460**
Error	183	0.8×10^{-5}		

Significant *P* values are given in bold. Minimum adequate models were constructed by sequentially removing non-significant interaction terms

throughout). Likewise, the adult environment, in the first place adult temperature, significantly affected the number of reproductively active females ($\chi^2_3 = 250$; $P < 0.0001$). When being reared in a common environment (23.5°C/15 h) and afterwards divided among the four adult treatments, the proportion of reproductively active females was highest in the 27°C/18 h (83%, $n = 154$), followed by the 27°C/12 h (77%, $n = 133$), 20°C/18 h (21%, $n = 126$), and finally in the 20°C/12 h treatment (5%, $n = 129$). However, 3-way ANOVAs revealed that, in the females that had been reared in a common environment, neither chill-coma recovery time ($F_{1,344} < 0.1$; $P = 0.922$) nor heat knock-down time ($F_{1,182} = 0.2$; $P = 0.653$) was significantly affected by the factor female reproductive status. Reproductive status was furthermore not involved in any significant interactions.

Table 2 Life-history traits (means ± 1 SE) in relation to temperature (20°C vs. 27°C) and photoperiod (12 h vs. 18 h photo phase) or temperature and sex in *Protophormia terraenovae*

	20°C		27°C	
	12 h	18 h	12 h	18 h
Larval time (days)	10.5 ± 0.1	11.0 ± 0.2	6.0 ± 0.1	5.9 ± 0.1
Growth rate (mg/day)	5.2 ± 0.1	4.5 ± 0.1	7.7 ± 0.1	7.5 ± 0.1
Pupal time (days)	10.2 ± 0.1	9.7 ± 0.1	5.2 ± 0.1	5.0 ± 0.1
Pupal mass (mg)	54.5 ± 0.8	48.8 ± 1.0	46.3 ± 0.5	43.7 ± 0.4
	20°C		27°C	
	Males	Females	Males	Females
Adult mass (mg)	27.3 ± 0.5	28.1 ± 0.5	26.3 ± 0.3	25.8 ± 0.4

Data were pooled across sexes or photoperiods to illustrate significant two-way interactions (cf. Table 1). Group sample sizes range between 38 and 57

To back up these results we additional performed *U* tests separately for each treatment group (see "Materials and methods"). Again, no indication for an effect of reproductive status on either chill-coma recovery time (all P values > 0.523) or heat knock-down time (all P values > 0.122) was found.

Temperature stress resistance and developmental plasticity (experiment 1)

Chill-coma recovery time was significantly affected by photoperiod, temperature, sex and the control variable age (Table 3a; Fig. 1a). Flies reared at the shorter compared to the longer day length showed reduced recovery times (1,239 ± 196 s, $n = 94$ vs. 1,488 ± 196 s, $n = 94$). Similarly, flies reared at the lower compared to the higher temperature exhibited shorter recovery times (843 ± 206 s, $n = 93$ vs. 1,884 ± 207 s, $n = 95$). Further, females were more cold-resistant than males (1,251 ± 196 s, $n = 89$ vs. 1,476 ± 195 s, $n = 99$). The significant effect of the factor age shows that cold stress resistance decreased from day 1 to day 3 after eclosion. Adding adult mass as covariate ($F_{1,181} = 6.7$; $P = 0.010$) to the statistical design presented in Table 3a revealed qualitatively identical results, except that the effect of photoperiod was not significant anymore ($P = 0.085$). This finding indicates that the response in chill-coma recovery is at least partly caused by photoperiod-mediated changes in adult mass (see above). Overall, larger individuals recovered earlier from cold exposure than smaller ones ($r = -0.36$, $P < 0.0001$, $n = 188$).

Regarding heat knock-down time, flies reared at the longer day length (901 ± 16 s; $n = 127$) resisted heat stress significantly longer compared to those reared at the shorter day length (841 ± 17 s; $n = 115$), while all other factors were non-significant (Table 3b; Fig. 1b). However, females tended to be more heat-resistant than males (892 ± 17 s, $n = 109$ vs. 849 ± 16 s; $n = 133$; $P = 0.058$). Adding adult mass as covariate ($F_{1,234} = 5.0$; $P = 0.027$) to the statistical design revealed a trend towards longer knock-down times in flies reared at 27°C (893 ± 17 s, $n = 117$) than in flies reared at 20°C (849 ± 17 s, $n = 122$; $F_{1,234} = 3.3$; $P = 0.071$), while all other results remained qualitatively identical. Overall, larger individuals were more heat-resistant than smaller ones ($r = 0.15$, $P = 0.021$, $n = 239$).

Table 3 ANOVA results for the effects of photoperiod, (developmental) temperature and sex on chill-coma recovery (a) and heat knock-down time (b) in *Protophormia terraenovae*

	df	MS	*F*	*P*
(a)				
Photoperiod	1	12,381	6.6	**0.0111**
Temperature	1	77,399	41.2	**<0.0001**
Sex	1	9,794	5.2	**0.0236**
Age	2	12,465	6.6	**0.0051**
Error	182	1,879		
(b)				
Photoperiod	1	218,805	7.08	**0.0083**
Temperature	1	35,820	1.16	0.2828
Sex	1	111,994	3.63	0.0582
Error	238	31,404		

In part (a) age at test day (varying between 1 and 3 days) was added as an additional factor, as for logistic reasons not all individuals could be measured at exactly the same age. Significant *P* values are given in bold. Minimum adequate models were constructed by sequentially removing non-significant interaction terms

Temperature stress resistance and adult acclimation (experiments 2 and 3)

After an acclimation period of 4 days, chill-coma recovery time was significantly affected by temperature and sex, but not by photoperiod in flies reared in a common environment (Table 4a). Flies acclimated to the lower temperature (887 ± 29 s, $n = 202$) showed shorter recovery times than flies acclimated to the higher temperature (1,543 ± 30 s, $n = 197$), and females (1,047 ± 28 s, $n = 219$) showed shorter recovery times than males (1,383 ± 31 s, $n = 180$). A significant temperature by sex interaction indicates that the sex difference was slightly more pronounced at 27°C (females by 25% faster) than at 20°C (females by 23% faster; Fig. 2a). Overall, variation in chill-coma recovery time in relation to photoperiod was quite variable across temperature by sex groups, with recovery time tending to be shorter at the shorter day length in two groups, longer at the shorter day length in one group, and very similar across both day lengths in the remaining group (significant three-way interaction; Fig. 2a).

Heat knock-down time also remained unaffected by photoperiod after 4 days of acclimation, but varied significantly across temperatures and sexes (Table 4b). Flies acclimated to 27°C (2,718 ± 50 s, $n = 198$) were more heat-resistant than flies acclimated to 20°C (2,268 ± 49 s, $n = 203$), and females (2,606 ± 50 s, $n = 208$) were more heat-resistant than males (2,380 ± 52 s, $n = 193$). The sex difference, however, was restricted to 20°C (significant temperature by sex interaction; Fig. 2b).

Using a longer acclimation period of 10 days revealed that, in addition to temperature and sex, photoperiod did significantly affect chill-coma recovery time (Table 4c). The shorter compared to the longer day length was associated with shorter recovery times (1,603 ± 48 s vs. 1,769 ± 48 s; $n = 181$ each). Also, the lower compared to the higher temperature caused shorter recovery times (995 ± 45 s, $n = 200$ vs. 2,377 ± 51 s; $n = 162$). Females (1,571 ± 44 s; $n = 208$) showed shorter recovery times than males (1,801 ± 52 s; $n = 154$). The effect of photoperiod was more pronounced at 27°C than at 20°C (significant photoperiod by temperature interaction; Fig. 3a). Heat knock-down time, in contrast, was still significantly affected by temperature and sex only, but not by

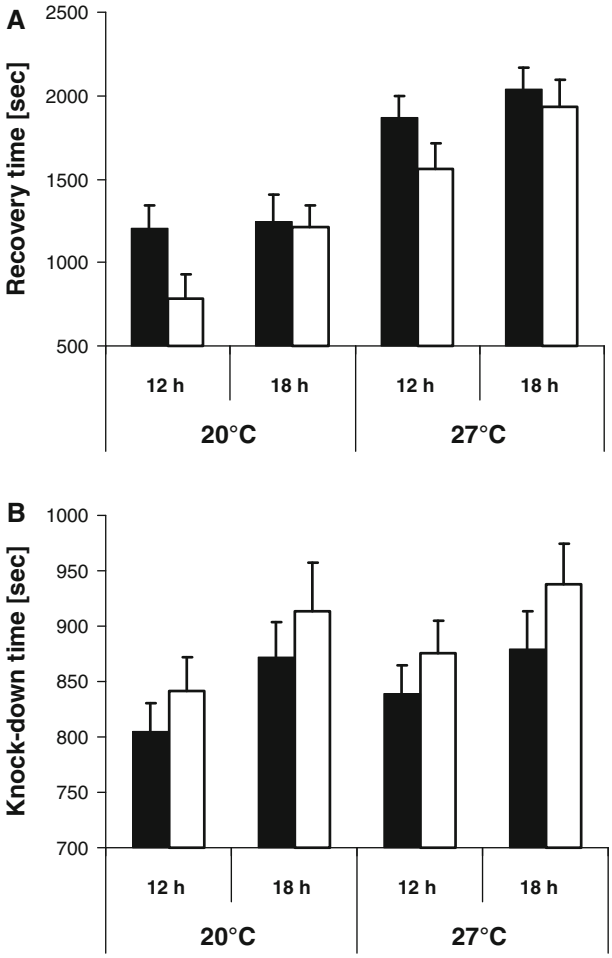

Fig. 1 Chill-coma recovery (**a**) and heat knock-down time (**b**) for *Protophormia terraenovae* flies in relation to temperature (20°C vs. 27°C), photoperiod (12 h vs. 18 h photo phase), and sex (*black bars*: males, *open bars*: females). All flies had been reared at the respective treatments. Sample sizes range between 17 and 38 individuals per sex and treatment group. Given are means + 1 SE

photoperiod (Table 4d). The higher compared to the lower temperature induced longer knock-down times (1,330 ± 19 s, $n = 118$ vs. 1,232 ± 16 s; $n = 137$), and females were more heat-resistant than males (1,338 ± 16 s, $n = 138$ vs. 1,206 ± 17 s; $n = 117$; Fig. 3b).

Discussion

Effects on life-history traits and reproductive diapause

Interestingly, photoperiod did not only affect temperature stress resistance (see below), but also all life-history traits investigated. Shorter day lengths induced shorter larval

Table 4 ANOVA results for the effects of photoperiod, adult temperature and sex on chill-coma recovery after 4 days of acclimation (a), on heat knock-down time after 4 days of acclimation (b), on chill-coma recovery after 10 days of acclimation (c), and on heat knock-down time after 10 days of acclimation (d) in *Protophormia terraenovae*

	df	MS	*F*	*P*
(a)				
Photoperiod	1	75,975	0.5	0.4962
Temperature	1	41,436,688	253.0	**<0.0001**
Sex	1	10,775,702	65.8	**<0.0001**
Photoperiod × temperature	1	165,683	1.0	0.3151
Photoperiod × sex	1	80,668	0.5	0.4832
Temperature × sex	1	1,120,227	6.8	**0.0093**
Photoperiod × temperature × sex	1	753,788	4.6	**0.0326**
Error	380	163,759		
(b)				
Photoperiod	1	20,113	<0.1	0.8384
Temperature	1	20,572,810	41.5	**<0.0001**
Sex	1	4,508,746	9.2	**0.0024**
Temperature × sex	1	3,189,015	6.5	**0.0106**
Error	370	482,756		
(c)				
Photoperiod	1	2,794,735	7.0	**0.0083**
Temperature	1	164,037,518	413.2	**<0.0001**
Sex	1	4,624,807	11.7	**0.0007**
Photoperiod × temperature	1	1,637,631	4.1	**0.0430**
Temperature × sex	1	1,144,269	2.9	0.0904
Error	356	396,985		
(d)				
Photoperiod	1	72,175	2.2	0.1423
Temperature	1	565,332	17.0	**<0.0001**
Sex	1	875,577	26.3	**<0.0001**
Error	251	33,314		

Significant *P* values are given in bold. Minimum adequate models were constructed by sequentially removing non-significant interaction terms

(accompanied by higher larval growth rates) but longer pupal development times. Owing to the opposing effects on larval versus pupal development time, effects of photoperiod on development time were overall negligible. The higher larval growth rates found at shorter day lengths may help compensating for environmental deficiencies in the cooler season (Nylin 1992; Gotthard et al. 1999). Evidently, the concomitantly lower temperatures prolong development times and reduce growth rates (Table 1), which is generally the case in ectotherms (e.g. Sinclair et al. 2003). Temperature by photoperiod interactions were present for all three developmental traits, indicating that differences among photoperiods were more pronounced at the lower temperature. This can be partly explained by the longer overall development time at lower temperatures, resulting in larger absolute differences (Steigenga and Fischer 2009).

Variation in body size showed strikingly similar patterns across photoperiods and temperatures: pupal as well adult mass were higher at the shorter day length and at the lower temperature and vice versa. Thus, both factors jointly contribute to the near universal pattern of larger body size at lower temperatures and in cooler seasons ('temperature size rule'; Atkinson 1994; Angilletta et al. 2004; Karl and Fischer 2008). The reasons for this

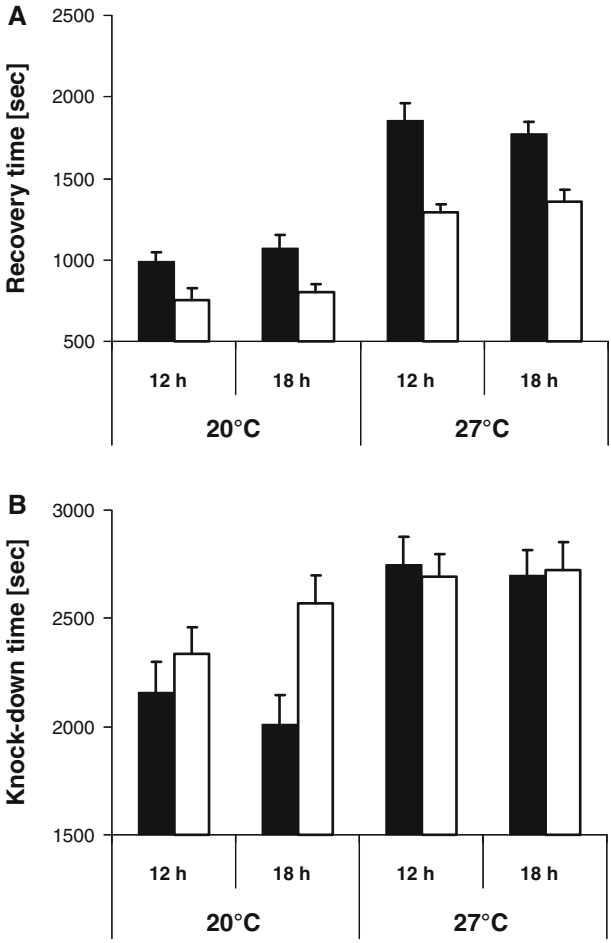

Fig. 2 Chill-coma recovery (**a**) and heat knock-down time (**b**) for *Protophormia terraenovae* flies in relation to temperature (20°C vs. 27°C), photoperiod (12 h vs. 18 h photo phase), and sex (*black bars*: males, *open bars*: females). All flies had been acclimated to the respective treatments for 4 days. Sample sizes range between 34 and 63 individuals per sex and treatment group. Given are means + 1 SE

recurrent pattern of phenotypic plasticity are hitherto poorly understood, and it might reflect a physiological constraint rather than an adaptation (Karl and Fischer 2008). It is interesting to note in this context that the developmental response to photoperiod in chill-coma recovery time was at least partly mediated through photoperiodic effects on body size. Thus, larger body size found at shorter photoperiods did help to increase cold stress resistance, which may favour an adaptive explanation. However, heat stress resistance was also positively related to body size, which was smaller at the longer day length. Consequently, our data do overall not support an adaptive explanation for photoperiod-mediated changes in body size. Generally, there is ample evidence that larger individuals are typically more stress-resistant than smaller ones, as could also be shown here (Blanckenhorn 2000; Karl et al. 2008).

The complete lack of sexual differences in life-history traits seems surprising, as in insects males often emerge before females thus having a shorter development facilitated by

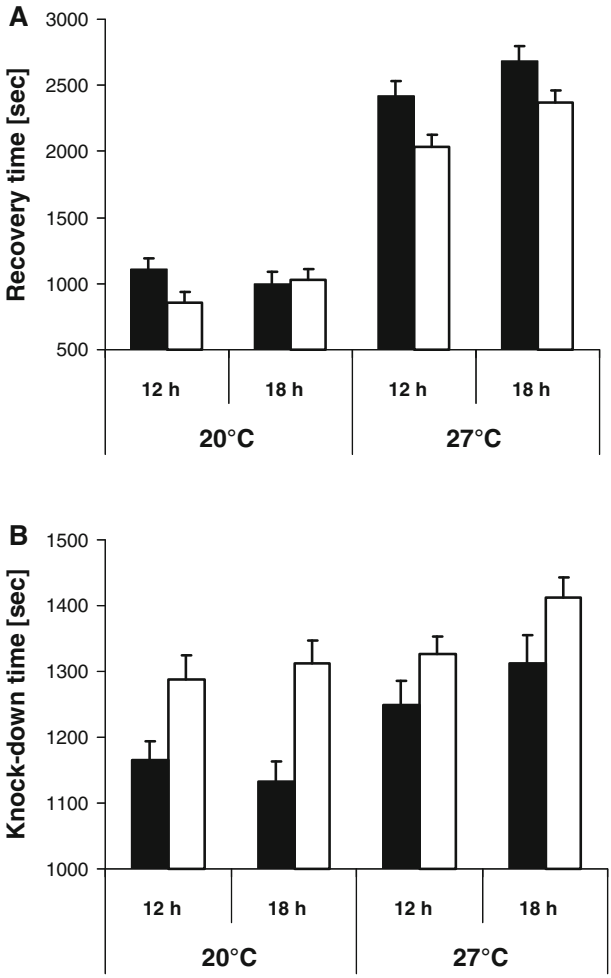

Fig. 3 Chill-coma recovery (**a**) and heat knock-down time (**b**) for *Protophormia terraenovae* flies in relation to temperature (20°C vs. 27°C), photoperiod (12 h vs. 18 h photo phase), and sex (*black bars*: males, *open bars*: females). All flies had been acclimated to the respective treatments for 10 days. Sample sizes range between 19 and 56 individuals per sex and treatment group. Given are means + 1 SE

higher growth rates, and because females are typically heavier than males (Honek 1993; Roff 2002). While the former pattern is caused by protandry selection in order to maximize male mating opportunities, the latter is caused by fecundity selection in females (Wiklund and Fagerström 1977; Honek 1993; Fischer and Fiedler 2000; Karl et al. 2008). The divergent results found here are probably related to the occurrence of multiple, overlapping generations a year in *P. terraenovae* (Grassberger and Reiter 2002). Under such conditions protandry is predicted to yield no benefits to males, as the availability of receptive females is not restricted to limited periods of time (Wiklund and Fagerström 1977). The similar body size across sexes suggests sexual selection on male body size as has been found for other dipterans (Kraushaar and Blanckenhorn 2002; Blanckenhorn et al. 2004).

Our treatments also induced substantial variation in the incidence of reproductive diapause. The lower temperature and the shorter day length strongly increased the incidence of reproductive diapause, with temperature having a much larger impact than photoperiod. These findings are in broad agreement with earlier ones using the same species (Numata and Shiga 1995), while in other dipterans the induction of ovarian

dormancy typically requires temperatures <15°C or day lengths <12 h (Tauber et al. 2007; Emerson et al. 2009). Interestingly, variation in the developmental as well as in the adult environment had very similar effects, although all latter individuals had been reared under conditions typically preventing diapause. Consequently, the vast majority of individuals were 're-programmed' in the adult stage. Developmental programming of diapause therefore seems to be fairly quickly and easily reversible in this species. However, our analyses on stress resistance traits showed that differences in cold and heat stress resistance were not related to developmental pathway, suggesting that such variation is related to the same cues inducing variation in pathways (i.e. temperature and photoperiod), but not to pathways per se. Note here that even within the groups having experienced conditions producing meaningful numbers of both pathways, there was not the slightest indication of any differences in stress resistance.

Effects on temperature stress resistance

Our study provides evidence that temperature stress resistance is affected by variation in photoperiod in the fly *P. terraenovae*: when exposed to a short as compared to a longer day length flies showed increased cold but decreased heat tolerance and vice versa. Thus, the flies responded to the reliable cue 'photoperiod' in a predictable manner, with increased cold tolerance coinciding with conditions indicating cooler seasons, while increased heat tolerance coincided with conditions indicating the warmer season. Hence, photoperiodic information seems to be used here as an indicator of the advancement of seasons very similar to life cycle decisions such as diapause induction (Denlinger 1991; Danks 1994, 2005; Kimura et al. 1994; Hori and Kimura 1998; Schmidt et al. 2005, 2008), suggesting adaptive phenotypic plasticity. In line with this interpretation it was recently shown that seasonal variation in heat and cold tolerance in a slug corresponds with changes in ambient temperatures, and is caused by both temperature and photoperiodic cues (Udaka et al. 2008).

Note that in our design the shorter day length may indicate either spring or autumn conditions. Either way though temperatures will be lower during these seasons compared to summer conditions. It is conceivable that the use of changing rather than constant photoperiods would have yielded even larger effects, but was prohibitive in our case as we deliberately focussed on short-term effects of photoperiod. The adaptive value of reduced chill-coma recovery or prolonged heat-knock down times could be related to higher levels of activity at suboptimal temperatures, providing more time for essential behaviors such as foraging, mate location and reproduction (Sørensen and Loeschcke 2002). Moreover, both traits are closely related to survival rates following temperature stress (Rako and Hoffmann 2006; Fischer et al. 2010).

In extension of previous studies (e.g. Lanciani et al. 1990, 1992; Kimura et al. 1994; Hori and Kimura 1998; Duman 2001; Danks 2005; Tsumuki and Hirai 2007; Bale and Hayward 2010) we here show a photoperiod-mediated plastic response independent of developmental pathway in cold but also in heat stress resistance, and further that such plastic responses cannot only be induced through developmental plasticity, but (for cold resistance) also in the adult stage. Thus the physiological mechanisms promoting cold-hardiness under a diapause-inducing photoperiod may not directly be linked to the process causing reproductive diapause (see also Higuchi and Kimura 1985; Denlinger 1991).

While in *experiment 1* any combination of developmental plasticity and adult acclimation could, on principle, be responsible for the pattern observed, *experiments 2 and 3* clearly reflect adult acclimation as individuals had been reared in a common environment.

However, an acclimation period of 4 days was obviously not enough to induce a plastic response to photoperiod (*experiment 2*), demonstrating that the results obtained from *experiment 1* basically show effects of the developmental environment. After 10 days of adult acclimation, however, a clear response to photoperiod was detected in cold tolerance (more pronounced at 27°C), although effect size was smaller than in *experiment 1* (reduction in recovery time by 10% as compared to 17%). The latter might be purely a matter of time, which remains to be tested though. This may also explain the lack of response in heat resistance even after 10 days. Our results demonstrate that phenotypic adjustment to different photoperiods may occur within relatively short periods, and that those responses are independent of reproductive activity or diapause. In so far our results resemble those obtained from other animals ranging from snails, slugs, amphibians, and fish through to mammals (Hoar 1956; Brattstrom and Lawrence 1962; Mahoney and Hutchinson 1969; Woiwode and Adelman 1992; Haim et al. 1998; Ansart et al. 2001; Goldman 2001; Udaka et al. 2008).

Consistently, higher developmental and acclimation temperatures increased heat but decreased cold tolerance and vice versa, as has been found in a variety of other insects (e.g. Hoffmann et al. 2003; Sørensen et al. 2005; Zeilstra and Fischer 2005; Chown and Terblanche 2007; Karl et al. 2008; Rajamohan and Sinclair 2009). *Experiment 2* clearly shows that plastic responses to temperature happen much faster than plastic responses to photoperiod. While there was no effect of photoperiod on temperature stress resistance after 4 days of acclimation, temperature variation caused clear changes. Typically, plastic responses to temperature in stress resistance traits can be induced within hours (rapid hardening response; e.g. Hoffmann et al. 2003; Loeschcke and Hoffmann 2007; Overgaard and Sørensen 2008; Rajamohan and Sinclair 2009; Fischer et al. 2010). The fact that responses to photoperiod take longer was not unexpected, as photoperiod unlike temperature does not show very rapid and sometimes unpredictable changes, but alters exclusively in a gradual manner. Seasonal adjustment in contrast to buffering detrimental effects of sudden heat or cold waves simply does not require particularly fast responses.

Throughout, females were more temperature stress resistant than males, which has also been found in some other studies (e.g. Willmer et al. 2000; Zeilstra and Fischer 2005). Sex was further two-times involved in interactions with temperature (*experiment 2*). Sex differences in chill-coma recovery time were more pronounced at the higher temperature, while the opposite was true for heat knock-down time. These data do not allow for any firm conclusions regarding such interactive effects.

Conclusions

Our study shows that variation in temperature stress resistance can be triggered by photoperiod, with shorter day lengths inducing more cold- and longer day lengths more heat-tolerant phenotypes. Such plastic changes can be induced during development but also in the adult stage. We suggest that this pattern represents adaptive seasonal plasticity. The mechanisms promoting variation in thermal resistance do not seem to be directly linked to the processes causing reproductive diapause. Note in this context that in lines selected for increased heat stress resistance a substantial number of phototransduction genes was found to be upregulated in *Drosophila*, suggesting the involvement of such genes in stress resistance or at least stress sensing (Nielsen et al. 2006; Sørensen et al. 2007). In *P. terraenovae* clock genes might be involved in the control of seasonal adaptation, especially with regard to the induction of reproductive diapause (Muguruma et al. 2010).

We therefore speculate that photoperiodic acclimation independent of developmental pathways might be a more general phenomenon in insects. While the effects of temperature for mediating plastic responses in stress resistance are becoming increasingly well understood (e.g. Hoffmann et al. 2003; Sørensen et al. 2003; Ayrinhac et al. 2004; Deere et al. 2006; Chown and Terblanche 2007; Marais et al. 2009; Fischer and Karl 2010), studying the impact of other environmental factors still deserves more future attention.

Acknowledgments This study was supported by a start-up fund from Greifswald University. We thank Philipp Braun for help during experiments.

References

Andersen LH, Kristensen TN, Loeschcke V, Toft S, Mayntz D (2010) Protein and carbohydrate composition of larval food affects tolerance to thermal stress and desiccation in adult *Drosophila melanogaster*. J Insect Physiol 56:336–340

Angilletta MJ Jr, Steury TD, Sears MW (2004) Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. Integr Comp Biol 44:498–509

Ansart A, Vernon P, Daguzan J (2001) Photoperiod is the main cue that triggers supercooling ability in the land snail, *Helix aspersa* (Gastropoda: Helicidae). Cryobiology 42:266–273

Atkinson D (1994) Temperature and organism size—a biological law for ectotherms. Adv Ecol Res 25:1–58

Ayrinhac A, Debat V, Gibert P, Kister A-G, Legout H, Moreteau B, Vergilino R, David JR (2004) Cold adaptation in geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more important than genetic variability. Funct Ecol 18:700–706

Bahrndorff S, Loeschcke V, Pertoldi C, Beier C, Holmstrup M (2009) The rapid cold hardening response of *Collembola* is influenced by thermal variability of the habitat. Funct Ecol 23:340–347

Bale JS, Hayward SAL (2010) Insect overwintering in a changing climate. J Exp Biol 213:980–994

Ballinger RE, Hawker J, Sexton OJ (1969) The effect of photoperiod acclimation on the thermoregulation of the lizard, *Sceloporus undulates*. J Exp Zool 171:43–48

Blanckenhorn WU (2000) The evolution of body size: what keeps animals small? Q Rev Biol 75:385–407

Blanckenhorn WU, Kraushaar U, Teuschl Y, Reim C (2004) Sexual selection on morphological and physiological traits and fluctuating asymmetry in the black scavenger fly *Sepsis cynipsea*. J Evol Biol 17:629–641

Brattstrom BH, Lawrence P (1962) The rate of thermal acclimation in anuran amphibia. Physiol Zool 85:148–156

Byrd J, Castner J (2001) Forensic entomology: the utility of arthropods in legal investigations. CRC Press, New York

Castañeda L, Lardies MA, Bozinovic F (2005) Interpopulational variation in recovery time from chill coma along a geographic gradient: a study in the common woodlouse, *Porcellio laevis*. J Insect Physiol 51:1346–1351

Chown SL, Terblanche JS (2007) Phyiological diversity in insects: ecological and evolutionary contexts. Adv Insect Physiol 33:50–152

Conn M (2006) Handbook of models for human aging. Elsevier, London

Dahlhoff EP, Rank NE (2007) The role of stress proteins in responses of a montane willow leaf beetle to environmental temperature variation. J Biosci 32:477–488

Danks HV (1994) Insect life cycle polymorphism. Kluwer, Dordrecht

Danks HV (2005) Key themes in the study of seasonal adaptations in insects. 1. Patterns of cold hardiness. Appl Entomol Zool 40:199–211

Deere JA, Sinclair BJ, Marshall DJ, Chown SL (2006) Phenotypic plasticity of thermal tolerances in five oribatid mite species from sub-Antarctic Marion Island. J Insect Physiol 52:693–700

Denlinger DL (1991) Relationship between cold hardiness and diapause. In: Lee RE, Denlinger DL (eds) Insects at low temperature. Chapman & Hall, New York, pp 174–198

Deutsch CA, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, Haak DC, Martin PR (2008) Impacts of climate warming on terrestrial ectotherms across latitude. Proc Natl Acad Sci USA 105:6668–6672

Duman JG (2001) Antifreeze and ice nucleator proteins in terrestrial arthropods. Annu Rev Physiol 63:327–357

Emerson KJ, Uyemura AM, McDaniel KL, Schmidt PS, Bradshaw WE, Holzapfel CM (2009) Environmental control of ovarian dormancy in natural populations of *Drosophila melanogaster*. J Comp Physiol A 195:825–829

Fischer K, Fiedler K (2000) Sex-related differences in reaction norms in the butterfly *Lycaena tityrus* (Lepidoptera: Lycaenidae). Oikos 90:372–380
Fischer K, Karl I (2010) Exploring plastic and genetic responses to temperature variation using Copper butterflies. Clim Res 43:17–30
Fischer K, Dierks A, Franke K, Geister TL, Liszka M, Winter S, Pflicke C (2010) Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. PLoS One 5:e15284
Gautier A, Schumann H (1973) Puparia of the subarctic or black blowfly *Protophormia terraenovae* (Robineau-Desvoidy, 1830) in a skull of a late eemian bison at Zemst, Brabant (Belgium). Palaeogeogr Palaeoclimatol Palaeoecol 14:119–125
Geister TL, Fischer K (2007) Testing the beneficial acclimation hypothesis: cool males—hot love? Behav Ecol 18:658–664
Goldman BD (2001) Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283–301
Gotthard K, Nylin S, Wiklund C (1999) Seasonal plasticity in two satyrine butterflies: state-dependent decision making in relation to daylength. Oikos 84:453–462
Grassberger M, Reiter C (2002) Effect of temperature on development of the forensically important Holarctic blow fly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae). Forensic Sci Int 128:177–182
Haim A, Shabtay A, Arad Z (1998) Thermoregulatory responses of mesic and xeric rodent species to photoperiod manipulations. Comp Biochem Physiol A 120:187–191
Higuchi C, Kimura MT (1985) Influence of photoperiod on low temperature acclimation for cold-hardiness in *Drosophila auraria*. Physiol Entomol 10:303–308
Hoar WS (1956) Photoperiodism and thermal resistance of goldfish. Nature 178:364–365
Hoffmann AA, Sørensen JG, Loeschcke V (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J Therm Biol 28:175–216
Honek A (1993) Intraspecific variation in body size and fecundity in insects: a general relationship. Oikos 66:483–492
Hori Y, Kimura MT (1998) Relationship between cold stupor and cold tolerance in *Drosophila* (Diptera: Drosophilidae). Environ Entomol 27:1297–1302
Karl I, Fischer K (2008) Why get big in the cold? Towards a solution of a life-history puzzle. Oecologia 155:215–225
Karl I, Janowitz SA, Fischer K (2008) Altitudinal life-history variation and thermal adaptation in the Copper butterfly *Lycaena tityrus*. Oikos 117:778–788
Kellermann VM, Van Heerwaarden B, Sgro CM, Hoffmann AA (2009) Fundamental evolutionary limits in ecological traits drive *Drosophila* species distributions. Science 325:1244–1246
Kimura MT, Ohtsu T, Yoshida T, Awasaki T, Lin LJ (1994) Climatic adaptations and distributions in the *Drosophila takahashii* species subgroup (Diptera: Drosophilidae). J Nat Hist 28:401–409
Kraushaar U, Blanckenhorn WU (2002) Population variation in sexual selection and its effect on size allometry in two dung fly species with contrasting sexual size dimorphism. Evolution 56:307–321
Lanciani CA, Giesel JT, Anderson JF, Emerson SS (1990) Photoperiod-induced changes in metabolic response to temperature in *Drosophila melanogaster*. Funct Ecol 4:41–45
Lanciani CA, Lipp KE, Giesel JT (1992) The effect of photoperiod on cold tolerance in *Drosophila melanogaster*. J Therm Biol 17:147–148
Loeschcke V, Hoffmann AA (2007) Consequences of heat hardening on a field fitness component in *Drosophila* depend on environmental temperature. Am Nat 169:175–183
Mahoney JJ, Hutchinson VH (1969) Photoperiod acclimation and 24-hour variations in the critical thermal maxima of a tropical and a temperate frog. Oecologia 2:143–161
Marais E, Terblanche JS, Chown SL (2009) Life stage-related differences in hardening and acclimation of thermal tolerance traits in the kelp fly, *Paratora dreuxi* (Diptera, Helcomyzidae). J Insect Physiol 55:336–343
Muguruma F, Goto SG, Numata H, Shiga S (2010) Effect of photoperiod on clock gene expression and subcellular distribution of PERIOD in the circadian clock neurons of the blow fly *Protophormia terraenovae*. Cell Tissue Res 340:497–507
Nielsen MM, Sørensen JG, Kruhoffer M, Justesen J, Loeschcke V (2006) Phototransduction genes are up-regulated in a global gene expression study of *Drosophila melanogaster* selected for heat resistance. Cell Stress Chaperones 11:325–333
Numata H, Shiga S (1995) Induction of adult diapause by photoperiod and temperature in *Protophormia terraenovae* (Diptera: Calliphoridae) in Japan. Environ Entomol 24:1633–1636
Nylin S (1992) Seasonal plasticity in life history traits: growth and development in *Polygonia c-album* (Lepidoptera: Nymphalidae). Biol J Linn Soc 47:301–323

Nylin S, Gotthard K (1998) Plasticity in life-history traits. Annu Rev Entomol 43:63–83
Overgaard J, Sørensen JG (2008) Rapid thermal adaptation during field temperature variations in *Drosophila melanogaster*. Cryobiology 56:159–162
Pigliucci M (2001) Phenotypic plasticity: beyond nature and nurture. John Hopkins University Press, Baltimore
Rajamohan A, Sinclair BJ (2009) Hardening trumps acclimation in improving cold tolerance of *Drosophila melanogaster* larvae. Physiol Entomol 34:217–223
Rako L, Hoffmann AA (2006) Complexity of the cold acclimation response in *Drosophila melanogaster*. J Insect Physiol 52:94–104
Roff DA (2002) The evolution of life-histories. Theory and analysis. Chapman & Hall, New York
Schmidt PS, Matzkin L, Ippolito M, Eanes WF (2005) Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. Evolution 59:1721–1732
Schmidt PS, Zhu C-T, Das J, Mariska B, Li Y, Eanes WF (2008) An amino acid polymorphism in the couch potato gene forms the basis for climatic adaptation in *Drosophila melanogaster*. Proc Natl Acad Sci USA 105:16207–16211
Sejerkilde M, Sørensen JG, Loeschcke V (2003) Effects of cold- and heat hardening on thermal resistance in *Drosophila melanogaster*. J Insect Physiol 49:719–726
Sinclair BJ, Vernon P, Klok CJ, Chown SL (2003) Insects at low temperatures: an ecological perspectives. Trends Ecol Evol 18:257–262
Sørensen JG, Loeschcke V (2002) Natural adaptation to environmental stress via physiological clock-regulation of stress resistance in *Drosophila*. Ecol Lett 5:16–19
Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6:1025–1037
Sørensen JG, Norry FM, Scannapieco AC, Loeschcke V (2005) Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. J Evol Biol 18:829–837
Sørensen JG, Nielsen MM, Loeschcke V (2007) Gene expression profile analysis of *Drosophila melanogaster* selected for resistance to environmental stressors. J Evol Biol 20:1624–1636
StatSoft Inc (2003) Statistica for windows. Version 6.1. Tulsa
Steigenga MJ, Fischer K (2009) Fitness consequences of variation in developmental temperature in a butterfly. J Therm Biol 34:244–249
Tauber E, Zordan M, Sandrelli F, Pegoraro M, Osterwalder N, Breda C, Daga A, Selmin A, Monger K, Benna C, Rosato E, Kyriacou CP, Costa R (2007) Natural selection favors a newly derived timeless allele in *Drosophila melanogaster*. Science 316:1895–1898
Terblanche JS, Marais E, Chown SL (2007) Stage-related variation in rapid cold hardening as a test of the environmental predictability hypothesis. J Insect Physiol 53:455–462
Tewksbury JJ, Huey RB, Deutsch CA (2008) Ecology: putting the heat on tropical animals. Science 320:1296–1297
Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, Collingham YC, Erasmus BFN, de Siqueira MF, Grainger A, Hannah L, Hughes L, Huntley B, van Jaarsveld AS, Midgley GF, Miles L, Ortega-Huerta MA, Peterson AT, Phillips OL, Williams SE (2004) Extinction risk from climate change. Nature 427:145–148
Tsumuki H, Hirai M (2007) Effects of photoperiod and temperature on endogenous ice nucleus production in larvae of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). Appl Entomol Zool 42:305–308
Udaka H, Goto SG, Numata H (2008) Effects of photoperiod and acclimation temperature on heat and cold tolerance in the terrestrial slug, *Lehmannia valentiana* (Pulmonata: Limacidae). Appl Entomol Zool 43:547–551
Wiklund C, Fagerström T (1977) Why do males emerge before females? A hypothesis to explain the incidence of protandry in butterflies. Oecologia 31:153–158
Willmer P, Stone G, Johnston IA (2000) Environmental physiology of animals. Blackwell, Oxford
Woiwode JG, Adelman IR (1992) Effects of starvation, oscillating temperatures, and photoperiod on the critical thermal maximum of hybrid striped × white bass. J Therm Biol 17:271–275
Zeilstra I, Fischer K (2005) Cold tolerance in relation to developmental and adult temperature in a butterfly. Physiol Entomol 30:92–95

6. Effects of inbreeding and selection on life history traits and temperature stress resistance

6.1 Effects of inbreeding on life-history and thermal performance in the tropical butterfly *Bicyclus anynana*

Anneke Dierks, Bianca Hoffmann, Stephanie S. Bauerfeind, and Klaus Fischer

Published as:

Dierks, A., Hoffmann, B., Bauerfeind, S.S. and Fischer, K. (2012). Effects of inbreeding on life history and thermal performance in the tropical butterfly *Bicyclus anynana*. Popul Ecol 54:83–90.

Popul Ecol (2012) 54:83–90
DOI 10.1007/s10144-011-0291-4

ORIGINAL ARTICLE

Effects of inbreeding on life history and thermal performance in the tropical butterfly *Bicyclus anynana*

Anneke Dierks · Bianca Hoffmann · Stephanie S. Bauerfeind · Klaus Fischer

Received: 4 April 2011 / Accepted: 22 September 2011 / Published online: 1 November 2011

Abstract Human-induced loss and fragmentation of natural habitats reduces population size and thereby presumably genetic diversity through inbreeding or genetic drift. Additionally, many species are confronted with increased temperature stress due to climate change, with reduced genetic diversity potentially interfering with a species' ability to cope with such conditions. While in general the detrimental impact of inbreeding has often been documented, its consequences for the ability to cope with temperature stress are still poorly understood. Against this background we here investigate the effects of inbreeding on egg hatching success, development and temperature stress tolerance in the tropical butterfly *Bicyclus anynana.* Specifically we test for an increased sensitivity to environmental stress in inbred individuals. Our results revealed that even comparatively low levels of inbreeding yield negative consequences for reproduction and development under beneficial conditions. Inbreeding also reduced cold tolerance in adult butterflies, while heat tolerance remained unaffected. We therefore conclude that acute stress tolerance may not be generally impaired by inbreeding.

Keywords Cold stress resistance · Heat stress resistance · Inbreeding depression · Life-history traits · Temperature stress tolerance

A. Dierks (✉) · B. Hoffmann · S. S. Bauerfeind · K. Fischer
Zoological Institute and Museum, University of Greifswald, Johann-Sebastian-Bach-Str. 11/12, 17489 Greifswald, Germany
e-mail: anneke.dierks@uni-greifswald.de

Introduction

Inbreeding, the mating between closely related individuals, has long been known to cause negative effects on fitness-related traits like reproduction, development and survival in both plants and animals (Darwin 1876; Charlesworth and Charlesworth 1987; Frankham et al. 2002). Such detrimental effects, known as inbreeding depression, have prompted research attention ever since the first detailed description in early 1876 by Charles Darwin (e.g., Darwin 1876; Crnokrak and Roff 1999; Armbruster et al. 2000; Carr and Eubanks 2002). Inbreeding depression is caused by increased levels of homozygosity, implying either an increased expression of deleterious recessive alleles (partial dominance hypothesis) or an increased homozygosity at loci with heterozygote advantage (overdominance hypothesis; Charlesworth and Charlesworth 1999; Pedersen et al. 2008; Vermeulen et al. 2008; Charlesworth and Willis 2009).

Despite the substantial knowledge on inbreeding in general, some issues have remained underexplored. For instance, due to an expected increase in the expression of deleterious recessive alleles under stress, inbred individuals are predicted to be more sensitive to environmental stress than outbred individuals (Lynch and Walsh 1998; Armbruster and Reed 2005; Fox and Reed 2010; Fox et al. 2011). Increased sensitivity to environmental stress in inbred individuals has been mainly investigated by comparing inbreeding depression between benign and stressful environments. Such experimental studies have indeed often found an increase in inbreeding depression in more stressful environments, though there are exceptions and even contrary findings (Bijlsma et al. 1999; Dahlgaard and Hoffmann 2000; Keller and Waller 2002; Armbruster and Reed 2005; Martin and Lenormand 2006; Waller et al.

2008; Fox and Reed 2010; Fox et al. 2011). In contrast, surprisingly few studies are available on inbreeding effects on stress resistance traits such as heat or cold tolerance (but Maynard Smith 1956; Ehiobu et al. 1989; Dahlgaard and Loeschcke 1997), at least in taxa other than *Drosophila* (Fox et al. 2011).

Studying the interplay between inbreeding and stress resistance is for the following reasons of crucial importance. If inbreeding depression is particularly pronounced under environmental stress, this will affect extinction risk in natural populations (Bijlsma et al. 2000). Moreover, inbreeding may limit evolutionary potential (Zhong et al. 2005; Liao and Reed 2009), thus reducing the ability to adapt to changing environments which is pivotal for the long-term persistence of any given species or population (Bubli et al. 1998; Bijlsma and Loeschcke 2005). While historically ecological factors were assumed to be of overwhelming importance for extinction risk in natural populations, a growing number of laboratory but also field studies suggests that the impact of inbreeding should not be ignored (Saccheri et al. 1998; Keller and Waller 2002; Kristensen et al. 2003).

The importance of the above considerations is likely to increase in the future, due to (1) a human-induced loss and fragmentation of natural habitats reducing population size and thereby increasing inbreeding and genetic drift in many threatened species (Bijlsma et al. 1997; Frankham et al. 2002), and (2) global warming and the associated changes in the frequency and severity of thermal stress (Chown and Terblanche 2007). Therefore, it is important to consider genetic and environmental stress together as both may interactively decrease population fitness (Kristensen et al. 2003), and to further examine such patterns in an array of plants and animals (Fox and Reed 2010; Fox et al. 2011).

Against this background we here study the effects of inbreeding on life-history traits and temperature stress resistance in the tropical butterfly *Bicyclus anynana* (Butler, 1879) under laboratory conditions. We therefore used three levels of inbreeding: two full-sib matings in two consecutive generations, one full-sib mating and an outbred control.

Materials and methods

Study organism

Bicyclus anynana (Butler, 1879) is a tropical, fruit-feeding butterfly ranging from southern Africa to Ethiopia (Larsen 1991). Adults feed on a variety of fallen and decaying fruits (Larsen 1991; Brakefield 1997). This species exhibits two seasonal morphs adapted to the wet and dry season, respectively, in its natural habitat and the associated changes in resting background and predation (Brakefield 1997; Lyytinen et al. 2004). Reproduction is basically limited to the warmer wet season, when oviposition plants are abundantly available and in which 2–3 generations occur. Reproduction finally ceases during the colder dry season and the butterflies do not mate before the first rain announces the beginning of the next wet season (Windig 1997; Brakefield 1997). A laboratory stock population was founded at Greifswald University, Germany, in 2008 from several thousand eggs derived from a well-established stock population at Bayreuth University, Germany. This population in turn originated from a stock population at Leiden University, The Netherlands, which was established in 1988 from 80 gravid females caught at a single location in Malawi. In total, our stock population has spent roughly 160 generations in the laboratory prior to the start of this experiment. 500–1000 individuals are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof et al. 2005).

Experimental design

To investigate the effects of inbreeding on an array of traits, three levels of inbreeding were established using a full-sib breeding design: outbred controls (C) resulting from matings between random butterflies ($F = 0$), inbreeding 1 (I1) resulting from matings between full sibs ($F = 0.25$), and inbreeding 2 (I2) resulting from matings between full sibs in two consecutive generations ($F = 0.375$). Assuming that the base population has an inbreeding level of zero, we calculated the expected inbreeding coefficients using the equation $F_t = (1 + 2 F_{t-1} + F_{t-2})/4$ as a measure of coancestry for full-sib matings (Falconer and Mackay 1996; Bijlsma et al. 2000; Kristensen et al. 2003). The different levels of inbreeding were established from the stock population as follows. Several hundred virgin stock butterflies, being initially kept separated by sex, were allowed to mate randomly in cylindrical hanging cages (30 × 38 cm) on day 4 after adult eclosion. Mating pairs were placed individually in translucent 1L plastic containers. After mating males were removed from the containers, while females were provided with fresh maize leaves for oviposition. The eggs produced by individual females over the following 10 days were transferred on a daily basis to elongated, sleeve-like cages containing a young maize plant for further development. Consequently, each 'sleeve' cage represented one full-sib family. Density per cage was standardized to 20–30 larvae, and maize plants were replaced as necessary. Unless otherwise stated, rearing took place in a climate room at 27°C, high humidity (ca. 70%) and a photoperiod of L12:D12. In order to synchronize adult eclosion, resulting pupae were temporarily transferred to 20°C (high humidity

and L12:D12 throughout) until all larvae had pupated. Resulting butterflies were used to set up the 'I2' treatment by mating one brother to one sister per full-sib family. Following full-sib matings, the above procedure was repeated once. Inbreeding effects were scored in the subsequent generation, i.e., after two full-sib matings in successive generations. Parallel to the second generation of the 'I2' treatment, the 'I1' treatment was set up as outlined above. Finally, the control treatment was set up by mating random stock individuals. This staggered design enabled us to compare all three inbreeding levels at the same time.

Data acquisition

Reproduction

Per inbreeding level 108 (C), 120 (I1) and 137 (I2) females were placed individually in 1L plastic containers and allowed to oviposit on fresh maize leaves for up to four days. Eggs laid on day 1 were removed, counted and placed into petri dishes lined with moist filter paper and containing a small cutting of maize for hatching caterpillars. This procedure was repeated until ca. 30 eggs per female had been collected. Petri dishes were checked daily for hatchlings, which were counted and subsequently transferred, using a fine paintbrush, for further development to translucent plastic boxes (125 ml) containing moistened filter paper and fresh cuttings of maize leaves in ample supply. Egg hatching success was determined as the proportion of eggs that hatched.

Development

Upon reaching the second instar, the above larvae were transferred, to sleeve-like rearing cages containing a young maize plant each. Larval densities were standardized to ca. 20 larvae per cage. A cage contained only larvae produced by single female and that had hatched on the same day. We kept 81 (C), 90 (I1), and 62 (I2) families, being reared at 27°C, L12:D12, and ca. 70% relative humidity, for scoring developmental traits. All cages were arranged within a total of six blocks, within which cages of all three inbreeding levels were represented and placed randomly. Blocks were used to account for slight temperature and humidity variation within the climate chamber. Cages were checked daily for pupae and pre-pupae, which were removed and placed individually into translucent plastic pots (125 ml) containing filter paper. Pots were again arranged on trays in a randomized block design. Every day pots were inspected for enclosed butterflies. We scored larval development time (from hatching to pupation), pupal development time (from pupation to adult eclosion), pupal mass (on the day following pupation, Sartorius LE225D), and larval growth rate (calculated as quotient of pupal mass and larval developmental time) for all individuals.

Temperature stress resistance

Temperature stress resistance was measured in an additional set of 53 (C), 68 (I1) and 53 (I2) families, having been set up in exactly the same way as outlined above. We used chill-coma recovery time (i.e., the time needed to regain mobility following cold exposure) and heat knock-down time (i.e., the time until being knocked down under heat stress). Both indices are considered reliable proxies of climatic cold and heat adaptation, respectively, and have been extensively used in *B. anynana* before (e.g., Geister and Fischer 2007; Fischer et al. 2010). For measuring cold resistance, 2–4 day old, virgin butterflies were placed individually in plastic cups and arranged on a tray in a randomized block design (maximum of 36 butterflies). Butterflies were then exposed for 19 h to 1°C in a climate cabinet (Sanyo MIR-553), inducing a chill coma. Preliminary studies showed that shorter exposure times lead to very quick recovery and longer ones to substantial mortality (data not shown). Afterwards the plastic boxes were transferred to a climate cabinet (Sanyo MIR-553) with a constant temperature of 20°C to determine recovery times while looking through the glass front door. Recovery time was defined as the time elapsed between taking the box out of the 1°C climate cabinet until a butterfly was able to stand on its legs. Only butterflies that had recovered within 1 h were included in further analyses, as most butterflies not recovering within this time period are dead (typically $<1\%$). Heat stress resistance was determined by using a knock-down assay. Again, 2–4 day old, virgin butterflies were arranged on a tray using a randomized block design (maximum of 20 butterflies per block). They were exposed to 45°C and continuously monitored. Heat knock-down time (defined as the time until a butterfly was no longer able to stand upright) for each individual was recorded. Throughout there was no re-use of butterflies, i.e., individuals were either used to measure heat or cold stress resistance.

Statistical analyses

Egg hatching success was analyzed using a one-way ANOVA. To investigate the effects of inbreeding on life-history traits (larval time, growth rate, pupal time, pupal mass) and thermal performance (chill-coma recovery time, heat knock-down time) general linear models were used with inbreeding level and sex as fixed factors, and family nested within inbreeding level as random factor. Block was included as additional factor when analyzing life-history traits. For temperature stress resistance traits, pupal mass

was added as covariate, and age at testing (day 2, 3, or 4 of adult life) was included as additional control factor. Significant differences between inbreeding levels were located with a Tukey HSD post hoc test. All statistical tests were performed by using JMP (4.0.0) or Statistica (6.1). Throughout, all means are given ±1 SE.

Results

Egg hatching success was significantly affected by inbreeding level, being highest in controls followed by inbreeding 1 and finally inbreeding 2 (C > I1 > I2; Tukey HSD after ANOVA; Fig. 1). Inbreeding level further significantly affected larval time, larval growth rate, pupal mass, and cold stress resistance, but not pupal time and heat stress resistance (Table 1; Fig. 2). Higher levels of inbreeding were associated with longer larval development times (C < I1 < I2; Tukey HSD after ANOVA), lower larval growth rates (C > I1 > I2, Tukey HSD), lower pupal mass (C > I1 > I2; Tukey HSD), and longer chill-coma recovery times (C < I1 = I2). Similarly, there was a slight tendency ($P = 0.07$) for an increase in pupal time with increasing inbreeding level (C: 7.29 ± 0.05 days, I1: 7.37 ± 0.04 days, I2: 7.42 ± 0.05 days).

Significant sexual differences were found in larval development time, larval growth rate, pupal mass, pupal development time, and cold stress resistance, but not in heat stress resistance (Table 1). Males compared to females showed shorter larval times (24.3 ± 0.2 vs. 26.7 ± 0.2 days), higher growth rates (0.21 ± 0.0021 vs. 0.19 ± 0.0019 mg/day), reduced pupal mass (149.7 ± 1.7 vs. 182.3 ± 1.6 mg), longer pupal times (7.5 ± 0.04 vs. 7.2 ± 0.3 days), and longer chill-coma recovery times (1216 ± 39 vs. 1137 ± 36 s). The latter variable was additionally affected by the covariate pupal mass (linear regression ns). Interactions between inbreeding level and sex were non-significant throughout (Table 1).

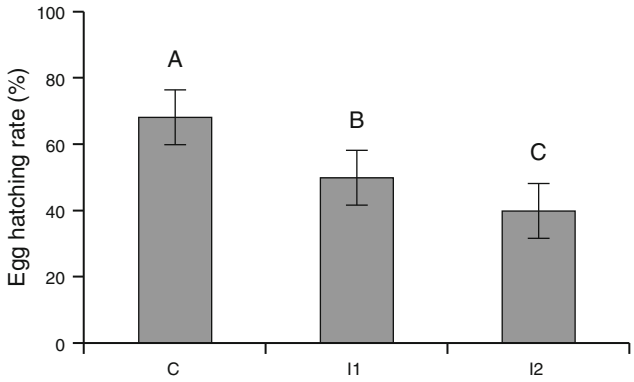

Fig. 1 Mean values (±1 SE) for egg hatching success across different inbreeding levels in *Bicyclus anynana* (*C* control, *I1* one full-sib mating, *I2* two full-sib matings). Different letters above bars indicate significant differences between inbreeding levels

Discussion

Inbreeding is known to have detrimental effects on fitness, especially so under stressful conditions (Dahlgaard and Hoffmann 2000; Armbruster and Reed 2005; Fox and Reed 2010). However, the degree to which inbreeding exerts negative effects on stress resistance traits per se, is currently largely unclear. We have therefore investigated effects of inbreeding on life-history as well as on stress resistance traits. Out of the seven traits measured, five were negatively affected by inbreeding, namely egg hatching success, larval time, larval growth rate, pupal mass, and cold stress resistance. These results are in broad agreement with earlier ones on various organisms, for instance longer development times (associated with reduced growth rate) in crickets, butterflies or beetles or reduced stress resistance in flies (Roff 1998; Haikola 2001; Fox and Scheibly 2006; Mikkelsen et al. 2010). Our results also confirm, that *B. anynana* carries a large genetic load (Saccheri et al. 1996, 2005; Van Oosterhout et al. 2000). Especially egg hatching rate has been shown to be extremely susceptible to inbreeding depression in *B. anynana* (Brakefield and Saccheri 1994; Saccheri et al. 1996, 2005; Van Oosterhout et al. 2000).

Regarding stress resistance traits, cold but not heat stress tolerance was affected by inbreeding, with control individuals recovering much faster from cold exposure compared to individuals from either inbreeding group. Thus far, very few studies have addressed variation in stress tolerance in relation to inbreeding. Similar to our findings, Dahlgaard and Loeschcke (1997) found no effect of inbreeding on heat-shock tolerance in *Drosophila* embryos. Mikkelsen et al. (2010) reported the same pattern found by us in *Drosophila melanogaster*, namely inbreeding depression in chill-coma recovery time but not in heat knock-down resistance. These and our findings indicate that stress tolerance may not be generally reduced by inbreeding, at least not if the environment experienced prior to testing was beneficial (as is also the case in our experiment). Note that the above negative results in other studies were obtained despite typically using much higher levels of inbreeding (4–5 full-sib matings; see also Dahlgaard et al 1995; Pedersen et al. 2005) compared to our study (1–2). Nevertheless, the rather moderate level of inbreeding used here, which ought to be ecologically more realistic, did negatively affect cold tolerance (and life-history traits, see above). Given the clear response even to moderate levels of inbreeding, there is essentially no evidence for confounding effects of laboratory adaptation on our results, despite a relatively long culture history of our

Table 1 ANOVA results for the effects of inbreeding level (control, one or two consecutive full-sib matings), family (random factor, nested within inbreeding level), sex and block (the latter for life-history traits only) on life-history and stress resistance traits in *Bicyclus anynana*

Trait	Factor	MS	*df*	*F*	*P*
Larval time	Inbreeding	206.6	2,214	10.5	**<0.0001**
	Family (inbreed.)	7575.1	214,1104	3.6	**<0.0001**
	Sex	1255.6	1,1104	127.4	**<0.0001**
	Inbreed. × sex	36.8	2,1104	1.9	0.1551
	Block	163.3	5,1104	3.3	**0.0056**
	Error	9.8546	1104		
Growth rate	Inbreeding	0.019	2,214	12.9	**<0.0001**
	Family (inbreed.)	0.531	214,1104	3.4	**<0.0001**
	Sex	0.027	1,1104	37.1	**<0.0001**
	Inbreed. × sex	0.002	2,1104	1.5	0.2186
	Block	0.021	5,1104	5.9	**<0.0001**
	Error	0.0007	1104		
Pupal mass	Inbreeding	9520	2,227	8.8	**0.0002**
	Family (inbreed.)	337331	227,115	2.7	**<0.0001**
	Sex	264302	1,115	488.5	**<0.0001**
	Inbreed. × sex	1460	2,115	1.3	0.2599
	Block	21474	5,115	7.9	**<0.0001**
	Error	541.03	1150		
Pupal time	Inbreeding	2.8	2,227	2.7	0.0706
	Family (inbreed.)	23.2	227,1146	0.2	0.9999
	Sex	19.5	1,1146	37.6	**<0.0001**
	Inbreed. × sex	0.7	2,1146	0.6	0.5339
	Block	4.7	5,1146	1.8	0.1069
	Error	0.5184	1146		
Cold tolerance	Inbreeding	1486625	2,72	5.4	**0.0063**
	Family (inbreed.)	284406	42,316	1.2	0.2287
	Sex	2816248	1,316	11.6	**0.0008**
	Inbreed. × sex	391045	2,316	1.6	0.2021
	Mass	1627117	1,316	6.7	**0.0102**
	Age at test	277454	1,316	1.1	0.2864
	Error	243307	316		
Heat tolerance	Inbreeding	148029	2,91	0.3	0.7705
	Family (inbreed.)	567768	42,189	1.0	0.4659
	Sex	408133	1,189	0.7	0.3956
	Inbreed. × sex	262984	2,189	0.5	0.6276
	Mass	232444	1,189	0.4	0.5213
	Age at test	1439563	1,189	2.6	0.1115
	Error	561870	209		

Pupal mass and age at testing were added as covariates in the analyses of stress resistance traits. $P < 0.05$ in bold

stock population. Possible effects of laboratory adaptation include genetic drift and inbreeding, which may result in reduced inbreeding depression because controls are already inbred to some extent, or because deleterious alleles have been purged already (Matos et al. 2000; Crnokrak and Barrett 2002; Meffert et al. 2006; Swindell and Bouzat 2006).

The sex differences found in life-history traits generally revealed expected patterns in line with previous findings (cf. Fischer et al. 2003, 2004, 2010). Females also showed a higher cold tolerance than males, while there was no sex difference in heat tolerance. A recent study on *B. anynana* involving 22 sex comparisons showed that, despite a few significant results, both sexes are in general equally stress-resistant in this species (Fischer et al. 2010; cf. Gilchrist et al. 1997; Jensen et al 2007; Karl et al. 2008). Interestingly, we could not detect any significant interactions between sex and inbreeding, indicating that inbreeding has very similar effects on males and females. Similar findings were obtained by Mikkelsen et al. (2010), who could not find significant interactions between sex and breeding regime for either heat, cold, or desiccation resistance.

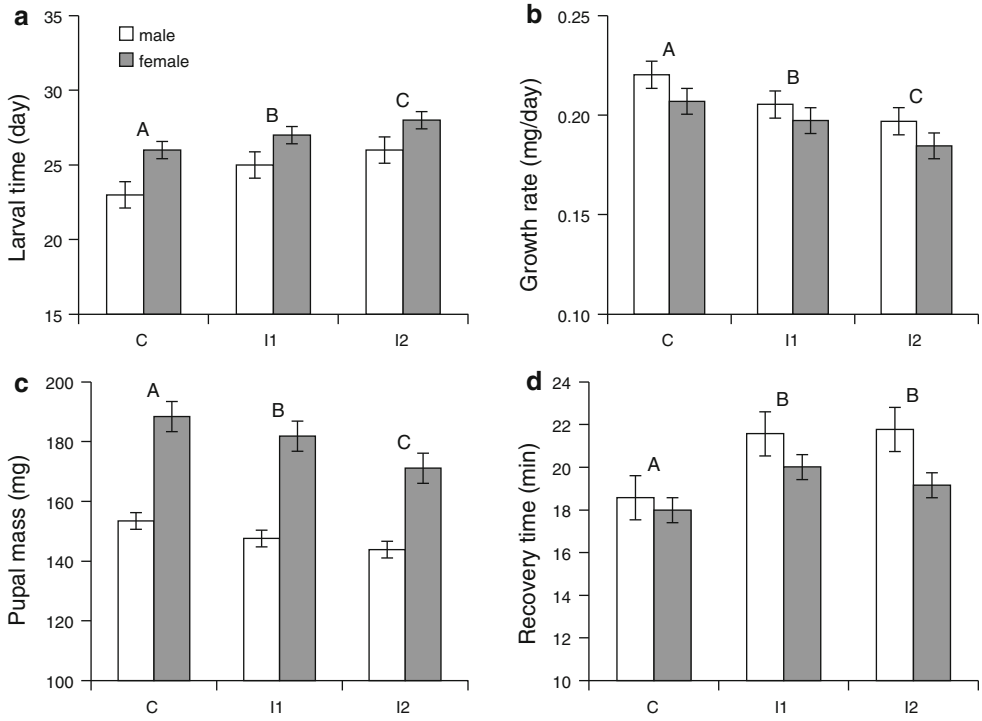

Fig. 2 Mean values (±1 SE) for **a** larval time, **b** growth rate, **c** pupal mass, and **d** chill-coma recovery time across different inbreeding levels in male and female *Bicyclus anynana* (*C* control, *I1* one full-sib mating, *I2* two full-sib matings). Different letters above bars indicate significant differences between inbreeding levels

Conclusions

Our results revealed that even comparatively low levels of inbreeding exert negative effects on various traits. However, we found no evidence for particularly pronounced inbreeding effects on temperature stress tolerance. Only one out of two stress resistance traits showed a significant response to inbreeding. The magnitude of inbreeding depression (measured as $\delta = 1 - w_{I2}/w_C$, where w_{I2} and w_C are average values for I2 and the outbred controls, respectively; Radwan and Drewniak 2001, cf. Charlesworth and Willis 2009) ranged between 8 and 41% reduction in performance for the life-history traits (8% for pupal mass, 8% for larval time, 11% for growth rate, and 41% for egg hatching rate) and was 12% for cold tolerance. It should be noted in this context that our assays to measure stress tolerance evidently involved severe stress: exposure to 1°C for 19 h and to 45°C until physical knock-down. We therefore conclude that acute stress tolerance may not be generally reduced by inbreeding, as long as the conditions experienced prior to testing did not involve stress (Dahlgaard and Loeschcke 1997). Nevertheless inbreeding may negatively affect an organism's ability to cope with thermal stress (cf. results on cold tolerance), which may additively contribute to the extinction risk of small populations in the coming decades for which the frequency and intensity of extreme weather events is predicted to strongly increase (Diffenbaugh et al. 2005, 2007). This notion, however, depends on the ecological relevance of our results, being obtained under laboratory conditions. Investigating inbreeding effects under such conditions has been repeatedly criticized for not reflecting variable natural environments (Chen 1993; Pray et al. 1994; Kristensen et al. 2008). Further, the importance of inbreeding in natural populations is generally somewhat controversial, amongst others due to purging (Crnokrak and Barrett 2002). However, accumulating evidence suggests that genetic factors are also involved in the extinction risk of natural populations (e.g., Saccheri et al. 1998; Keller and Waller 2002; Kristensen et al. 2008).

Acknowledgments We thank Susann Lienik and Luise Schultheiß for help with the experiment and two anonymous reviewers for very valuable comments on an earlier draft. This work was supported by a start-up grant from Greifswald University.

References

Armbruster P, Reed DH (2005) Inbreeding depression in benign and stressful environments. Heredity 95:235–242. doi:10.1038/sj.hdy.6800721

Armbruster P, Hutchinson RA, Linvell T (2000) Equivalent inbreeding depression under laboratory and field conditions in a tree-hole-breeding mosquito. Proc R Soc B 267:1939–1945. doi:10.1098/rspb.2000.1233

Brakefield PM (1997) Phenotypic plasticity and fluctuating asymmetry as responses to environmental stress in the butterfly *Bicyclus anynana*. In: Bijlsma RR, Loeschke V (eds) Environmental stress: adaptation and evolution. Birkhäuser, Basel, pp 105–146

Brakefield PM, Saccheri IJ (1994) Guidelines in conservation genetics and the use of population cage experiments with butterflies to investigate the effects of genetic drift and

inbreeding. In: Loeschcke V, Tomiuk J, Jain SK (eds) Conservation genetics. Birkhaeuser, Basel, pp 165–179
Bijlsma R, Loeschcke V (2005) Environmental stress, adaptation and evolution: an overview. J Evol Biol 18:744–749. doi:10.1111/j.1420-9101.2005.00962.x
Bijlsma R, Bundgaard J, Boerema AC, Van Putten WF (1997) Genetic and environmental stress, and the persistence of populations. In: Bijlsma R, Loeschke V (eds) Environmental stress. Adaptation and evolution. Birkhäuser, Basel, pp 193–207
Bijlsma R, Bundgaard J, Van Putten WF (1999) Environmental dependence of inbreeding depression and purging in *Drosophila melanogaster*. J Evol Biol 12:1125–1137. doi:10.1046/j.1420-9101.1999.00113.x
Bijlsma R, Bundgaard J, Boerema AC (2000) Does inbreeding affect the extinction risk of small populations? Predictions from *Drosophila*. J Evol Biol 13:502–514. doi:10.1046/j.1420-9101.2000.00177.x
Bubli OA, Imasheva AG, Loeschke V (1998) Selection for knock-down resistance to heat in *Drosophila melanogaster* at high and low larval densities. Evolution 52:619–625
Carr DE, Eubanks MD (2002) Inbreeding alters resistance to insect herbivory and host plant quality in *Mimulus guttatus* (Scrophulariaceae). Evolution 56:22–30. doi:10.1111/j.0014-3820.2002.tb00846.x
Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. Annu Rev Ecol Syst 18:237–268
Charlesworth B, Charlesworth D (1999) The genetic basis of inbreeding depression. Genet Res 74:329–340
Charlesworth D, Willis JH (2009) The genetics of inbreeding depression. Genetics 10:783–796. doi:10.1038/nrg2664
Chen X (1993) Comparison of inbreeding and outbreeding in hermaphroditic *Arianta arbustorum* (L.) (land snail). Heredity 71:456–461. doi:10.1038/hdy.1993.163
Chown SL, Terblanche JS (2007) Physiological diversity in insects: ecological and evolutionary contexts. Adv Insect Physiol 33:50–152. doi:10.1016/S0065-2806(06)33002-0
Crnokrak P, Roff DA (1999) Inbreeding depression in the wild. Heredity 83:260–270. doi:10.1038/sj.hdy.6885530
Crnokrak P, Barrett SCH (2002) Purging the genetic load: a review of the experimental evidence. Evolution 56:2347–2358. doi:10.1111/j.0014-3820.2002.tb00160.x
Dahlgaard J, Hoffmann AA (2000) Stress resistance and environmental dependency of inbreeding depression in *Drosophila melanogaster*. Conserv Biol 14:1187–1192. doi:10.1046/j.1523-1739.2000.99206.x
Dahlgaard J, Loeschcke V (1997) Effects of inbreeding in three life stages of *Drosophila buzzatii* after embryos were exposed to a high temperature stress. Heredity 78:410–416. doi:10.1038/hdy.1997.64
Dahlgaard J, Krebs RA, Loeschcke V (1995) Heat-shock tolerance and inbreeding in *Drosophila buzzatii*. Heredity 74:157–163
Darwin CR (1876) The effects of cross and self fertilisation in the vegetable kingdom. John Murray, London. doi:10.1017/CBO9780511694202
Diffenbaugh PJS, Trapp RJ, Giorgi F (2005) Fine-scale processes regulate the response of extreme events to global climate change. Proc Natl Acad Sci USA 102:15774–15778. doi:10.1073/pnas.0506042102
Diffenbaugh PJS, Giorgi F, Gao X (2007) Heat stress intensification in the Mediterranean climate change hotspot. Geophys Res Lett 34:L11706. doi:10.1029/2007GL030000
Ehiobu NG, Goddard ME, Taylor JF (1989) Effect of rate of inbreeding on inbreeding depression in *Drosophila melanogaster*. Theor Appl Genet 77:123–127
Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics. Longman, Essex
Fischer K, Brakefield PM, Zwaan BJ (2003) Plasticity in butterfly egg size: why larger offspring at lower temperatures? Ecology 84:3138–3147. doi:10.1890/02-0733
Fischer K, Zeilstra I, Hetz SK, Fiedler K (2004) Physiological costs of growing fast: does accelerated growth rate reduce pay-off in adult fitness? Evol Ecol 18:343–353. doi:10.1007/s10682-004-2004-3
Fischer K, Dierks A, Franke K, Geister TL, Liszka M, Winter S, Pflicke C (2010) Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. PLoS One 5:e15284. doi:10.1371/journal.pone.0015284
Frankham R, Ballou JD, Briscoe DA (2002) Introduction to conservation genetics. Cambridge University Press, Cambridge. doi:DOI10.1017/S0016672304216913
Fox CW, Reed DH (2010) Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. Evolution 65:246–258. doi:10.1111/j.1558-5646.2010.01108.x
Fox CW, Scheibly KL (2006) Variation in inbreeding depression among populations of the seed beetle, *Stator limbatus*. Entomol Exp Appl 121:137–144
Fox CW, Stillwell RC, Wallin WG, Curtis CL, Reed DH (2011) Inbreeding-environment interactions for fitness: complex relationships between inbreeding depression and temperature stress in a seed-feeding beetle. Evol Ecol 25:25–43. doi:10.1007/s10682-010-9376-3
Geister TL, Fischer K (2007) Testing the beneficial acclimation hypothesis: temperature effects on mating success in a butterfly. Behav Ecol 18:658–664. doi:10.1093/beheco/arm024
Gilchrist GW, Huey RB, Partridge L (1997) Thermal sensitivity of *Drosophila melanogaster:* evolutionary responses of adults and eggs to laboratory natural selection at different temperatures. Physiol Zool 70:403–414
Haikola S (2001) Inbreeding depression and the maintenance of genetic load in *Melitaea cinxia* metapopulations. Conserv Genet 2:325–335
Jensen D, Overgaard J, Sørensen JG (2007) The influence of developmental stage on cold shock resistance and ability to cold-harden in *Drosophila melanogaster*. J Insect Physiol 53:179–186. doi:10.1016/j.jinsphys.2006.11.008
Karl I, Schmitt T, Fischer K (2008) Phosphoglucose isomerase genotype affects life-history traits and cold stress resistance in a Copper butterfly. Funct Ecol 22:887–894. doi:10.1111/j.1365-2435.2008.01438.x
Keller LF, Waller DM (2002) Inbreeding effects in wild populations. Trends Ecol Evol 17:230–341. doi:10.1038/sj.hdy.6885530
Kristensen TN, Dahlgaard J, Loeschcke V (2003) Effects of inbreeding and environmental stress on fitness—using *Drosophila buzzatii* as a model organism. Conserv Genet 4:453–465
Kristensen TN, Loeschke V, Hoffmann AA (2008) Linking inbreeding effects in captive populations with fitness in the wild: release of replicated *Drosophila melanogaster* lines under different temperatures. Conserv Biol 22:189–199. doi:10.1111/j.1523-1739.2007.00816.x
Larsen TB (1991) The butterflies of Kenya. Oxford University Press, Oxford
Liao W, Reed DH (2009) Inbreeding-environment interactions increase extinction risk. Anim Conserv 12:54–61. doi:10.1098/rspb.2008.0426
Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates, Sunderland
Lyytinen A, Brakefield PM, Lindström L, Mappes JA (2004) Does predation maintain eyespot plasticity in *Bicyclus anynana*? Proc R Soc B 271:279–283. doi:10.1098/rspb.2003.2571
Martin G, Lenormand T (2006) The fitness effect of mutations across environments: a survey in light of fitness landscape models. Evolution 60:2413–2427. doi:10.1554/06-162.1

Matos M, Rose MR, Rocha Pité MT, Rego C, Avelar T (2000) Adaptation to the laboratory environment in *Drosophila subobscura*. J Evol Biol 13:9–19. doi:10.1046/j.1420-9101.2000.00116.x

Maynard Smith J (1956) Acclimatization to high temperatures in inbred and outbred *Drosophila subobscura*. J Genet 54:497–505

Meffert LM, Regan JL, Hicks SK, Mukana N, Day SB (2006) Testing alternative methods for purging genetic load using the housefly (*Musca domestica* L.). Genetica 128:419–427. doi:10.1007/s10709-006-7667-y

Mikkelsen K, Loeschke V, Kristensen TN (2010) Trait specific consequences of fast and slow inbreeding: lessons from captive populations of *Drosophila melanogaster*. Conserv Genet 11:497–488. doi:10.1007/s10592-009-0030-y

Pedersen KS, Kristensen TN, Loeschke V (2005) Effects of inbreeding and rate of inbreeding in *Drosophila melanogaster*—Hsp70 expression and fitness. J Evol Biol 18:756–762. doi:10.1111/j.1420-9101.2005.00884.x

Pedersen KS, Kristensen TN, Loeschke V (2008) Metabolomic signature of inbreeding at benign and stressful temperatures in *Drosophila melanogaster*. Genetics 180:1–11. doi:10.1534/genetics.108.089144

Pray LA, Schwartz JM, Goodnight CJ, Stevens L (1994) Environmental dependency of inbreeding depression: implications for conservation biology. Conserv Biol 8:562–568. doi:10.1046/j.1523-1739.1994.08020562.x

Radwan J, Drewniak M (2001) Inbreeding depression for size but not for symmetry in *Drosophila melanogaster*. Hereditas 134:85–89

Roff DA (1998) Effects of inbreeding on morphological and life history traits of the sand cricket, *Gryllus firmus*. Heredity 81:28–37

Saccheri IJ, Brakefield PM, Nichols RA (1996) Severe inbreeding depression and rapid fitness rebound in the butterfly *Bicyclus anynana* (Satyridae). Evolution 50:2000–2013

Saccheri IJ, Kuussaari M, Kankare M, Vikman P, Fortelius W, Hanski I (1998) Inbreeding and extinction in a butterfly metapopulation. Nature 392:491–494. doi:10.1038/33136

Saccheri IJ, Lloyd HD, Helyar SJ, Brakefield PM (2005) Inbreeding uncovers fundamental differences in the genetic load affecting male and female fertility in a butterfly. Proc R Soc B 272:39–46. doi:10.1098/rspb.2004.2903

Swindell WR, Bouzat JL (2006) Ancestral inbreeding reduces the magnitude of inbreeding depression in *Drosophila melanogaster*. Evolution 60:762–767. doi:10.1111/j.0014-3820.2006.tb01154.x

Van Oosterhout C, Zijlstra WG, Van Heuven MK, Brakefield PM (2000) Inbreeding depression and genetic load in laboratory metapopulations of the butterfly *Bicyclus anynana*. Evolution 54:218–225. doi:10.1111/j.0014-3820.2000.tb00022.x

Van't Hof AE, Zwaan BJ, Saccheri IJ, Daly D, Bot ANM, Brakefield PM (2005) Characterization of 28 microsatellite loci for the butterfly *Bicyclus anynana*. Mol Ecol Notes 5:169–172. doi:10.1111/j.1471-8286.2004.00870.x

Vermeulen CJ, Bijlsma R, Loeschke V (2008) QTL mapping of inbreeding-related cold sensitivity and conditional lethality in *Drosophila melanogaster*. J Evol Biol 21:1236–1244. doi:10.1111/j.1420-9101.2008.01572.x

Waller DM, Dole J, Bersch AJ (2008) Effects of stress and phenotypic variation on inbreeding depression in *Brassica rapa*. Evolution 62:917–931. doi:10.1111/j.1558-5646.2008.00325.x

Windig JJ (1997) The calculation and significance testing of genetic correlations across environments. J Evol Biol 10:853–874. doi:10.1111/j.1420-9101.1997.tb00002.x

Zhong D, Pai A, Yan G (2005) Costly resistance to parasitism: evidence from simultaneous quantitative trait loci mapping for resistance and fitness in *Tribolium castaneum*. Genetics 169:2127–2135. doi:10.1534/genetics.104.038794

6.2 Response to selection on cold tolerance is constrained by inbreeding

Anneke Dierks, Birgit Baumann, and Klaus Fischer

Published as:

Dierks, A., Baumann, B. and Fischer, K. (2012). Response to selection on cold tolerance is constrained by inbreeding. Evolution. DOI: 10.1111/j.1558-5646.2012.01604.x.

doi:10.1111/j.1558-5646.2012.01604.x

RESPONSE TO SELECTION ON COLD TOLERANCE IS CONSTRAINED BY INBREEDING

Anneke Dierks,[1,2] Birgit Baumann,[1] and Klaus Fischer[1]

[1]*Zoological Institute and Museum, University of Greifswald, J.-S.-Bachstraße 11/12, D-17489 Greifswald, Germany*

[2]*E-mail: anneke.dierks@uni-greifswald.de*

Received October 20, 2011
Accepted January 26, 2012
Data Archived: Dryad: doi:10.5061/dryad.vj86fq35

The evolutionary potential of any given population is of fundamental importance for its longer term prospects. Modern land-use practices often result in small and isolated populations, increasing the risk of extinction through reduced genetic diversity as a consequence of inbreeding or drift. Such genetic erosion may also interfere with a population's evolutionary potential. In this study, we investigate the consequences of inbreeding on evolutionary potential (the ability to increase cold resistance) in a laboratory population of the tropical butterfly *Bicyclus anynana*. To explore constraints on evolution, we applied artificial selection to chill-coma recovery time, starting from three levels of inbreeding (outbred control, one or two full-sibling matings). Ten generations of selection produced highly divergent phenotypes, with the lines selected for increased cold tolerance showing about 28% shorter recovery times after cold exposure relative to unselected controls. Correlated responses to selection in 10 different life-history and stress-resistance traits were essentially absent. Inbred lines showed a weaker response to selection, indicating reduced evolutionary potential and thereby constraints on evolution. Inbreeding depression was still measurable in some traits after the course of selection. Traits more closely related to fitness showed a clear fitness rebound, suggesting a trait-specific impact of purging. Our findings have important implications for the longer term survival of small populations in fragmented landscapes.

KEY WORDS: **Artificial selection, evolutionary potential, extinction risk, genetic load, purging, stress resistance.**

Biotic and abiotic conditions are not constant over time, so the long-term persistence of any given species or population depends on its ability to respond to environmental change (Bijlsma and Loeschcke 2005, 2012; Malcolm 2011). In particular, thermal stress often imposes severe challenges through negatively affecting fitness-related traits, warranting phenotypic adjustment (Frankham 1995; Hoffmann et al. 2003; Umina et al. 2005). Responses to environmental challenges may occur quickly through phenotypic plasticity, that is, within a set genotype, or through genetic adaptation needing longer periods of time. Here, allele frequencies change as a result of the selection pressure exerted by the environment (e.g., Bijlsma and Loeschcke 2005; David et al. 2005; Sørensen et al. 2005).

Sufficient standing genetic variation is supposed to be of crucial importance for adaptation to new environmental conditions (Falconer and Mackay 1996; Roff 1997; Bijlsma and Loeschke 2005; Bouzat 2010). This is because adaptation primarily seems to rely on the presence of beneficial variants within populations rather than the production of new variants by mutation (Blows and Hoffmann 2005; Orr and Unckless 2008; Bijlsma and Loeschcke 2012). However, in modern landscapes, evolutionary adaptation may be hampered by an increasing loss and fragmentation of natural habitats (Saunders et al. 1991; Watling and Donnelli 2006; Fischer and Lindenmayer 2007). The concomitant reduction in effective population size may result in reduced genetic variation, and thus evolutionary potential through reduced gene pools, inbreeding, and/or genetic drift (Bijlsma et al. 1997; Frankham et al. 2002, Frankham 2005; Willi et al. 2006; Keyghobadi 2007; Vandergast et al. 2007; Mikkelsen et al. 2010; Bijlsma and Loeschcke 2012). Indeed, several studies have shown that

increased fragmentation may increase the incidence of inbreeding in natural populations (Saccheri et al. 1998; Lennartsson 2002; Andersen et al. 2004; Leimu et al. 2010).

Apart from interfering with a population's evolutionary potential, inbreeding exerts direct detrimental effects on, for example, survival rate, reproduction, and development (Darwin 1876; Charlesworth and Charlesworth 1987; Frankham et al. 2002; Frankham 2005). Consequently, inbreeding is expected to increase the extinction risk of small populations (Frankham 1995; Bijlsma et al. 2000; Swindell and Bouzat 2006; Bouzat 2010). Although the importance of ecological factors has been emphasized in the past (Lande 1988; Lynch et al. 1995), new evidence suggests that genetic factors (e.g., inbreeding, drift) may also be involved in the extinction of natural populations (Saccheri et al. 1998; Crnokrak and Roff 1999; Hedrick and Kalinowski 2000; Keller and Waller 2002; Bouzat 2010).

Adding further complexity, survival rates of inbred populations are difficult to predict due to "purging," that is, selection against deleterious recessive alleles (Templeton and Read 1984; Keller et al. 1994; Hedrick and Kalinowski 2000; Crnokrak and Barrett 2002). Though beneficial effects of purging have been repeatedly documented (e.g., Saccheri et al. 1996; Meffert et al. 1999; Van Oosterhout et al. 2000; Swindell and Bouzat 2006; Fox et al. 2008), such effects are often small and also contrary results are known (e.g., Van Oosterhout et al. 2000; Frankham et al. 2001; Reed et al. 2003; Meffert and Regan 2006; Mikkelsen et al. 2010). Therefore, our understanding of the effectiveness of purging is still limited. Some recent studies actually indicate that purging may have limited success in variable environments when applied to small populations (Jamieson et al. 2003; Boakes et al. 2007). Differences in the effectiveness of purging may arise from, for example, variation in the rate of inbreeding, stochastic effects associated with bottlenecks, the specific genetic background, the environment in which inbreeding occurs, immigration, mutation, and population size (Hedrick and Kalinowski 2000; Wang 2000; Leberg and Firmin 2008; Mikkelsen et al. 2010).

Against the above background, we here explore the consequences of inbreeding on evolutionary potential, measured as response to selection. Although a negative impact of genetic erosion on evolutionary potential is predicted by theory (e.g., through inbreeding), empirical evidence for such effects seems to be exceedingly scarce (Keyghobadi 2007; Anderson et al. 2010). This is despite the fact that many studies have investigated effects of inbreeding (and bottlenecks) on genetic and phenotypic variance, as only a few studies have assessed the consequence thereof for changes in adaptive potential (Turelli and Barton 2006; Bouzat 2010). Especially studies investigating effects of inbreeding on evolutionary responses are largely absent (but see Wade et al. 1996), whereas a few more have investigated the impact of bottlenecks (e.g., Lopez-Fanjul and Villaverde 1989; Swindell and Bouzat 2005; Van Heerwaarden et al. 2008). This is unfortunate as, due to this, we (1) know very little about the strength of the constraints imposed by genetic erosion on adaptive evolution, and (2) as inbreeding may affect genetic variance in a way not predicted by additive theory (Wade et al. 1996; Bouzat 2010). For instance, heritability of several traits was found to increase rather than decrease after inbreeding or population bottlenecks (Bryant et al. 1986; Lopez-Fanjul and Villaverde 1989; Meffert 1995; Wade et al. 1996; Van Heerwaarden et al. 2008). Consequently, nonadditive effects may play a crucial role. Furthermore, the assessment of genetic variation and its reduction is frequently based on neutral genetic markers, although correlations between molecular genetic diversity and quantitative genetic variation may be weak, and although heterozygosity of such markers may show weak correlations with inbreeding coefficients (Hedrick 2001; Pemberton 2004; Gilligan et al. 2005; Bijlsma and Loeschcke 2012).

We therefore measured evolutionary potential here as a response to artificial selection on cold stress resistance, namely chill-coma recovery time. Artificial selection provides a powerful tool to identify constraints on short-term evolutionary change (Brakefield 2003), which is the principal aim of our study. Selection was started from three ecologically relevant levels of inbreeding, caused by full-sibling matings ($F = 0.00/0.25/0.375$; cf. Mikkelsen et al. 2010). In parallel, we test for longer lasting effects of inbreeding depression and for effects of purging, by measuring inbreeding depression in several fitness-related traits, such as fecundity, development time, longevity, and temperature stress resistance after the course of selection. We used the tropical butterfly *Bicyclus anynana* (Butler, 1879) as a model organism, as it has been intensively used in studies using artificial selection and on inbreeding (e.g., Brakefield and Saccheri 1994; Saccheri et al. 1996; Beldade et al. 2002; Joron and Brakefield 2003; Fischer 2006; Bauerfeind and Fischer 2007; Dierks et al. 2012; Prudic et al. 2011).

Material and Methods

STUDY ORGANISM

Bicyclus anynana is a tropical, fruit-feeding butterfly distributed from southern Africa to Ethiopia (Larsen 1991). The adults feed on a diversity of fallen and decaying fruits (Larsen 1991; Brakefield 1997). This species exhibits two seasonal morphs as an adaptation to the wet and dry season in its natural environment, and the associated changes in resting background and predation (Brakefield 1997; Lyytinen et al. 2004; Bauerfeind and Fischer 2007). As morphs are gradually replaced during seasonal transitions, both phenotypes may occur simultaneously (Brakefield and Reitsma 1991). A laboratory stock population was founded at

Greifswald University (Germany), in 2007 from several thousand eggs derived from a well-established stock population at Bayreuth University (Germany). This population in turn originated from a stock population at Leiden University (the Netherlands), which was established in 1988 from 80 gravid females caught at a single location in Malawi. Several hundred individuals are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof et al. 2005; Bauerfeind and Fischer 2007).

SELECTION PROCEDURE

Selection lines for increased cold tolerance (CT) and according unselected control (UC) lines were derived from three groups differing in the level of inbreeding, using the above-mentioned outbred laboratory stock throughout. The three levels of inbreeding were established using a full-sibling breeding design (cf. Dierks et al. 2012, also for further details): outbred controls (C) resulting from random mating, inbreeding 1 (I1) resulting from mating between full-siblings, and inbreeding 2 (I2) resulting from mating between full-siblings in two consecutive generations. To set up the inbreeding treatments, several hundred virgin stock butterflies were allowed to mate randomly in cylindrical hanging cages (30 × 38 cm) on day 4 after adult eclosion. Mating pairs were removed and placed individually in translucent 1L plastic containers, where females were allowed to lay eggs on fresh maize leaves for 10 days. The eggs produced by individual females were afterwards transferred to elongated, sleeve-like cages containing a young maize plant for further development. Consequently, each "sleeve" cage represented one full-sibling family. Density per cage was standardized to 20–30 larvae, and maize plants were replaced as necessary. Resulting butterflies were used to set up the "I2" treatment by mating one brother to one sister, per full-sibling family. Following this first full-sibling mating, the above procedure was repeated once. Parallel to the second generation of the "I2" treatment, the "I1" treatment was set up as outlined above. Finally, the control treatment was set up by mating random virgin males and females from the stock population. This staggered design resulted in a synchronized eclosion of butterflies from all three inbreeding levels, with about 120 full-sibling families each.

To initiate artificial selection, butterflies were pooled across families within inbreeding levels. Four groups were set up per inbreeding level, two for selection on increased CT, and two as UCs. This design resulted in a total of 12 lines (3 inbreeding levels × 4; see Table 1). To set up the six UC lines, two times 40 males and 40 females were randomly selected per inbreeding level. To set up the six selection lines, between 448 and 492 individuals per inbreeding level (1416 individuals in total) were scored in the F_0 generation for cold stress resistance on day 1 following adult eclosion (for details see below). Prior to testing, butterflies were marked individually by writing a number on both hind wings. Following testing, butterflies were transferred to 20°C to keep them in good shape (instead of 27°C; see below) until all butterflies had been measured. Per inbreeding level, the 80 males and 80 females being most cold tolerant were selected and randomly divided between two groups to establish the CT lines. After all CT measurements had been completed, selected males and females were allowed to mate randomly within their lines, having been kept separated by sex before. In subsequent generations, about 300 individuals were reared per line and generation, from which 120 males and 120 females were scored for CT in the selection lines, and 40 males and 40 females in the UC lines. Throughout, 40 males and 40 females were selected to found the next generation per line, being either the most cold tolerant ones (CT lines) or being selected at random (UC lines). Selection was continued for 10 generations.

Unless otherwise stated, butterflies were reared in a single climate room at 27°C (exclusively inducing wet season phenotypes),

Table 1. Overview over the 12 selection lines with codes indicating inbreeding level (C, I1, I2), selection regime (CT, UC), and replicate number (1, 2).

Line	Code	Inbreeding level	Selection regime	Replicate
1	C-CT1	Outbred control	Cold tolerance	1
2	C-CT2	Outbred control	Cold tolerance	2
3	C-UC1	Outbred control	Unselected control	1
4	C-UC2	Outbred control	Unselected control	2
5	I1-CT1	Inbreeding 1	Cold tolerance	1
6	I1-CT2	Inbreeding 1	Cold tolerance	2
7	I1-UC1	Inbreeding 1	Unselected control	1
8	I1-UC2	Inbreeding 1	Unselected control	2
9	I2-CT1	Inbreeding 2	Cold tolerance	1
10	I2-CT2	Inbreeding 2	Cold tolerance	2
11	I2-UC1	Inbreeding 2	Unselected control	1
12	I2-UC2	Inbreeding 2	Unselected control	2

high relative humidity (70%), and a photoperiod of L12:D12 (24-h light cycle). Larvae were reared in population cages (50 × 50 × 50 cm), being fed on potted maize plants provided in ample supply. Plants were replaced as necessary. Adults were kept in cylindrical hanging cages (30 × 38 cm), being fed on moist banana. Throughout, cages were arranged in a randomized block design within the climate cabinet in order to balance potential slight temperature and humidity variation (Bauerfeind and Fischer 2007; Fischer et al. 2010).

CT ASSAYS

To score cold stress resistance, we used chill-coma recovery time, which is the time needed to regain mobility following cold exposure. This trait is considered a reliable proxy of climatic cold adaptation and has been used successfully in *B. anynana* before (e.g., Geister and Fischer 2007; Fischer et al. 2010). For measuring chill-coma recovery time, one-day old butterflies were placed individually in translucent plastic cups (125 mL) and arranged on a tray in a randomized block design (maximum of 72 butterflies per block). Butterflies were then exposed to 1°C in a climate cabinet for 19 h (Sanyo MIR-553), inducing a chill coma. Preliminary studies have shown that results are largely independent of the method used to induce a chill coma, and that the above method produced highly repeatable results (Fischer et al. 2010). After cold exposure, the trays were transferred to a room with a constant temperature of 20°C to determine recovery times. Recovery time was defined as the time elapsed between taking the trays out of the 1°C climate cabinet until a butterfly was able to stand on its legs (Geister and Fischer 2007; Fischer et al. 2010). Blocks were observed for a maximum of 60 min. Butterflies that did not regain mobility within this time span were either given the maximum recovery time of 60 min (if still alive) or were excluded from further analyses (if dead; typically $< 1\%$). CT was always scored one day after adult eclosion.

CORRELATED RESPONSES TO SELECTION

To score responses and correlated responses to selection, as well as whether effects of inbreeding are still measurable after the course selection, we analyzed reproductive traits (generation 11), cold and heat stress resistance, developmental traits, longevity and survival rates (all generation 12, i.e., after two generations without selection), as detailed below.

Reproductive traits

Fecundity and egg hatching success were measured in about 50 females per line. On day 2 after adult eclosion, females were mated to random males within lines. Mating couples were transferred individually to translucent plastic pots (1L) containing a fresh leaf of maize for oviposition. After mating, males were removed and females were allowed to oviposit for four days, being fed with moist bananas. Typically, during this period of time, 35–40% of the lifetime fecundity is realized (Bauerfeind and Fischer 2005). On days 2 and 4 of oviposition, all eggs laid were removed, counted and about 30 eggs per female were placed into a petri dish lined with moist filter paper and a maize leaf for hatchlings to feed on. The numbers of hatchlings and unsuccessful eggs were counted over the following eight days (note that egg development at 27°C is typically four days).

Developmental traits

Developmental traits were measured using 10 replicate cages per line, resulting in a total of 120 cages. All eggs within one cage were collected within a single 12-h light period from at least 100 fecund females. Maize plants were replaced as necessary, and densities within cages were standardized to 30–35 larvae per line on day 12 of larval development (thus aiming at 300 individuals per line). Cages were once again arranged in a randomized block design within the climate cell. They were checked daily for pupae and prepupae, which were removed and placed individually into translucent 125-mL plastic pots containing filter paper. We scored larval development time (from egg laying to pupation, thus including egg development), pupal development time (from pupation to adult eclosion), pupal mass (measured on the day following pupation, Sartorius LE225D), larval growth rate (pupal mass/larval developmental time), and sex for all individuals.

Cold and heat stress resistance

Cold stress resistance was measured as described above. Heat stress resistance was determined by measuring heat knock-down time, which is a widely used and well-established proxy of heat adaptation (e.g., Huey et al. 1992; Sørensen et al. 2005; Karl et al. 2008; Fischer et al. 2010). One day after eclosion, butterflies were placed in translucent cups (60 mL), being arranged on a tray in a randomized block design (maximum of 40 butterflies per block). They were afterwards exposed to 45°C in a climate cabinet (Sanyo MIR-553). To measure heat knock-down time, which was defined as the time until a butterfly was not able to stand upright anymore, butterflies were continuously monitored through the glass doors of the cabinets. Throughout, there was no reuse of animals, that is, individuals were used for measuring cold or heat stress resistance. Chill-coma recovery time was measured in 91–116, and heat knock-down time in 73–103 individuals per line.

Survival and longevity

The animals exposed to heat stress during heat knock-down assays were also used to score survival rates 48 h after heat exposure. Longevity was scored in the animals that had been used for measuring CT as well as in an additional group of untreated animals

(having experienced neither cold nor heat stress). Three different treatments were applied to both of the latter groups, namely a control group (being fed with banana ad libitum), a starvation group (being provided water only), and a desiccation group (being provided neither food nor water). Cages (1 per line) were checked daily for dead butterflies.

STATISTICAL ANALYSES

Realized heritabilities (h^2) were calculated by fitting least square regressions to chill-coma recovery time (relative to UCs) on cumulated selection differentials, with heritabilities being estimated as the slope of the regression lines. Analyses of covariance were used to compare the slopes of regression lines, using selection regime as a fixed factor, replicate line as a random factor, and cumulated selection differential as covariate. General linear models were used to test for differences in fecundity, egg hatching success, chill-coma recovery time, heat knock-down time, and life-history traits (larval time, growth rate, pupal time, pupal mass). Selection regime and inbreeding level were used as fixed factors throughout, and replicate line (nested within selection regime) as random factor. Sex was added as a fixed factor when appropriate. Significant differences between groups were located with the Tukey honestly significant difference (HSD) posthoc test. Survival rates after heat exposure were analyzed using a nominal logistic regression on binary data (dead or alive). Longevity data were analyzed separately for cold-stressed and unstressed butterflies using Cox proportional hazards. All statistical tests were performed by using JMP (4.0.0) or Statistica (6.1). Unless otherwise stated, least square means ± 1 standard error (SE) are given throughout.

Results

All data are available from the Dryad data repository (doi:10.5061/dryad.vj86fq35).

RESPONSE TO SELECTION

After 10 generations of selection, a significant response to selection was observed with the lines selected for increased CT showing an on average by 28.9% shorter chill-coma recovery time compared to UCs (CT: 1527 ± 15 sec < UC: 2148 ± 33 sec; $F_{1,2} = 41.4$, $P = 0.036$; Fig. 1). Realized heritabilities (h^2) ranged between 0.01 and 0.16 (mean 0.07 ± 0.02) and were significant in seven of 12 line by sex combinations (Table 2). The outbred control lines showed the highest values (0.098 ± 0.03; three out of four significant), followed by the I2 lines (0.083 ± 0.03; three out of four significant), and finally the I1 lines (0.035 ± 0.02; one out of four significant). Furthermore, realized heritabilities were on average higher in females (0.10 ± 0.02) than in males (0.05 ± 0.02), being significant in five of six

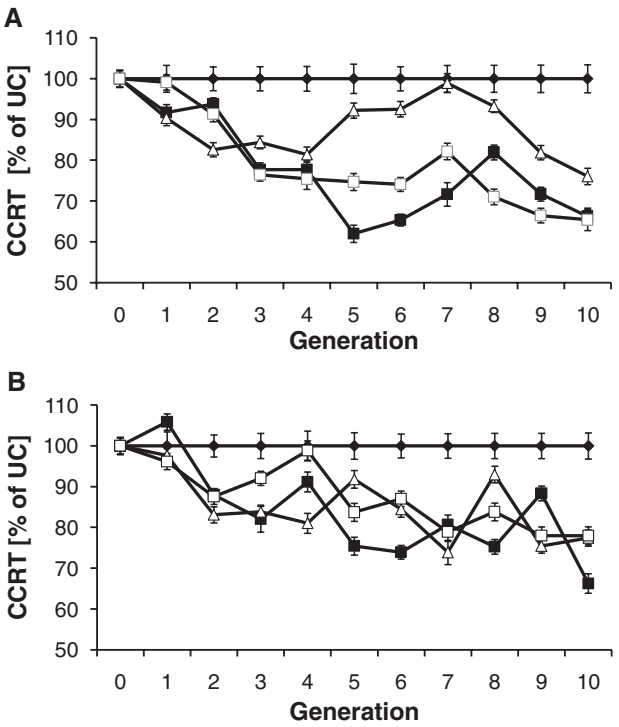

Figure 1. Response to selection on reduced chill-coma recovery time (CCRT) in *Bicyclus anynana* over 10 generations. Note that unselected control lines were set to 100%, and that the data for all other lines are presented relative to the unselected controls. Population means (±SE) are given for each generation. Responses are shown separately for replicate lines 1 (A) and 2 (B; cf. Table 1). Data are pooled across sexes. Unselected control (UC) lines: black diamonds. Selected noninbred lines (C-CT): black squares. Selected inbred lines 1 (1 full-sibling mating, I1-CT): white triangles. Selected inbred lines 2 (2 full-sibling mating, I2-CT): white squares.

Table 2. Realized heritabilities (h^2) of chill-coma recovery time for replicated selection lines of *Bicyclus anynana*, being set up from different inbreeding levels. Least square regressions were fitted to trait values (relative to unselected controls) on cumulated selection differentials, with heritabilities being estimated as the slope of the regression lines. Significant *P*-values are given in bold.

Line	Sex	R^2	h^2	*P* value
C-CT1	Male	0.16	0.037	0.2258
C-CT1	Female	0.77	0.159	**0.0004**
C-CT2	Male	0.59	0.104	**0.0058**
C-CT2	Female	0.55	0.093	**0.0088**
I1-CT1	Male	0.01	0.007	0.8315
I1-CT1	Female	0.11	0.034	0.3230
I1-CT2	Male	0.00	0.006	0.8592
I1-CT2	Female	0.42	0.093	**0.0302**
I2-CT1	Male	0.59	0.079	**0.0061**
I2-CT1	Female	0.74	0.141	**0.0007**
I2-CT2	Male	0.10	0.017	0.3429
I2-CT2	Female	0.71	0.096	**0.0012**

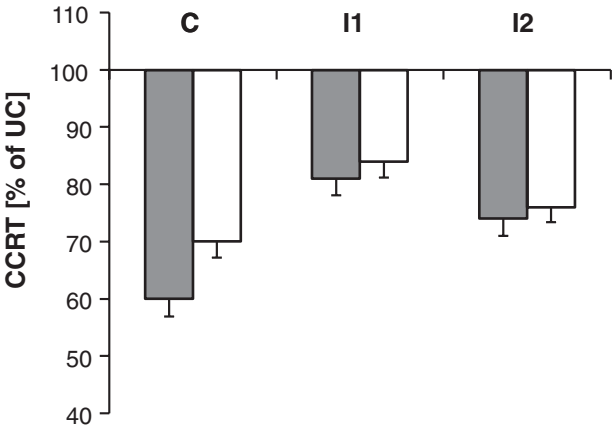

Figure 2. Least square means (−1 SE) for chill-coma recovery time (CCRT, relative to unselected controls set to 100%) in *Bicyclus anynana* across different inbreeding levels (C: control; I1: one full-sibling mating; I2: two full-sibling matings) and replicate lines (gray bars: replicate 1; white bars: replicate 2).

and two of six line by sex combinations, respectively. The slopes of regressions fitted to chill-coma recovery time on cumulated selection differentials differed among inbreeding levels (interaction between inbreeding level and cumulated selection differential: $F_{2,116} = 4.9$; $P = 0.0090$), but not among replicate lines ($F_{1,116} = 0.4$; $P = 0.53$).

CORRELATED RESPONSES TO SELECTION AND EFFECTS OF INBREEDING AND SEX

Stress resistance, reproductive, and developmental traits

After two generations without selection, CT (1490 ± 50 sec) and UC (2046 ± 51 sec) lines differed still significantly with the CT lines showing a by 27.2% reduced chill-coma recovery time (Table 3). The response to selection was strongest in the C lines (by 34.6% reduced chill-coma recovery time), followed by the I2 (25.3%), and finally the I1 (17.2%) lines (significant selection regime × inbreeding level interaction; Fig. 2). Correlated responses to selection were largely absent, as indicated by a lack of significant main effects of selection regime on heat tolerance, egg number, egg hatching success, larval time, pupal time, pupal mass, and growth rate (Table 3 and Fig. 3). Selection regime was, however, involved in interactions for larval time, pupal time, and growth rate. The significant interaction between selection regime and inbreeding level for larval time indicates that CT lines had longer larval times than UC lines in the inbreeding treatment I2, with the opposite pattern being found in inbreeding treatments C and I1 (Fig. 3D). Longer larval times in the I2-CT lines were evident in I2 females only (CT: 28.2 days > UC: 27.5 days), but not in I2 males (CT: 26.6 days = UC: 26.7 days; Tukey HSD after analysis of variance; significant selection regime × inbreeding level × sex interaction). Pupal time was longer in the CT than in the UC lines in the noninbred controls, with the opposite pattern being found in the two inbreeding treatments (significant selection regime × inbreeding level interaction; Fig. 3E). Finally, although larval growth rate decreased with increasing inbreeding level in the CT lines, there was no such tendency in the UC lines (significant selection regime × inbreeding level interaction; Fig. 3G). The rather constant growth rates across inbreeding levels in the UC lines are caused by females (C: 18.9% days^{-1}; I1: 18.8% days^{-1}; I2: 19.0% days^{-1}), whereas in UC males growth rates tended to decrease with increasing inbreeding level (C: 18.8% days^{-1}; I1: 18.8% days^{-1}; I2: 18.6% days^{-1}; significant selection regime × inbreeding level × sex interaction).

Inbreeding level, in contrast, significantly affected egg number, pupal time, pupal mass, and larval growth rate, but not chill-coma recovery time, heat knock-down time, egg hatching success, and larval time (Fig. 3). Inbreeding was associated with lower egg numbers (C: 45 ± 2 > I1: 40 ± 2 > I2: 34 ± 2; Tukey HSD), shorter pupal times (C: 6.7 ± 0.03 days > I2: 6.6 ± 0.03 days > I1: 6.4 ± 0.03 days; Tukey HSD), lower pupal masses (C: 172.4 ± 0.9 mg = I1: 171.6 ± 0.9 mg > I2: 161.0 ± 0.9 mg; Tukey HSD), and lower larval growth rates (C: 18.9 ± 0.05% days^{-1} = I1: 18.9 ± 0.05% days^{-1} > I2: 18.7 ± 0.05% days^{-1}; Tukey HSD).

Significant sex differences were present in all traits investigated, except growth rate and heat tolerance, with males showing shorter chill-coma recovery times compared to females (males: 1702 ± 34 sec < females: 1834 ± 34 sec), shorter larval times (males: 26.7 ± 0.06 days < females: 27.8 ± 0.04 days), longer pupal times (males: 6.8 ± 0.03 days > females: 6.4 ± 0.02 days), and lower pupal masses (males: 149.1 ± 0.9 mg < females: 187.6 ± 0.6 mg). The sex differences in chill-coma recovery time were restricted to the UC lines (UC: males 9.8% faster than females; CT: males 0.2% slower; significant selection regime × sex interaction). The general pattern of shorter recovery times in males than in females persisted throughout, except in the C-CT lines (significant selection regime × inbreeding level × sex interaction; Fig. 4). Variation between replicate lines was nonsignificant throughout, although there were significant differences among replicate cages for larval time, pupal time, pupal mass, and larval growth rate.

Heat stress survival and longevity

Inbreeding level, replicate line, and sex, but not selection regime, significantly affected survival rates after heat exposure (Table 4). Survival rates were highest in the outbred control lines (29.1%, $n = 374$) followed by inbreeding levels I2 (22.4%, $n = 371$) and finally I1 (18.0%, $n = 311$). Further, survival rates were higher in females compared to males (26.2%, $n = 557$ vs. 20.4%, $n = 499$), except for a higher survival rate in I2 males

Table 3. Nested analyses of variance for the effects of selection regime (fixed), inbreeding level (fixed), and replicate line (random) on stress resistance and life-history traits in *Bicyclus anynana*. Replicate line was nested within selection regime throughout. Sex (fixed) and cage (random effect, nested within replicate line and selection regime) were added as factor when appropriate. Significant *P*-values are given in bold.

Trait	Factor	df	MS	*F*	*P* value
Cold tolerance	Selection regime	1,2	282,228.0	73.3	0.0134
	Replicate [Sel. Reg.]	2,1242	3847.6	2.6	0.0718
	Inbreeding level	2,1242	861.5	0.6	0.5539
	Sex	1,1242	7175.1	4.9	**0.0267**
	Sel. Reg. × Inbr. Lev.	2,1242	8781.5	6.0	**0.0025**
	Sel. Reg. × Sex	1,1242	7787.0	5.3	**0.0210**
	Inbr. Lev. × Sex	2,1242	224.6	0.2	0.8752
	Sel. × Inbr. × Sex	2,1242	4747.7	3.3	**0.0388**
	Error	1242	1457.5		
Heat tolerance	Selection regime	1,2	80.0	2.1	0.2749
	Replicate [Sel. Reg.]	2,1029	36.9	0.1	0.9129
	Inbreeding level	2,1029	112.1	0.3	0.7584
	Sex	1,1029	53.9	0.1	0.7154
	Sel. Reg. × Inbr. Lev.	2,1029	61.3	0.2	0.8596
	Sel. Reg. × Sex	1,1029	92.4	0.2	0.6331
	Inbr. Lev. × Sex	2,1029	60.6	0.2	0.8611
	Sel. × Inbr. × Sex	2,1029	169.7	0.4	0.6579
	Error	1029	405.2		
Egg number	Selection regime	1,2	293.7	0.2	0.6746
	Replicate [Sel. Reg.]	2,559	1240.8	1.7	0.1903
	Inbreeding level	2,559	4969.3	6.7	**0.0014**
	Sel. Reg. × Inbr. Lev.	2,559	1169.5	1.6	0.2093
	Error	559	745.5		
Egg hatching	Selection regime	1,2	1963.9	3.4	0.2057
	Replicate [Sel. Reg.]	2,540	575.4	1.0	0.3708
	Inbreeding Level	2,540	845.6	1.5	0.2330
	Sel. Reg. × Inbr. Lev.	2,540	326.3	0.6	0.5695
	Error	540	578.9		
Larval time	Selection regime	1,2	3.4	0.4	0.6063
	Replicate [Sel. Reg.]	2,36	10.9	0.3	0.7281
	Cage [Sel. & Repl.]	36,1855	38.6	18.6	**<0.0001**
	Inbreeding level	2,1855	0.8	0.4	0.6960
	Sex	1,1855	463.3	223.2	**<0.0001**
	Sel. Reg. × Inbr. Lev.	2,1855	14.2	6.9	**0.0011**
	Sel. Reg. × Sex	1,1855	2.1	1.0	0.3153
	Inbr. Lev. × Sex	2,1855	1.8	0.9	0.4108
	Sel. × Inbr. × Sex	2,1855	8.3	4.0	**0.0183**
	Error	1855	2.1		
Pupal time	Selection Regime	1,2	0.1	0.1	0.8784
	Replicate [Sel. Reg.]	2,38	1.8	1.3	0.2867
	Cage [Sel. and Repl.]	36,1852	36.2	3.7	**<0.0001**
	Inbreeding level	2,1852	9.7	23.3	**<0.0001**
	Sex	1,1852	59.8	143.2	**<0.0001**
	Sel. Reg. × Inbr. Lev.	2,1852	8.6	20.6	**<0.0001**
	Sel. Reg. × Sex	1,1852	0.4	1.0	0.3097
	Inbr. Lev. × Sex	2,1852	0.1	0.2	0.7884
	Sel. × Inbr. × Sex	2,1852	0.4	1.1	0.3429
	Error	1852	0.4		

Continued.

Table 3. Continued.

Trait	Factor	df	MS	*F*	*P* value
Pupal mass	Selection regime	1,2	4308	3.4	0.1923
	Replicate [Sel. Reg.]	2,42	1433	1.9	0.1605
	Cage [Sel. and Repl.]	36,1855	795	1.9	**0.0013**
	Inbreeding level	2,1855	21,475	50.7	**<0.0001**
	Sex	1,1855	568,263	1342.0	**<0.0001**
	Sel. Reg. × Inbr. Lev.	2,1855	829	2.0	0.1416
	Sel. Reg. × Sex	1,1855	271	0.6	0.4240
	Inbr. Lev. × Sex	2,1855	1200	2.8	0.0590
	Sel. × Inbr. × Sex	2,1855	550	1.3	0.2733
	Error	1855	424		
Growth rate	Selection regime	1,2	74	<0.1	0.9697
	Replicate [Sel. Reg.]	2,36	46,860	0.3	0.7559
	Cage [Sel. and Repl.]	36,1854	187,693	14.9	**<0.0001**
	Inbreeding level	2,1854	77,166	6.1	**0.0022**
	Sex	1,1854	29,541	2.3	0.1259
	Sel. Reg. × Inbr. Lev.	2,1854	52,093	4.1	**0.0162**
	Sel. Reg. × Sex	1,1854	12,354	1.0	0.3222
	Inbr. Lev. × Sex	2,1854	1074	<0.1	0.9183
	Sel. × Inbr. × Sex	2,1854	63,650	5.1	**0.0065**
	Error	1854	12,601		

MS = mean squares

compared to I2 females (significant inbreeding level × sex interaction; Fig. 5).

Longevity was significantly affected by selection regime in untreated animals, being longer in CT (8.7 ± 0.3 days; $n = 502$) than in UC lines (8.0 ± 0.3 days, $n = 485$; Table 5). This effect, however, was restricted to banana-fed individuals from the I1 and I2 groups. Longevity was even shorter in CT than UC butterflies in the outbred control group, and while there was essentially no difference in relation to selection regime in the starvation and desiccation groups (significant selection regime × inbreeding level × feeding regime interaction; Fig. 6A). Furthermore, a similar effect was absent in the animals exposed to cold stress. In untreated animals, a significant selection regime × sex interaction indicated that CT males lived longer than UC males (6.3 ± 4.2 days, $n = 85 > 5.4 \pm 3.0$ days, $n = 69$), which was not the case in females (8.1 ± 5.7 days, $n = 144 = 8.1 \pm 4.7$ days, $n = 171$).

Longevity in both un- and cold-stressed individuals differed significantly across feeding treatments and sexes. Banana-fed control butterflies lived significantly longer (unstressed: 11.9 ± 0.5 days, $n = 147$; cold-stressed: 14.9 ± 0.45 days, $n = 304$) than butterflies exposed to starvation (unstressed: 6.7 ± 0.2 days, $n = 160$; cold-stressed: 6.7 ± 0.14 days, $n = 327$) and finally desiccation (unstressed: 3.9 ± 0.1 days, $n = 162$; cold-stressed: 4.2 ± 0.05 days, $n = 356$). Females lived longer than males (unstressed: females 8.1 ± 0.3 days, $n = 315 >$ males 5.9 ± 0.3 days, $n = 154$; cold-stressed: females 8.7 ± 0.2 days, $n = 513 >$ males 8.0 ± 0.3 days, $n = 474$). However, a significant interaction between sex and feeding regime in cold-stressed butterflies indicates wide variation in sex differences across feeding treatments, being smallest in the banana-fed control group (3%; females: 14.7 ± 6.1 days, $n = 148$; males: 15.1 ± 9.1 days, $n = 156$), largest in the starvation group (36%; females: 8.1 ± 2.5 days, $n = 173 >$ males: 5.2 ± 1.4 days, $n = 154$), and intermediate in the desiccation group (17%; females: 4.6 ± 1.0 days, $n = 192 >$ males: 3.8 ± 0.7 days, $n = 164$). Although the pattern of longer female life span was consistent across inbreeding levels in both latter groups mentioned above, this was not the case in the banana-fed control animals (significant inbreeding level × sex × feeding regime interaction; Fig. 6B). Here, males lived longer than females in the I2 but shorter in the I1 group, while there was no difference in the outbred control group. In unstressed butterflies a different pattern emerged, with sex differences being least pronounced in the desiccation group (significant sex × feeding regime interaction; banana-fed control: females: 13.1 ± 5.9 days, $n = 101 >$ males: 9.3 ± 5.0 days, $n = 46$; starvation: females: 7.5 ± 2.2 days, $n = 106 >$ males: 5.0 ± 1.1 days, $n = 54$; desiccation: females: 4.0 ± 1.1 days, $n = 108 >$ males: 3.8 ± 1.2 days, $n = 54$).

Discussion

RESPONSE TO SELECTION

As expected, based on studies using *Drosophila* (e.g., Anderson et al. 2005; Mori and Kimura 2008; Bertoli et al. 2010;

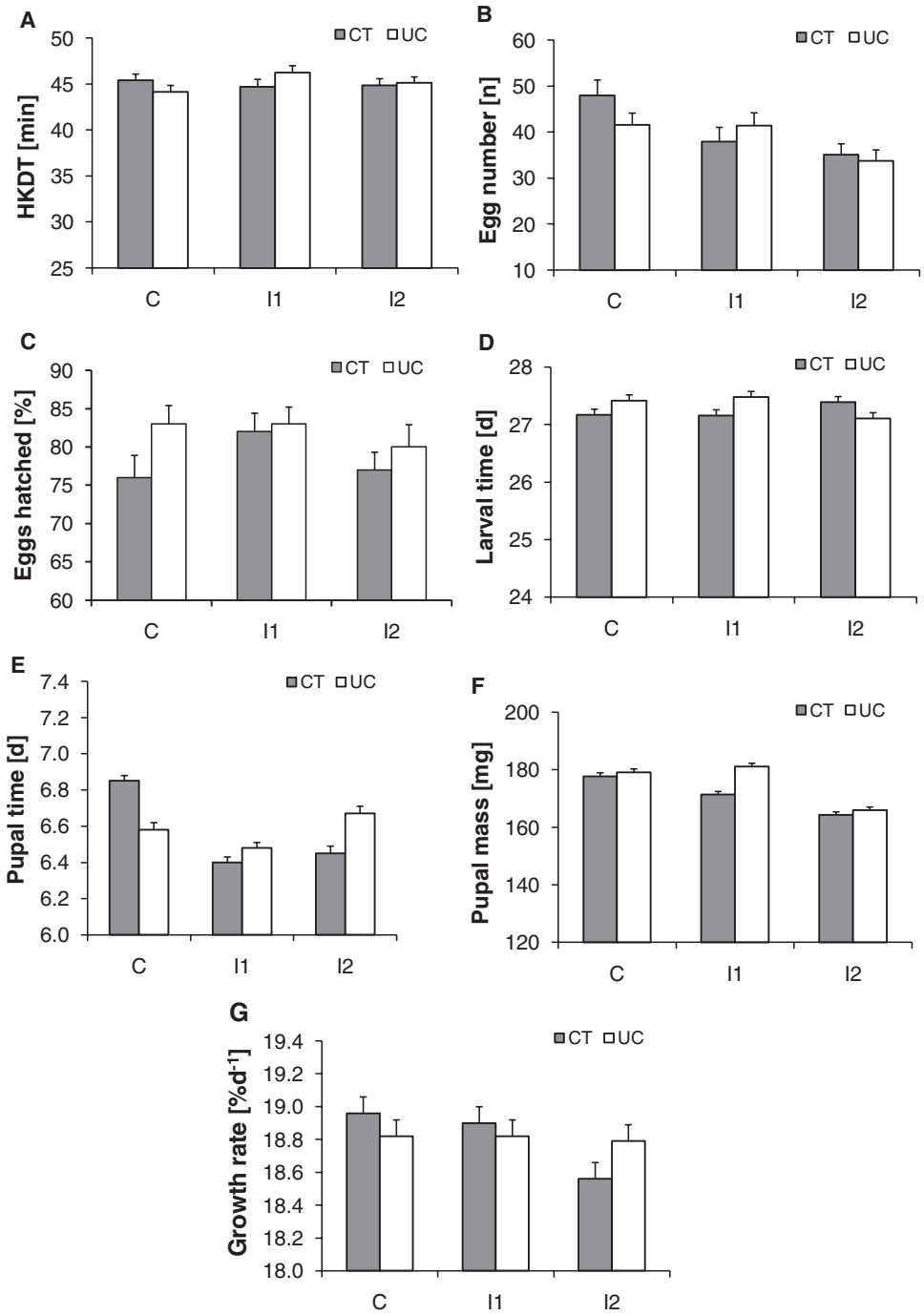

Figure 3. Least square means (+1 SE) for heat knock-down time (HKDT; A), egg number (B), egg hatching success (C), larval time (D), pupal time (E), pupal mass (F), and larval growth rate (G) in relation to inbreeding level (C: outbred control; I1: one full-sibling mating; I2: two full-sibling matings) and selection regimes (CT: lines selected for increased cold tolerance; UC: unselected control lines) in *Bicyclus anynana*.

Udaka et al. 2010), artificial selection on increased cold stress resistance yielded a significant response in *B. anynana*. However, the response to selection differed across inbreeding levels, with the outbred control lines showing a stronger response (a 35% quicker recovery from chill coma) than the inbred lines (I2: 25%; I1: 17%). Apart from the significant interaction between selection regime and inbreeding level, this conclusion is further supported by lower realized heritabilities in the inbred lines, and by significant differences in the slopes of regressions fitted to chill-coma recovery time on cumulated selection differentials (significant

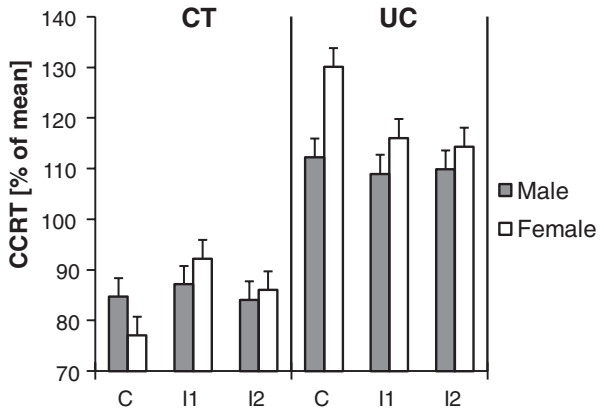

Figure 4. Least square means (+1 SE) for chill-coma recovery times (CCRT) across different inbreeding levels (C: outbred control; I1: one full-sibling mating, I2: two full-sibling matings), sexes, and selection regimes (CT: lines selected for increased cold tolerance; UC: unselected control lines) in *Bicyclus anynana*.

Table 4. Nominal logistic regression for the effects of selection regime, replicate line, inbreeding level, and sex on survival rates after heat exposure in *Bicyclus anynana*. Replicate line was nested within selection regime. Significant *P*-values are given in bold.

Factor	df	χ^2	*P* value
Selection regime	1	1.8	0.1797
Replicate [Sel. Reg.]	2	18.2	**0.0001**
Inbreeding level	2	11.7	**0.0028**
Sex	1	5.6	**0.0181**
Sel. Reg. × Inbr. Lev.	2	3.2	0.2057
Sel. Reg. × Sex	1	0.2	0.6944
Inbr. Lev. × Sex	2	8.0	**0.0183**
Sel. Reg. × Inbr. Lev. × Sex	2	1.5	0.4747

interaction term). Such detrimental effects of inbreeding on adaptive potential have been predicted by theory, but remained experimentally largely untested (but see Wade et al. 1996, who detected a reduced response to selection for increased pupal weight following inbreeding).

The reduced response to selection could on principal be caused by either additive or nonadditive (dominance, epistasis) genetic effects. On the one hand, neutral quantitative genetic theory predicts that inbreeding will decrease the additive genetic variance of quantitative traits (and thereby their evolutionary potential) proportional to the inbreeding coefficient (Falconer and Mackay 1996; Frankham et al. 2002; Roff and Emerson 2006; Van Heerwaarden et al. 2008). In line with these predictions, realized heritabilities were lower in the inbred compared to the outbred control groups, suggesting a contribution of additive effects reducing trait heritability (e.g., Saccheri et al. 2001; England et al. 2003; Kristensen et al. 2005; Swindell and Bouzat 2005; Bakker et al. 2010). Note in this context that our design, through deliberately mitigating the effects of inbreeding by random mating in subsequent generations (thus mimicking a short-term population bottleneck), should have restored additive genetic variance to large extents, especially because a rather large number of families was involved. Nevertheless, significant inbreeding depression was still measurable after the course of selection (see Table 6 and below).

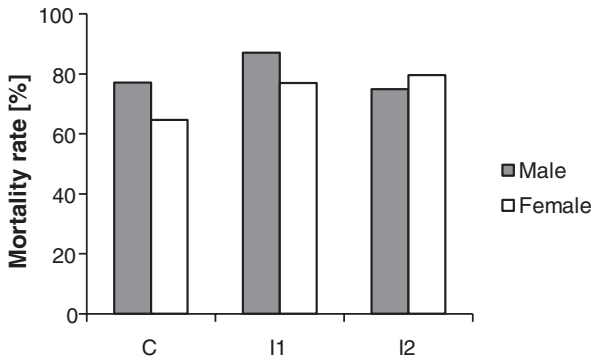

Figure 5. Mortality rates after heat exposure across different inbreeding levels (C: outbred control; I1: one full-sibling mating; I2: two full-sibling mating) and sexes in *Bicyclus anynana*.

Thus, on the other hand, nonadditive effects might also be involved. This notion is supported by the fact that the weakest response to selection was found in inbreeding treatment 1 but not 2, as would have been predicted by additive theory. Some empirical and theoretical studies suggest that, if nonadditive genetic effects are present, additive genetic variance may actually increase rather than decrease through inbreeding (e.g., Bryant et al. 1986; Lopez-Fanjul and Villaverde 1989; Meffert 1995; Wade et al. 1996; Saccheri et al. 2001; Turelli and Barton 2006; Van Heerwaarden et al. 2008; Bouzat 2010). Such patterns are likely caused by chance increases in the frequencies of recessive deleterious alleles, and/or by the release of additive (co)variance as the number of polymorphic loci and thereby potential interlocus interactions decline (Lopez-Fanjul et al. 2002; Van Bushkirk and Willi 2006; Van Heerwaarden et al. 2008; Anderson et al. 2010). This is particularly likely for life-history traits closely related to fitness, as is the case here, because they are more likely to have a nonadditive genetic architecture than, for example, morphological traits (Roff and Emerson 2006; Van Bushkirk and Willi 2006; Willi et al. 2006). Accordingly, a recent meta-analysis found strong support for nonlinear changes in genetic variation with increasing inbreeding levels in life-history traits (Van Bushkirk and Willi 2006). Heritability and additive variance increased with increasing levels of inbreeding by a factor up to 4, with a maximum at $F = 0.4$ (Van Bushkirk and Willi 2006). Note that the latter value is virtually identical to our inbreeding level 2, which might explain its stronger response to selection compared to inbreeding level 1. Thus, although additive effects may have caused the

Table 5. Cox Proportional Hazards for the effects of selection regime, replicate line, inbreeding level, sex, and feeding regime on longevity in unstressed (A) and cold-stressed (B) *Bicyclus anynana* butterflies. Throughout, replicate was nested within selection regime. Significant *P*-values are given in bold.

(A)	Factor	df	χ^2	*P* value
	Selection regime	1	1.8	0.1816
	Replicate [Sel. Reg.]	2	0.2	0.9161
	Inbreeding Level	2	2.8	0.2467
	Sex	1	42.6	**<0.0001**
	Feeding Regime	2	233.9	**<0.0001**
	Sel. Reg. × Inbr. Lev.	2	2.8	0.2509
	Sel. Reg. × Sex	1	8.0	**0.0047**
	Inbr. Lev. × Sex	2	0.8	0.6688
	Sel. × Feed. Reg.	2	2.0	0.3612
	Inbr. Lev. × Feed. Reg.	4	2.5	0.6400
	Sex × Feed. Reg.	2	15.3	**0.0005**
	Sel. × Inbr. × Fed. Reg.	4	1.6	0.8172
	Sel. × Inbr. × Sex	2	3.4	0.1824
	Sel. × Sex × Feed.	2	3.3	0.1948
	Inbr. × Sex × Feed.	4	4.7	0.3222
	Sel. × Inbr. × Sex × Feed.	4	3.3	0.5080
(B)	Factor	df	χ^2	*P* value
	Selection regime	1	4.1	**0.0431**
	Replicate [Sel. Reg.]	2	3.0	0.2312
	Inbreeding level	2	3.6	0.1674
	Sex	1	77.5	**<0.0001**
	Feeding regime	2	784.9	**<0.0001**
	Sel. Reg. × Inbr. Lev.	2	5.7	0.0593
	Sel. Reg. × Sex	1	0.0	0.8772
	Inbr. Lev. × Sex	2	1.8	0.4007
	Sel. × Feed. Reg.	2	1.8	0.4162
	Inbr. Lev. × Feed. Reg.	4	3.4	0.4942
	Sex × Feed. Reg.	2	68.6	**<0.0001**
	Sel. × Inbr. × Fed. Reg.	4	15.4	**0.0039**
	Sel. × Inbr. × Sex	2	1.2	0.5410
	Sel. × Sex × Feed.	2	0.2	0.8886
	Inbr. × Sex × Feed.	4	9.6	**0.0476**
	Sel. × Inbr. × Sex × Feed.	4	2.2	0.6910

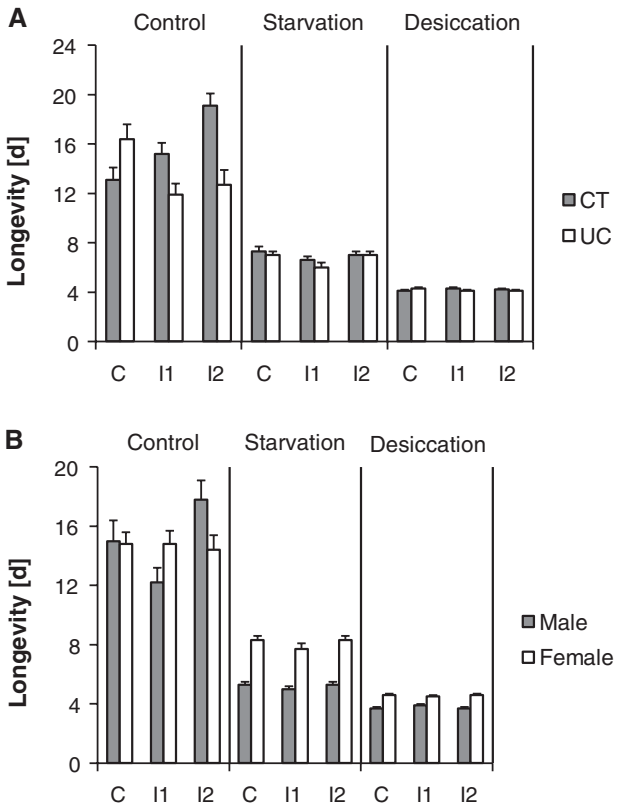

Figure 6. Means (+1 SE) for longevity across selection regimes (CT: lines selected for increased cold tolerance; UC: unselected control lines), feeding regimes (control: banana-fed; starvation: water-fed; desiccation: neither food nor water) and different inbreeding levels (C: control, I1: one full-sibling mating, I2: two full-sibling mating; A), and across sexes, selection, and feeding regimes (B) after cold exposure in *Bicyclus anynana*.

overall weaker response to selection in the inbred lines, inbreeding level 2 may have partially benefitted from nonadditive effects, releasing novel additive variance.

Whether changes in additive variance or trait heritabilities induced by nonadditive effects might confer enhanced adaptability is currently debated. Note that essentially all empirical studies having investigated such changes did not address the issue of the consequences for adaptive capacities. Moreover, a recent study showed statistically detectable increases in additive genetic variance after a bottleneck, caused by both dominance and epistatic effects. However, this change did not affect the response to selection, which was essentially absent (Van Heerwaarden et al. 2008). Reasons underlying this finding may be that the novel additive variance released through dominance and epistatic effects is (1) dependent on genetic background, and therefore vulnerable to rapid elimination by natural selection (Van Heerwaarden et al. 2008), and that it (2) relies on the action of rare alleles likely to be deleterious, thus being accompanied by inbreeding depression, which has indeed generally been found (also in our study; cf. Lopez-Fanjul and Villaverde 1989; Wade et al. 1996; Lopez-Fanjul et al. 2000). In summary, these findings question a positive role of increased additive variance after inbreeding or bottlenecks for the rate of adaptive evolution. However, based on the paucity of data available to date, it is premature to draw any general conclusions regarding the effects of nonadditive effects on evolutionary potential (Van Bushkirk and Willi 2006; Van Heerwaarden et al. 2008).

Alternatively, the differences between inbreeding level 1 and 2 in our study could be caused by chance effects. Several studies reported large variation across inbred lines despite identical

inbreeding coefficients (e.g., Fowler and Whitlock 1999; Reed et al. 2002; Kristensen et al. 2003; Wright et al. 2008), with some inbred lines even outperforming outbred controls. Such lineage effects probably result from the number of founding individuals carrying deleterious recessive alleles (Fowler and Whitlock 1999; Reed et al. 2002; Wright et al. 2008), and are an ubiquitous feature in studies on inbreeding depression (Armbruster and Reed 2005). The twofold higher realized heritabilities for females compared to males should also be noted. Although formal testing is not possible, the pattern is striking. Such sexual differences in the evolution of CT have also been found in a few other studies, although heritabilities were lower rather than higher in females (Krebs and Loeschcke 1997; Vermeulen et al. 2008).

CORRELATED RESPONSES TO SELECTION

Out of 10 traits investigated, only a single one showed at least some evidence for a correlated response to selection: longevity after cold exposure. Although this trait does reflect a measure of cold stress resistance, and might therefore be closely related to the trait under selection (chill-coma recovery time), the overall evidence is weak. This is because positive effects of selection were restricted to inbred, banana-fed butterflies. Note in this context that the significant interactions involving the factor selection regime also did not show conclusive evidence for any correlated responses. In line with our findings, studies on *Drosophila* also yielded mainly negative or inconclusive results regarding correlated responses (e.g., Watson and Hoffmann 1996; Anderson et al. 2005; Mori and Kimura 2008; Bertoli et al. 2010). For instance, although in some selection studies, there was a positive genetic correlation between CT and longevity, others found no association (Norry and Loeschcke 2002; Bubliy and Loeschcke 2005) or opposite patterns (Anderson et al. 2005; Mori and Kimura 2008). Furthermore, the lack of a correlated response in heat resistance is consistent with some studies on *Drosophila*, suggesting that different mechanisms are involved in cold versus heat tolerance (Anderson et al. 2005; Mori and Kimura 2008; Bertoli et al. 2010). In summary, cold adaptation obviously operates at least largely independent from other traits including heat tolerance.

EFFECTS OF INBREEDING

Interestingly, effects of inbreeding were still measurable after the course of selection, that is, 11–12 generations following the full-sibling mating, a time period during which butterflies were allowed to mate randomly (thus outbreeeding). Although egg number, pupal mass, larval growth rate, and survival after heat exposure were all negatively affected by inbreeding, suggesting enduring inbreeding depression, pupal time was shorter rather than longer in inbred individuals. However, make note of the overall small differences of 0.3 days across inbreeding levels in this trait. The other traits, including cold and heat tolerance, larval time, egg hatching success, and longevity showed no inbreeding depression. Therefore, the results obtained still reflect some of the patterns obtained immediately after inbreeding (Dierks et al. 2012; Table 6). In the earlier study egg hatching success, larval time, larval growth rate, pupal mass, and cold stress resistance were negatively affected by inbreeding, while heat stress resistance, and pupal time remained unaffected. Thus, two of the traits having been also examined in the earlier study were still negatively influenced by inbreeding (larval growth rate and pupal mass, though effect size was smaller now), whereas inbreeding depression was not measurable anymore in cold stress resistance, larval time, and egg hatching success. Note that egg number and survival after heat exposure, which were also negatively affected by inbreeding after selection, were not measured in the first experiment.

Table 6. Effects of inbreeding on various traits before (i.e., immediately after full-sibling matings) and after the selection experiment (i.e., 11–12 generations later). Complete (no effects of inbreeding measurable anymore after selection) and partial (effects still significant, though effect size has decreased) fitness rebounds are also indicated. "−": negatively affected; "0": not affected, "+": positively affected (though note the very small effect size; see text); "∗": Not measured prior to selection. T: temperature.

Trait	Before	After	Rebound
Egg hatching success	−	0	Yes
Fecundity	∗	−	
Larval development T	−	0	Yes
Pupal development T	0	+	
Larval growth rate	−	−	Partly
Pupal mass	−	−	Partly
Longevity	∗	0	
Heat survival	∗	−	
Heat knock-down T	0	0	
Chill-coma recovery T	−	0	Yes

Bicyclus anynana, and especially its egg hatching success, has earlier been found to be extremely susceptible to inbreeding depression, suggesting that this species carries a large genetic load (Saccheri et al. 1996; Dierks et al. 2012). This may explain why inbreeding effects were still measurable after 12 generations of random mating. Nevertheless, clear fitness rebounds were observed in cold stress resistance, larval time, egg hatching success, and, to a lesser extent, in growth rate and pupal mass. Saccheri et al. (1996) also discovered a rapid fitness rebound in the average egg hatching rate following inbreeding. The trait specificity of fitness rebounds may suggest a contribution of purging (e.g., Frankham et al. 2001; Pedersen et al. 2005; Mikkelsen et al. 2010). Such effects should be most pronounced in traits closely related to fitness, which may apply here for instance to egg hatching success (Ehiobu et al. 1989; Pedersen et al. 2005). The prolonged

negative effects of inbreeding on fecundity suggests, in line with Saccheri et al. (1996), that inbreeding depression is far less severe for fecundity than for fertility.

SEX DIFFERENCES AND EFFECTS OF FEEDING REGIME ON LONGEVITY

With one exception (larval growth rate), the sex differences found in life-history traits are in agreement with earlier findings (Fischer et al. 2003, 2004, 2010; Dierks et al. 2012). Although larval growth rate though is typically higher in males than in females, we found no significant difference here. This most likely reflects an experimental artifact, as growth rates were measured in a subset of animals only, including a disproportionally high number of individuals with a relatively long development (thus, we missed the fastest males). Regarding stress resistance traits, males showed a higher CT than females, which is the opposite of the pattern found prior to selection (Dierks et al. 2012). We assume this to be a chance effect, as a study on *B. anynana*, including 22 sex comparisons in temperature stress resistance, showed a significant result in only four cases, indicating that both sexes are equally stress resistant in general (Fischer et al. 2010).

Food stress reduced life span in *B. anynana* as expected (Bauerfeind et al. 2009). Females were, in general, the longer lived sex (with one exception in the cold-stressed, banana-fed I2 group), as has been demonstrated in *B. anynana* before (Bauerfeind et al. 2009). The sex difference became larger when cold-stressed butterflies were exposed to food stress, but smaller in unstressed butterflies. We suppose that these differences are a result of different reproductive and activity patterns of males and females (see also Brakefield and Reitsma 1991; Bauerfeind and Fischer 2005; Fischer et al. 2008; Bauerfeind et al. 2009).

Conclusions

Our study shows a clear response to selection on cold stress resistance, which was smaller in inbred compared to outbred populations. Correlated responses to selection were essentially absent. We believe that our results have large implications for the survival of small populations in fragmented landscapes, and therefore for many species inhabiting cultivated landscapes. Small populations are evidently prone to a loss of genetic diversity through drift and/or inbreeding, and are at the same time under pressure through human-induced deterioration of their habitats. Importantly, we here experimentally demonstrate that increased levels of inbreeding indeed reduces evolutionary potential, and therefore the ability to cope with environmental change. In this context, our findings on long-lasting detrimental effects of relatively mild and putatively ecologically relevant levels of inbreeding seem also important, as this will further increase the risk of extinction.

ACKNOWLEDGMENTS

We thank V. Loeschcke (Aarhus) and two anonymous reviewers for insightful comments on an earlier draft of this manuscript, and L. Schultheiß, S. Arndt, S. Bauerfeind, N. Kölzow, K. Franke, S. Geisthardt, H. Höltje, and C. Michalowsky for help with the experiment. This work was supported by a start-up grant from Greifswald University.

LITERATURE CITED

Andersen, L. W., K. Fog, and C. Damgaard. 2004. Habitat fragmentation causes bottlenecks and inbreeding in the European tree frog (*Hyla arborea*). Proc. R. Soc. Lond. B 271:1293–1302

Anderson, A. R., A. A. Hoffmann, and S. W. McKechnie. 2005. Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: physiology and life-history traits. Genet. Res. 85:15–22.

Anderson, S., M. Ellmer, T. H. Jorgensen, and A. Palme. 2010. Quantitative genetic effects of bottlenecks: experimental evidence from a wild plant species, *Nigella degenii*. J. Hered. 101:298–307.

Armbruster, P., and D. H. Reed. 2005. Inbreeding depression in benign and stressful environments. Heredity 95:235–242.

Bakker, J., M. E. C. van Rijswijk, F. J. Weissing, and R. Bijlsma. 2010. Consequences of fragmentation for the ability to adapt to novel environments in experimental *Drosophila* metapopulations. Conserv. Genet. 11:449–462.

Bauerfeind, S. S., and K. Fischer. 2005. Effects of adult-derived carbohydrates, aminoacids and micronutrients on female reproduction in a fruit-feeding butterfly. J. Insect Physiol. 51:545–554.

———. 2007. Maternal body size as an evolutionary constraint on egg size in a butterfly. Evolution 61:2374–2385.

Bauerfeind, S. S., J. E. C. Perlick, and K. Fischer. 2009. Disentangling environmental effects on adult life span in a butterfly across the metamorphic boundary. Exp. Gerontol. 44:805–811.

Beldade, P., K. Koops, and P. M. Brakefield. 2002. Developmental constraints versus flexibility in morphological evolution. Nature 416:844–847.

Bertoli, C. I., A. C. Scannapieco, P. Sambucetti, and F. M. Norry. 2010. Direct and correlated responses to chill-coma recovery selection in *Drosophila buzzatii*. Entomol. Exp. Appl. 134:154–159.

Bijlsma, R., and V. Loeschcke. 2005. Environmental stress, adaptation and evolution: an overview. J. Evol. Biol. 18:744–749.

———. 2012. Genetic erosion impedes adaptive responses to stressful environments. Evol. Appl. 5:117–129.

Bijlsma, R., J. Bundgaard, A. C. Boerema, and W. F. Van Putten. 1997. Genetic and environmental stress, and the persistence of populations. Pp. 193–207 *in* R. R. Bijlsma, and V. Loeschcke, eds. Environmental stress, adaption and evolution. Birkhäuser, Basel.

Bijlsma, R., J. Bundgaard, and A. C. Boerema. 2000. Does inbreeding affect the extinction risk of small populations? Predictions from *Drosophila*. J. Evol. Biol. 13:502–514.

Blows, M. W., and A. A. Hoffmann. 2005. A reassessment of genetic limits to evolutionary limits. Ecology 86:1371–1384.

Boakes, E. H., J. Wang, and W. Amos. 2007. An investigation of inbreeding depression and purging in captive pedigreed populations. Heredity 98:172–182.

Bouzat, J. L. 2010. Conservation genetics of population bottlenecks: the role of chance, selection, and history. Conserv. Genet. 11:463–478.

Brakefield, P. M. 1997. Phenotypic plasticity and fluctuating asymmetry as responses to environmental stress in the butterfly *Bicyclus anynana*. Pp. 105–146 *in* R. R. Bijlsma and V. Loeschcke, eds. Environmental stress: adaptation and evolution. Birkhäuser, Basel.

———. 2003. Artificial selection and the development of ecologically relevant phenotypes. Ecology 84:1661–1671.

Brakefield, P. M., and N. Reitsma.1991. Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. Ecol. Entomol. 16:291–303.

Brakefield, P. M., and I. J. Saccheri. 1994. Guidelines in conservation genetics and the use of population cage experiments with butterflies to investigate the effects of genetic drift and inbreeding. Pp. 165–179 *in* V. Loeschcke, J. Tomiuk, and S. K. Jain, eds. Conservation genetics. Birkhäuser, Basel.

Bryant, E. H. S., A. McCommas, and L. M. Combs. 1986. The effect of an experimental bottleneck upon quantitative genetic variation in the housefly. Genetics 114:191–121.

Bubliy, O. A., and V. Loeschcke. 2005. Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. J. Evol. Biol. 18:789–803.

Charlesworth, D., and B. Charlesworth. 1987. Inbreeding depression and its evolutionary consequences. Annu. Rev. Ecol. Syst. 18:237–268.

Crnokrak, P., and S. Barrett. 2002. Purging the genetic load: a review of the experimental evidence. Evolution 56:2347–2358.

Crnokrak, P., and D. A. Roff. 1999. Inbreeding depression in the wild. Heredity 83:260–270.

Darwin, C. R. 1876. The effects of cross and self fertilisation in the vegetable kingdom. John Murray, London.

David, J. R., L. O. Araripe, M. Chakir, H. Legout, B. Lemos, G. Petavy, C. Rohmer, D. Joly, and B. Moreteau. 2005. Male sterility at extreme temperatures: a significant but neglected phenomenon for understanding *Drosophila* climatic adaptations. J. Evol. Biol. 18:838–846.

Dierks, A., B. Hoffmann, S. S. Bauerfeind, and K. Fischer. 2012. Effects of inbreeding on life history and thermal performance in the tropical butterfly *Bicyclus anynana*. Popul. Ecol. 54:83–90.

Ehiobu, N. G., M. E. Goddard, and J. F. Taylor. 1989. Effect of rate of inbreeding on inbreeding depression in *Drosophila melanogaster*. Theor. Appl. Genet. 77:123–127.

England, P. R., G. H. R. Osler, L. M. Woodworth, M. E. Montgomery, D. A. Briscoe, and R. Frankham. 2003. Effects of intense versus diffuse population bottlenecks on microsatellite genetic diversity and evolutionary potential. Conserv. Genet. 4:595–604.

Falconer, D. S., and T. F. C. Mackay.1996. Introduction to quantitative genetics. 2nd ed. Longman Scientific, New York.

Fischer, J., and D. B. Lindenmayer. 2007. Landscape modification and habitat fragmentation: a synthesis. Global Ecol. Biogeogr. 16:265–280.

Fischer, K. 2006. Reduced male mating vigor in selection lines of the butterfly *Bicyclus anynana*. J. Insect Behav. 19:657–668.

Fischer, K., P. M. Brakefield, and B. J. Zwaan. 2003. Plasticity in butterfly egg size: why larger offspring at lower temperatures? Ecology 84:3138–3147.

Fischer, K., I. Zeilstra, S. K. Hetz, and K. Fiedler. 2004. Physiological costs of growing fast: does accelerated growth rate reduce pay-off in adult fitness? Evol. Ecol. 18:343–353.

Fischer, K., J. Perlick, and T. Galetz. 2008. Residual reproductive value and male matingsuccess: older males do better. Proc. R. Soc. Lond. B 275:1517–1524.

Fischer, K., A. Dierks, K. Franke, T. L. Geister, M. Liszka, S. Winter, and C. Pflicke. 2010. Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. PLoS One 5:e15284.

Fowler, K., and M. C. Whitlock. 1999. The distribution of phenotypic variance with inbreeding. Evolution 53:1143–1156.

Fox, C. W., K. L. Scheibly, and D. H. Reed. 2008. Experimental evolution of the genetic load and its implications for the genetic basis of inbreeding depression. Evolution 62:2236–2249.

Frankham, R. 1995. Inbreeding and extinction: a threshold effect. Conserv. Biol. 9:792–799.

———. 2005. Genetics and extinction. Biol. Conserv. 126:131–140.

Frankham, R., D. M. Gilligan, D. Morris, and D. A. Briscoe. 2001. Inbreeding and extinction: effects of purging. Conserv. Genet. 2:279–284.

Frankham, R., J. D. Ballou, and D. A. Briscoe. 2002. Introduction to conservation genetics. Cambridge Univ. Press, Cambridge, UK.

Geister, T. L., and K. Fischer. 2007. Testing the beneficial acclimation hypothesis: temperature effects on mating success in a butterfly. Behav. Ecol. 18:658–664.

Gilligan, D. M., D. A. Briscoe, and R. Frankham. 2005. Comparative losses of quantitative and molecular variation in finite populations of *Drosophila melanogaster*. Genet. Res. 85:47–55.

Hedrick, P. W. 2001. Conservation genetics: where are we now? Trends Ecol. Evol. 16:629–636.

Hedrick, P. W., and S. T. Kalinowski. 2000. Inbreeding depression in conservation biology. Annu. Rev. Ecol. Syst. 31:139–162.

Hoffmann, A. A., R. J. Hallas, J. A. Dean, and M. Schiffer. 2003. Low potential for climatic stress adaptation in a rainforest *Drosophila* species. Science 301:100–102.

Huey, R. B., W. D. Crill, J. G. Kingsolver, and K. E. Weber. 1992. A method for rapid measurement of heat or cold resistance of small insects. Funct. Ecol. 6:489–494.

Jamieson, I. G., M. S. Roy, and M. Lettinek. 2003. Sex specific consequences of recent inbreeding in an ancestrally inbred population of New Zealand Takahe. Conserv. Biol. 17:708–716.

Joron, M., and P. M. Brakefield. 2003. Captivity masks inbreeding effects on male mating success in butterflies. Nature 424:191–194.

Karl, I., T. Schmitt, and K. Fischer. 2008. Phosphoglucoseisomerase genotype affects life-history traits and cold stress resistance in a Copper butterfly. Funct. Ecol. 22:887–894.

Keller, L. F., and D. M. Waller. 2002. Inbreeding effects in wild populations. Trends Ecol. Evol. 17:230–341.

Keller, L. F., P. Arcese, J. N. M. Smith, W. M. Hochachka, and S. C. Stearns. 1994. Selection against inbred song sparrows during a natural population bottleneck. Nature 372:356–357.

Keyghobadi, N. 2007. The genetic implications of habitat fragmentation for animals. Can. J. Zool. 85:1049–1064.

Krebs, R. A., and V. Loeschcke. 1997. Estimating heritability in a threshold trait: heat-shock tolerance in *Drosophila buzzatii*. Heredity 79:252–259.

Kristensen, T. N., J. Dahlgaard, and V. Loeschcke. 2003. Effects of inbreeding and environmental stress on fitness – using *Drosophila buzzatii* as a model organism. Conserv. Genet. 4:453–465.

Kristensen, T. N., A. C. Sørensen, D. Sorensen, K. S. Pedersen, J. G. Sørensen, and V. Loeschcke. 2005. A test of quantitative genetics theory using *Drosophila*—effects of inbreeding and the rate of inbreeding on heritabilities and variance components. J. Evol. Biol. 18:736–770.

Lande, R. 1988. Genetics and demography in biological conservation. Science 241:1455–1460.

Larsen, T. B. 1991. The butterflies of Kenya. Oxford Univ. Press, Oxford, UK.

Leberg, P. L., and B. D. Firmin. 2008. Role of inbreeding depression and purging in captive breeding and restoration programmes. Mol. Ecol. 17:334–343.

Leimu, R., P. Vergeer, F. Angeloni, and N.J. Ouborg. 2010. Habitat fragmentation, climate change, and inbreeding in plants. Ann. N Y Acad. Sci. 1195:84–98.

Lennartsson, T. 2002. Extinction thresholds and disrupted plant-pollinator interactions in fragmented plant populations. Ecology 83:3060–3072.

Lopez-Fanjul, C., and A. Villaverde. 1989. Inbreeding increases genetic variation for viability in *Drosophila melanogaster*. Evolution 43:1800–1804.

Lopez-Fanjul, C., A. Fernandez, and M. A. Toro. 2000. Epistasis and the conversion of non-additive to additive genetic variance at population bottlenecks. Theor. Popul. Biol. 58:49–59.

Lopez-Fanjul, C., A. Fernandez, and M. A. Toro. 2002. The effect of epistasis on the excess of the additive and nonadditive variances after population bottlenecks. Evolution 56:865–876

Lynch, M. J., J. Connery, and R. Bürger. 1995. Mutational meltdowns in sexual populations. Evolution 49:1067–1080.

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

Malcolm, J. W. 2011. Smaller gene networks permit longer persistence in fast-changing environments. PLoS One 6:e14747.

Meffert, L. M. 1995. Bottleneck effects on genetic variance for courtship repertoire. Genetics 139:365–374.

Meffert, L. M., and J. L. Regan. 2006. Reversed selection responses in small populations of the housefly (*Musca domestica* L.). Genetica 127:1–9.

Meffert, L. M., J. L. Regan, and B. W. Brown. 1999. Convergent evolution of the mating behaviour of founder-flush populations of the housefly. J. Evol. Biol. 12:859–868.

Mikkelsen, K., V. Loeschcke, and T. N. Kristensen. 2010. Trait specific consequences of fast and slow inbreeding: lessons from captive populations of *Drosophila melanogaster*. Conserv. Genet. 11:497–488.

Mori, N., and M. T. Kimura. 2008. Selection for rapid and slow recovery from chill- and heat-coma in *Drosophila melanogaster*. Biol. J. Linn. Soc. 95:72–80.

Norry, F. M., and V. Loeschcke. 2002. Longevity and resistance to cold stress in cold-stress selected lines and their controls in *Drosophila melanogaster*. J. Evol. Biol. 15:775–783.

Orr, H. A., and R. L. Unckless. 2008. Population extinction and the genetics of adaptation. Am. Nat. 172:160–169.

Pedersen, K. S., T. N. Kristensen, and V. Loeschcke. 2005. Effects of inbreeding and rate of inbreeding in *Drosophila melanogaster*—Hsp70 expression and fitness. J. Evol. Biol. 18:756–762.

Pemberton, J. 2004. Measuring inbreeding depression in the wild: the old ways are the best. Trends Ecol. Evol. 19:613–615.

Prudic, K. L., C. Jeon, H. Cao, and A. Monteiro. 2011. Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation. Science 331:73–75.

Reed, D. H., D. A. Briscoe, and R. Frankham. 2002. Inbreeding and extinction: the effect of environmental stress and lineage. Conserv. Genet. 3:301–307.

Reed, D. H., E. H. Lowe, D. A. Briscoe, and R. Frankham. 2003. Inbreeding and extinction: effects of rate of inbreeding. Conserv. Genet. 4:405–410.

Roff, D. A. 1997. Evolutionary quantitative genetics. Chapman & Hall, New York.

Roff, D. A., and K. Emerson. 2006. Epistasis and dominance: evidence for differential effects in life-history versus morphological traits. Evolution 60:1981–1990.

Saccheri, I. J., P. M. Brakefield, and R. A. Nichols. 1996. Severe inbreeding depression and rapid fitness rebound in the butterfly *Bicyclus anynana* (Satyridae). Evolution 50:2000–2013.

Saccheri, I. J., M. Kuussaari, M. Kankare, P. Vikman, W. Fortelius, and I. Hanski. 1998. Inbreeding and extinction in a butterfly metapopulation. Nature 392:491–494.

Saccheri, I. J., R. A. Nichols, and P. M. Brakefield. 2001. Effects of bottlenecks on quantitative genetic variation in the butterfly *Bicyclus anynana*. Genet. Res. 77:167–181.

Saunders, D. A., R. J. Hobbs, C. R. Margules. 1991. Biological consequences of ecosystem fragmentation—a review. Conserv. Biol. 5:18–32.

Sørensen, J. G., F. M. Norry, A. S. Scannapieco, and V. Loeschcke. 2005. Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. J. Evol. Biol. 18:829–837.

Swindell, W. R., and J. L. Bouzat. 2005. Modelling the adaptive potential of isolated populations: experimental simulations using *Drosophila*. Evolution 59:2159–2169.

———. 2006. Selection and inbreeding depression: effects of inbreeding and inbreeding environment. Evolution 60:1014–1022.

Templeton A. R., and B. Read. 1984. Factors eliminating inbreeding depression in a captive herd of Speke's gazelle (*Gazella spekei*). Zool. Biol. 3:177–199.

Turelli, M., and N. H. Barton. 2006. Will population bottlenecks and multilocus epistasis increase additive genetic variance? Evolution 60:1763–1776.

Udaka, H., C. Ueda, and S. G. Goto. 2010. Survival rate and expression of heat-shock protein 70 and frost genes after temperature stress in *Drosophila melanogaster* lines that are selected for recovery time from temperature coma. J. Insect Physiol. 56:1889–1894.

Umina, P. A., A. R. Weeks, M. R. Kearny, S. W. McKechnie, and A. A. Hoffmann. 2005. A rapid shift in a classic clinal pattern in *Drosophila* reflecting climate change. Science 308:691–693.

Van Bushkirk, J., and Y. Willi. 2006. The change in quantitative genetic variation with inbreeding. Evolution 60:2428–2434.

Van Heerwaarden, B., Y. Willi, T. N. Kristensen, and A. A. Hoffmann. 2008. Population bottlenecks increase additive genetic variance but do not break a selection limit in rain forest *Drosophila*. Genetics 179:2135–2146.

Van Oosterhout, C., W. G. Zijlstra, M. K. Van Heuven, and P. M. Brakefield. 2000. Inbreeding depression and genetic load in laboratory metapopulations of the butterfly *Bicyclus anynana*. Evolution 54:218–225.

Vandergast, A. G., A. J. Bohonak, D. B. Weissman, and R. N. Fisher. 2007. Understanding the genetic effects of recent habitat fragmentation in the context of evolutionary history: phylogeography and landscape genetics of a southern California endemic Jerusalem cricket (Orthoptera: Stenopelmatidae: Stenopelmatus). Mol. Ecol. 16:977–992.

Van't Hof, A. E., B. J. Zwaan, I. J. Saccheri, D. Daly, A. N. M. Bot, and P. M. Brakefield. 2005. Characterization of 28 microsatellite loci for the butterfly *Bicyclus anynana*. Mol. Ecol. Notes 5:169–172.

Vermeulen, C. J., R. Bijlsma, and V. Loeschcke. 2008. QTL mapping of inbreeding-related cold sensitivity and conditional lethality in *Drosophila melanogaster*. J. Evol. Biol. 21:1236–1244.

Wade, M. J., S. M. Shuster, and L. Stevens. 1996. Inbreeding: its effect on response to selection for pupal weight and the heritable variance in fitness in the flour beetle, *Tribolium castaneum*. Evolution 50: 723–733.

Wang, J. 2000. Effects of population structures and selection strategies on purging of inbreeding depression due to deleterious mutations. Genet. Res. 76:75–86.

Watling, J. I., and M. A. Donnelly. 2006. Fragments as islands: a synthesis of faunal responses to habitat patchiness. Conserv. Biol. 20:1016–1025.

Watson, M. J. O., and A. A. Hoffmann. 1996. Acclimation, cross-generation effects, and the response to selection for increased cold resistance in *Drosophila*. Evolution 50:1182–1192.

Willi, Y., J. V. Buskirk, and A. A. Hoffmann. 2006. Limits to the adaptive potential of small populations. Annu. Rev. Ecol. Evol. Syst. 37:433–458.

Wright, L. I., T. Tregenza, and D. J. Hosken. 2008. Inbreeding, inbreeding depression and extinction. Conserv. Genet. 9:833–843.

Associate Editor: P. Witkopp

6.3 Does selection on increased cold tolerance in the adult stage confer resistance throughout development?

Anneke Dierks*, Nadine Kölzow, Kristin Franke, and Klaus Fischer

Zoological Institute and Museum, University of Greifswald, J.-S.-Bachstraße 11/12, D-17489 Greifswald, Germany

Running title: Correlated responses to selection across life stages

***Corresponding author**

Anneke Dierks

Zoological Institute and Museum

University of Greifswald

Johann-Sebastian-Bach-Str. 11/12

D-17489 Greifswald, Germany

Tel.: +49-3834-864266

Fax: +49-3834-864252

Email address: anneke.dierks@uni-greifswald.de

Published as:

Dierks, A., Kölzow, N., Franke, K. and Fischer, K. (in press). Does selection on increased cold tolerance in the adult stage confer resistance throughout development? Journal of Evolutionary Biology

Abstract

Artificial selection is a powerful approach to unravel constraints on genetic adaptation. While it has been frequently used to reveal genetic trade-offs among different fitness-related traits, only a few studies have targeted genetic correlations across developmental stages. Here we test whether selection on increased cold tolerance in the adult stage increases cold resistance throughout ontogeny in the butterfly *Bicyclus anynana*. We used lines selected for decreased chill-coma recovery time and according controls, which had originally been set up from three levels of inbreeding (outbred control, one or two full-sib matings). Four generations after having terminated selection, a significant response to selection was found in one day-old butterflies (the age at which selection took place). Older adults showed a very similar though weaker response. Nevertheless, cold resistance did not increase in either egg, larval or pupal stage in the selection lines, but was even lower compared to control lines for eggs and young larvae. These findings suggest a cost of increased adult cold tolerance, presumably reducing resource availability for offspring provisioning and thereby stress tolerance during development, which may substantially affect evolutionary trajectories.

Key words: artificial selection; cold resistance; genetic correlation; inbreeding depression; resource allocation; survival rate

Introduction

At least at evolutionary time scales, all organisms are faced with environmental change such as e.g. climatic alterations, new diseases or new predators (Frankham & Kingsolver 2004; Frankham 2005). Therefore, the ability to respond to such changes is of crucial importance to ensure a species' longer term persistence. This can be either achieved through phenotypic plasticity, i.e. quick environmentally-induced adjustments within a set genotype, or through genetic adaptation involving changes in allele frequencies (Bijlsma & Loeschcke 2005; David *et al.* 2005; Sørensen *et al.* 2005). Though phenotypic plasticity allows for maximal flexibility, it does not seem to be generally favoured by selection, suggesting that plastic responses involve non-trivial costs (DeWitt *et al.* 1998; Relyea 2002; Pigliucci 2005). In contrast, it has been repeatedly suggested that genetic adaptation may come at reduced costs, thus being favoured in stable environments (DeWitt *et al.* 1998; Relyea 2002; Aubret & Shine 2010). This notion, however, has been challenged by some recent findings (Meyer & Di Giulio 2003; Bourguet *et al.* 2004).

Perhaps the most obvious limit to genetic adaptation are negative genetic correlations (caused by antagonistic pleiotropy), in which a beneficial genetic change in one trait is linked to a detrimental change in another (Stearns 1989; Roff 2002). Over recent decades our knowledge on genetic trade-offs has much increased, especially due to the use of artificial selection experiments, representing the most straight-forward approach (e.g. Bell & Koufopanou 1986; Brakefield 2003; Czesak & Fox 2003; Bubliy & Loeschcke 2005; Bertoli *et al.* 2010 Fischer *et al.* 2006; Bauerfeind & Fischer 2007). For instance, artificial selection in *Drosophila melanogaster* revealed a strong trade-off between larval development time and adult weight (Nunney 1996), and between cold tolerance and starvation resistance (Hoffmann *et al.* 2005). In general, there is agreement that such negative genetic correlations among traits related to fitness have the potential to maintain genetic variation within populations, and more importantly to bias or constrain evolutionary change (Cheverud 1984; Roff 2002).

While such genetic trade-offs have been quite frequently measured among different traits (see above), hardly any data is available on potential trade-offs across developmental stages (but e.g. Tucic 1979; Cheverud *et al.* 1983; Loeschcke & Krebs 1996). Throughout ontogeny, though, selection at any age is expected to result in correlated responses in all other life-history stages. Such genetic influences may on principle yield (1) consistent effects throughout development, (2) divergent patterns across developmental stages, or (3) phenotypic effects in one or two consecutive ages only (Cheverud *et al.* 1983).

Against this background, we here investigate genetic links between the expression of a specific phenotype at different time points in the ontogeny of the butterfly *Bicyclus anynana*. To this end we used lines artificially selected for an ecologically important trait (Kristensen *et al.* 2011), increased cold tolerance in the adult stage and according control lines (Dierks *et al.* 2012a, 2012b). We predict that increased cold tolerance should prevail throughout development (i.e. also in the egg, larval, and pupal stage; cf. Tucic 1979). This prediction rests on the straight-forward assumption of genetically positively correlated gene expression, which should be beneficial throughout ontogeny if cold tolerance is selected for in the adult stage. Note in this context that at least some studies indicate that the costs involved in thermal adaptation may be minor (Anderson *et al.* 2005; Bertoli *et al.* 2010; Dierks *et al*. 2012b). On the other hand, thermal sensitivity and tolerance may change during ontogeny, as morphology and physiology will undergo fundamental changes (Chen *et al.* 1987; Block *et al.* 1990; Tsutsayeva & Sevryukova 2001; Jensen *et al.* 2007). For instance, Krebs and Loeschcke (1997) found that pupae of *Drosophila buzzatii* were most heat-resistant, followed by eggs, first- and finally third-instar larvae. Furthermore, using selection for increased heat-shock resistance in adult and larval *Drosophila buzzatii*, Loeschcke & Krebs (1996) found no evidence for correlated responses across developmental stages.

The selection lines used here were initially set up from different levels of inbreeding (F = 0.00 / 0.25 / 0.375), in order to investigate effects of selection regime and inbreeding on the response to selection (Dierks *et al.* 2012b). Although inbreeding effects are beyond the

scope of this paper and although lines went through several generations of outbreeding prior to our current experiments, we nevertheless also report the respective results here for completeness.

Material and Methods

Study organism

We used the butterfly *Bicyclus anynana* Butler, 1879 (Lepidoptera, Nymphalidae), a species that is distributed in the tropical areas of Southern Africa up to Ethiopia (Larsen 1991), for this study. Adults feed on a diversity of fallen and decaying fruits (Larsen 1991; Brakefield 1997). This species exhibits two seasonal morphs as an adaptation to the wet and dry season in its natural environment, and the associated changes in resting background and predation (Brakefield 1997; Lyytinen *et al.* 2004; Bauerfeind & Fischer 2007). Due to its amenability to experimental manipulations *B. anynana* has been used frequently in studies on life-history evolution and developmental plasticity before (e.g. Brakefield & Mazzotta 1995; van Oosterhout *et al*. 2000; Bauerfeind *et al*. 2009; Fischer *et al.* 2010). The origins of our laboratory stock population, which has been founded at Greifswald University, Germany, in 2007, lead back to a stock population at Leiden University, The Netherlands. The latter population has been established in 1988 from over 80 gravid females, collected at Nkatha Bay, Malawi. Since then, several hundred individuals are reared in each generation to avoid inbreeding and to maintain high levels of heterozygosity (Van't Hof *et al.* 2005; Bauerfeind & Fischer 2007).

Inbreeding treatments and selection regime

To investigate effects of selection in the adult stage throughout development, we used in total 12 selection lines, which had been selected for increased cold stress tolerance starting from different levels of inbreeding (cf. Dierks *et al.* 2012a, 2012b; also for further details). First, three levels of inbreeding had been established using a full-sib breeding design: inbreeding 1 (I1) with individuals resulting from matings between full sibs, inbreeding 2 (I2)

resulting from matings between full sibs in two consecutive generations, and outbred controls (C) resulting from random mating. To initiate artificial selection, butterflies within inbreeding levels were pooled across families (120 each). Per inbreeding level, four groups were set up, two for selection on increased cold tolerance, and two as unselected controls , resulting in a total of 12 lines (3 inbreeding levels x 4). Per generation and line 40 males and 40 females were selected to found the next generation, being either the most cold-tolerant ones or being selected at random (Control lines). Selection was applied to chill-coma recovery time, i.e. the time needed to regain mobility following cold exposure, on day 1 following adult eclosion. This trait is considered a reliable proxy of climatic cold adaptation, and has been used successfully in *B. anynana* before (e.g. Geister & Fischer 2007; Fischer *et al*. 2010). Selection was continued for 10 generations, yielding highly divergent phenotypes with the lines selected for increased cold tolerance showing a by 28 % shorter chill-coma recovery time compared to unselected controls (Dierks *et al*. 2012b). Lines had been kept without selection under standard rearing conditions (see below) for 4 generations prior to this experiment. Several hundred butterflies were reared per line in each generation.

General rearing

Unless otherwise stated, butterflies were reared in a climate room at 27°C, high relative humidity (70%), and a photoperiod of L12:D12 (24-h light cycle) throughout. Larvae were reared in population cages (50 x 50 x 50 cm) and fed on young maize plants. Adults were kept in cylindrical hanging cages (30 x 38 cm) and provided with banana and moist cotton wool. Cages were arranged in a randomized block design within the climate cabinet in order to balance potential slight temperature and humidity variation (Bauerfeind & Fischer 2007; Fischer *et al*. 2010; Dierks *et al.* 2012a).

Experimental design

The effects of (inbreeding and) selection on increased cold tolerance in the adult stage was tested in various developmental stages, specifically in (1) eggs (tested one day after oviposition), (2) second and fourth instar larvae, (3) one and five day-old pupae, and (4) one

(control) and seven day-old adults. As proxies of cold tolerance we used chill-coma recovery time for adults, and survival rates for the other developmental stages (see further below). Adequate cold stress conditions were selected based on pilot experiments on each developmental stage (data not shown), aiming at survival rates of ca. 50 %. Throughout, each individual was tested only once. Eggs for the experiments outlined below were collected from 100 males and 100 females per line, which were allowed to mate randomly.

Experiment 1: Egg stage. Two-hundred eggs per line were collected, being randomly distributed over 10 replicate petri dishes (20 eggs each) containing moistened filter paper. The petri dishes were exposed to -1°C for 90 minutes on the day following oviposition, and were afterwards returned to 27°C. Unsuccessful (dead) eggs were counted six days after the cold shocks had been performed (note that egg development is ca. 4 days at 27°C). Throughout petri dishes containing eggs from the different lines were arranged in a randomized block design.

Experiment 2: Larval stage. For measuring survival rates of larvae, second and fourth instar larvae were placed individually in translucent plastic cups (125 ml), and were exposed for 9 hours to -5°C in a climate cabinet (Sanyo MIR-553). Twenty-four hours after their return to 27°C, during which larvae had access to a fresh leaf of maize for feeding, surviving larvae were counted. 60 second and 50 fourth instar larvae were tested per line.

Experiment 3: Pupal stage. One or five days after pupation, pupae were individually placed in translucent plastic cups (125 ml), and were exposed for 2 hours to -3.5°C in a climate cabinet (Sanyo MIR-553). After their return to 27°C, cups were checked daily for eclosed butterflies. We scored the number of successfully eclosed individuals. We tested 28-39 male and 31-37 female one day-old pupae, and 30-48 male and 30-48 female five day-old pupae per line.

Experiment 4: Adult stage. We used chill-coma recovery time for measuring cold tolerance of butterflies. This method produces highly repeatable results, has been used successfully in *B. anynana* before, and is considered a reliable proxy of climatic cold adaptation (e.g. Geister &

Fischer 2007; Fischer *et al.* 2010). Unmated one and seven day-old butterflies were placed individually in translucent plastic cups (125 ml) and arranged on a tray in a randomized block design (maximum of 72 butterflies per block). We exposed them for 19 hours to 1°C in a climate cabinet (Sanyo MIR-553) to induce a chill coma. Afterwards they were transferred to a room with a constant temperature of 20°C to determine recovery times. Recovery time was defined as the time elapsed between taking the trays out of the 1°C climate cabinet until a butterfly was able to stand on its legs (Geister & Fischer 2007; Fischer *et al*. 2010). Observation time was restricted to a maximum of 60 minutes. Butterflies, which did not recover within this time span, were given the maximum recovery time of 60 minutes (if still alive) or were excluded from further analyses (if dead; typically < 1%). We tested 30-45 males and 30-40 females per line and treatment.

Statistical analyses

Survival rates of eggs, larvae and pupae were analyzed using nominal logistic regressions on binary data (dead or alive). Selection regime, inbreeding level, and sex (only for pupae) were used as fixed factors, and replicate (nested within selection regime and inbreeding level) as random factor. Similar general linear models were applied to test for differences in chill-coma recovery time in the adult stage. Significant differences between groups were located with the Tukey HSD posthoc test. Throughout, means are given ± 1 SE. All statistical tests were performed by using JMP (4.0.0) or Statistica (6.1).

Results

Survival rates

Selection regime significantly affected survival rates of eggs, and tended to affect survival rates of young larvae, but did not influence survival rates of pupae or older larvae (Table 1; Fig. 1). Contrary to expectations, though, survival rates after cold exposure of eggs (Control lines: 57.3 ± 2.1 % > Cold-tolerant lines: 43.7 ± 2.3 %) and young larvae (Control lines: 58.6 ± 2.0 %; Cold-tolerant lines: 53.9 ± 4.4 %) were higher in the control lines than in the cold-tolerant lines. Inbreeding level significantly affected survival rates of eggs, young larvae and young pupae, but not of old larvae and old pupae. Egg survival rate was highest in the I1 followed by the I2 and finally the C treatment (I1: 56.9 ± 2.4 %; I2: 53.9 ± 2.8 %; C: 40.8 ± 2.9 %). Like in eggs, young larvae survival was highest in the I1 followed by the I2 and finally the C treatment (I1: 59.6 ± 2.8%; I2: 57.5 ± 2.6%; C: 51.7 ± 6.3%). Pupal survival was also highest in the I1, followed by the C and finally the I2 treatment (I1: 76.0 ± 4.4 %; C: 71.0 ± 3.0 %; I2: 59.4 ± 4.5 %). Variation between replicate lines was significant in eggs, 2nd instar larvae, 4th instar larvae and five day-old pupae. The significant interaction between selection regime and inbreeding level for 4th instar larvae reflects that negative effects of selection were confined to the I1 and I2 treatments, while an opposite pattern was found in the outbred controls (Fig. 1c). Additionally, a significant interaction between inbreeding level and sex for one day-old pupae indicates that survival rates of males compared to females were higher in the I1 and I2, but lower in C treatment (I1: 82.6 % > 68.9 %; I2: 63.4 % > 55.3 %; C: 67.9 % < 74.2 %).

Table 1: Nominal logistic regressions for the effects of selection regime and inbreeding level on survival rates after cold exposure in *Bicyclus anynana*. Throughout, replicate line (random factor) was nested within selection regime and inbreeding level. Sex was added as factor in the analyses of pupal survival rates only (note that sex determination of eggs and larvae was not possible). Significant *P*-values are given in bold.

Developmental stage	**Factor**	***df***	***Chi²***	***P***
Eggs	Selection regime	1	26.35	**<0.0001**
	Inbreeding level	2	41.36	**<0.0001**
	Replicate [Sel., Inbr.]	6	12.84	**0.0457**
	Sel. x Inbr.	2	5.47	0.0649
Larvae (2nd instar)	Selection regime	1	3.65	0.0561
	Inbreeding level	2	9.91	**0.0070**
	Replicate [Sel., Inbr.]	6	12.70	**0.0481**
	Sel. x Inbr.	2	3.89	0.1429
Larvae (4th instar)	Selection regime	1	1.88	0.1705
	Inbreeding level	2	0.58	0.7502
	Replicate [Sel., Inbr.]	6	15.91	**0.0142**
	Sel. x Inbr.	2	10.23	**0.0060**
Pupae (day 1)	Selection regime	1	0.01	0.9452
	Inbreeding level	2	7.68	**0.0215**
	Sex	1	3.21	0.0734
	Replicate [Sel., Inbr.]	6	11.84	0.0656
	Sel. x Inbr.	2	4.47	0.1077
	Sel. x Sex	1	0.00	0.9820
	Inbr. x Sex	2	8.61	**0.0135**
	Sel. x Inbr. x Sex	2	2.08	0.3534
Pupae (day 5)	Selection regime	1	0.14	0.7122
	Inbreeding level	2	4.68	0.0963
	Sex	1	0.37	0.5419
	Replicate [Sel., Inbr.]	6	24.59	**0.0004**
	Sel. x Inbr.	2	2.46	0.2930
	Sel. x Sex	1	0.22	0.6414
	Inbr. x Sex	2	3.73	0.1553
	Sel. x Inbr. x Sex	2	2.71	0.2580

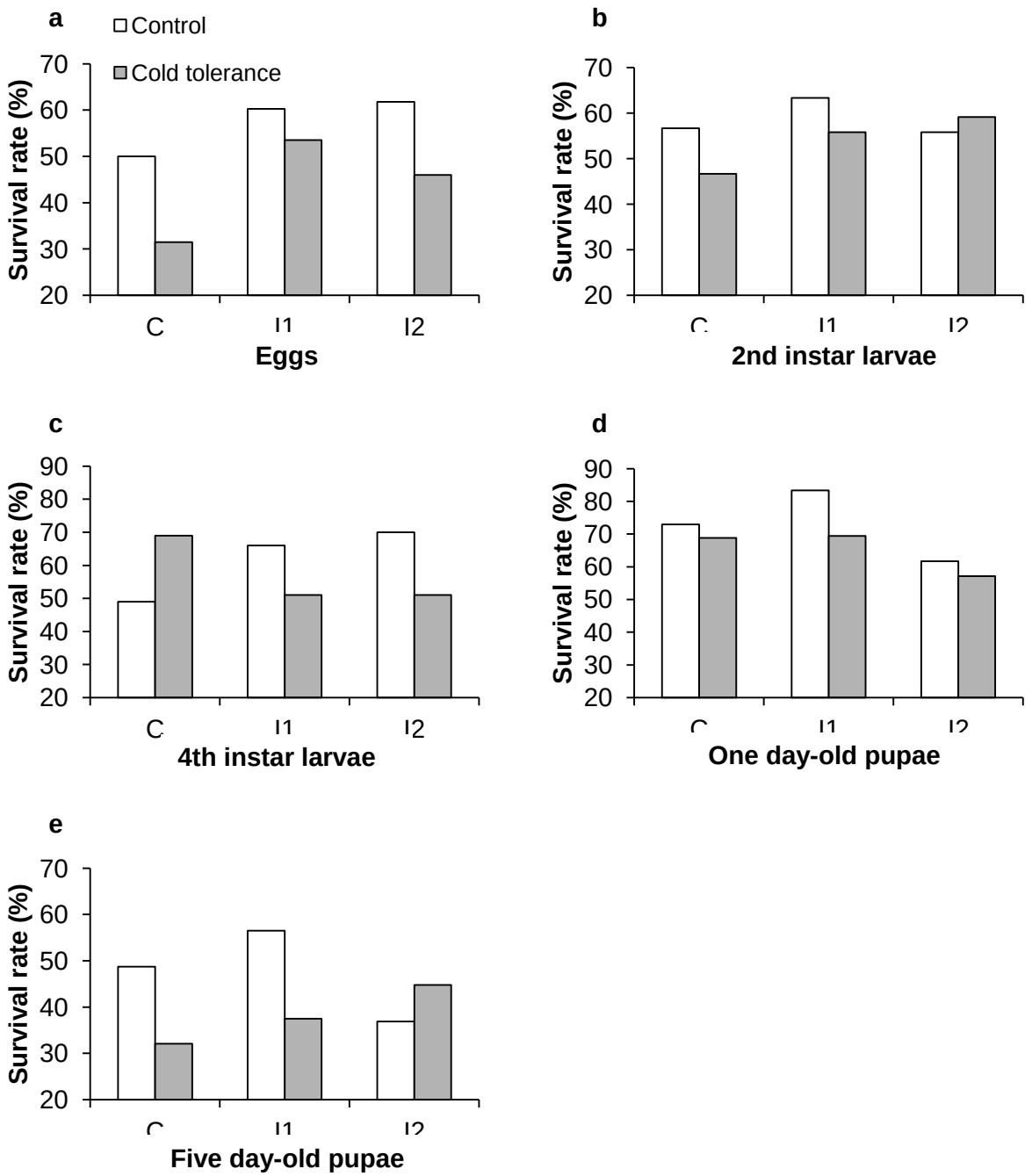

Fig. 1: Survival rates of eggs (a), 2nd instar larvae (b), 4th instar larvae (c), one day-old pupae (d), and five day-old pupae (e) in *Bicyclus anynana* in relation to inbreeding level (C: outbred control; I1: one full-sib mating; I2: two full-sib matings) and selection regime (CT: lines selected for increased cold tolerance; UC: unselected control lines).

Chill-coma recovery time

After 4 generations without selection, chill-coma recovery time was significantly shorter in one day-old cold-tolerant than in one day-old control butterflies (by 15.7 %; 1152 ± 24 s < 1366 ± 25 s; Table 2). Despite similar effect size (recovery time by 15.0 % shorter in cold-tolerant lines than in control lines), there was no significant effect of selection regime in seven day-old butterflies (1864 ± 42 s = 2191 ± 40 s, note the increase in SEs; Fig. 2). The latter pattern is at least partly caused by males, in which cold-tolerant and control individuals had similar recovery times (Cold-tolerant lines 1857 ± 65.1 s = Control lines 2042 ± 51.3 s), whilst the difference was still significant in females (Cold-tolerant lines 1870 ± 55.0 s < Control lines 2336 ± 58.6 s; Tukey HSD after ANOVA; significant selection regime by sex interaction). For one day-old butterflies, the significant inbreeding treatment by sex interaction indicates that recovery times tended to decrease with increasing inbreeding level in males, but to increase in females (males: C: 1291 ± 44.5 s, I1: 1251 ± 48.2 s, I2: 1181 ± 46.6 s; females: C: 1161 ± 46.3 s, I1: 1209 ± 49.5 s, I2: 1308 ± 48.9 s).

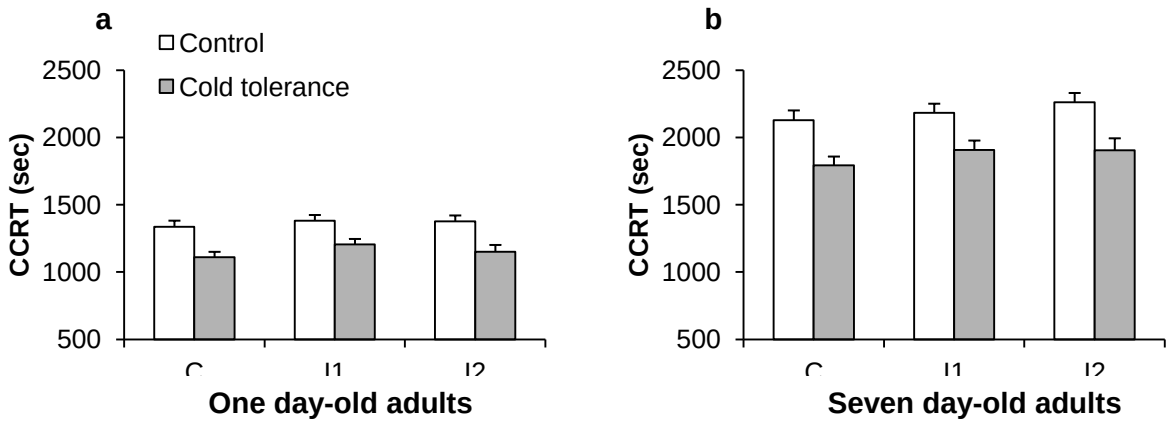

Fig. 2: Means (+ 1 SE) for chill-coma recovery time (CCRT) in one (a) and seven day-old (b) *Bicyclus anynana* butterflies in relation to inbreeding level (C: outbred control; I1: one full-sib mating; I2: two full-sib matings) and selection regime.

Table 2: Nested analyses of variance for the effects of selection regime (fixed), inbreeding level (fixed), sex (fixed), and replicate line (random) on cold stress resistance in one and seven day-old *Bicyclus anynana* butterflies. Throughout, replicate line was nested within selection regime and inbreeding level. Significant *P*-values are given in bold.

Age	Factor	*MS*	*df*	*F*	*P*
One day-old	Selection regime	49354.4	1,6	20.55	**0.0037**
	Inbreeding level	319.5	2,6	0.13	0.8780
	Replicate [Sel., Inbr.]	2418.9	6,889	1.52	0.1688
	Sex	323.9	1,889	0.20	0.6522
	Sel. x Inbr.	1694.6	2,6	0.71	0.5301
	Sel. x Sex	108.6	1,889	0.07	0.7941
	Inbr. x Sex	9231.8	2,889	5.80	**0.0032**
	Sel. x Inbr. x Sex	683.2	2,889	0.43	0.6513
	Error	1593.0	889		
Seven day-old	Selection regime	5401.3	1,6	2.50	0.1648
	Inbreeding level	1043.5	2,6	0.48	0.6391
	Replicate [Sel., Inbr.]	2161.6	6,753	1.59	0.1459
	Sex	1339.5	1,753	0.99	0.3206
	Sel. x Inbr.	89.7	2,6	0.04	0.9596
	Sel. x Sex	9066.4	1,753	6.69	**0.0099**
	Inbr. x Sex	2783.6	2,753	2.05	0.1291
	Sel. x Inbr. x Sex	541.2	2,753	0.40	0.6710
	Error	1356.0	753		

Discussion

Response to selection on increased cold tolerance in the adult stage

In line with studies using *Drosophila* (e.g. Anderson *et al.* 2005; Mori & Kimura 2008; Bertoli *et al.* 2010; Udaka *et al*. 2010), artificial selection on increased cold tolerance yielded a significant response in *B. anynana* (Dierks *et al.* 2012b). When measured directly after the course of selection, however, chill-coma recovery time was by 28.9% shorter in the selection as compared to the control lines (Dierks *et al.* 2012b), while the difference was only 15.7% in

the current study undertaken four generations later (as measured in one day-old butterflies, the age at which selection was carried out). This rather rapid convergence towards control line levels indicates that the selected genes and alleles had not yet become fixed in the selection lines, and furthermore (for obvious reasons) a lack of a selective pressure maintaining increased cold tolerance under laboratory conditions. Perhaps, there was even selection against increased cold tolerance, which would be expected if significant costs were involved (Marshall & Sinclair 2010; Duncan *et al*. 2011; Stoks & De Block 2011). However, a previous study could not detect any such costs in an array of traits (Dierks *et al*. 2012b).

In seven as compared to one day-old butterflies, a similar pattern was observed (by 15.0% faster recovery in the selection lines), though the difference between selection and control lines was here significant in females only. The lack of a significant main effect of selection regime thus seems to be caused by a differential response across the sexes in combination with an increase in variance and the low statistical power of ANOVAs involving random factors (Charmantier *et al.* 2006; Descamps *et al*. 2008). Note in this context the striking effect of age, with chill-coma recovery time being 61% longer in seven day-old than in one day-old butterflies. As individual performance is expected to decrease with age because of senescence, i.e. a decline in physiological functions (Descamps *et al*. 2008), this effect was expected. Under laboratory conditions, *B. anynana* typically lives and reproduces for 3-4 weeks (Ferkau and Fischer 2006, Fischer 2007), but survival in the field can be expected to be much shorter, averaging at around 10 days in many butterfly species (Brakefield and Reitsma 1991; Fischer and Fiedler 2001). Therefore, a difference in 6 days is considered to be ecologically highly relevant. Previous studies on effects of age on chill coma recovery times though yielded contradictory results, thus challenging the notion of a linear correlation between age and cold tolerance (Fischer *et al*. 2010; Lalouette *et al*. 2010). In summary, artificial selection applied in the adult stage clearly increased cold tolerance in this stage. However, does this also confer increased cold tolerance in other developmental stages?

Effects on cold tolerance during development

In our study, we found no evidence for increased cold tolerance in either the egg, larval or pupal stage as a consequence of selection on shorter chill-coma recovery time in the adult stage. Note that we have necessarily measured different traits, namely cold stress survival during egg, larval, and pupal development, and chill-coma recovery time in the adult stage. However, both traits are clearly related, for which there is direct evidence in *B. anynana* (Fischer *et al*. 2010). While positive effects were thus lacking throughout, increased cold tolerance in the adult stage was even associated with reduced cold stress survival for eggs, with according tendencies being found in young larvae (except in the I2 treatment), and furthermore in all other developmental stages investigated (though not in all groups). Similarly, Watson & Hoffmann (1996) found a negative effect of selection for increased cold tolerance in *Drosophila*, with selected lines having a lower fecundity compared to control lines. In our study, increased cold tolerance in the adult stage seems to come at a cost in terms of a reduced cold tolerance during development.

In another study using *Drosophila*, in contrast, positive correlated responses to selection on increased cold tolerance were found, with an increase in cold tolerance across developmental stages after 52 generations of selection (Tucic 1979). Note though that in this study selection was applied to all stages (eggs, larvae, pupae, adults) using pre-defined survival rates. Effects were strongest in the adult stage and moreover more pronounced in the stages closer to the ones in which selection took place (Tucic 1979). Against this background our results seem counter-intuitive, which might be caused by: (1) the involvement of different mechanisms in cold stress survival versus chill-coma recovery time, although both traits are related (Anderson *et al*. 2005; Hoffmann *et al*. 2002; Fischer *et al*. 2010); (2) the involvement of different mechanisms facilitating cold tolerance in the adult stage and during development (note that Gilchrist et al. 1997 revealed clear effects of thermal experimental evolution in the adult but not in the egg stage); (3) the presence of

resource-allocation trade-offs with an enhanced investment into adult cold tolerance leaving less resources available for offspring provisioning.

Distinguishing between these possibilities will be an important task for future research. However, based on the (at least partly) negative effects of increased adult cold tolerance on offspring performance during development, being most pronounced in early developmental stages, we favor for the time being a resource-based explanation. A wealth of studies indicates that allocation trade-offs, with energy-demanding functions competing for limited energy resources, play an important role in life-history evolution (Stearns 1989, 1992; Roff 2002, 2007; Roff & Fairbairn 2007). Consequently, resources allocated to increased cold tolerance in the adult stage may not be available anymore for offspring provisioning, which may in turn diminish subsequent offspring performance. Such effects should be most pronounced in early developmental stages, which is indeed the case here.

Effects of inbreeding

As mentioned above, results on inbreeding are included here because of the initial set up of the selection experiment, which had included effects of inbreeding on the response to selection (Dierks *et al.* 2012b). After 14 generations of random mating, we found significant differences between inbreeding groups in the survival rates of eggs, 2nd instar larvae and one day-old pupae. While in pupae survival rates were significantly lower in the I2 compared to the other treatments, egg and 2nd instar larvae survival was lower in the control compared to the inbred groups. Whether these differences reflect effects of initial inbreeding though is questionable, given the rather long period of random mating which should on principle set off any inbreeding effects (see also Phillips et al. 2001). Therefore, it seems more likely that the above differences result from random genetic drift within lines.

Note in this context that inbreeding yielded pronounced negative effects on e.g. egg hatching success immediately after full-sib matings (Dierks *et al*. 2012a), but that such effects were not detectable anymore after the course of selection (Dierks *et al.* 2012b). Additionally non-

additive effects may play a major role in inbreeding depression of *B. anynana* (Dierks *et al.* 2012b).

Conclusions

Our study shows that, despite a significant response to selection on increased cold tolerance in the adult stage, there was no correlated positive response in other developmental stages. In contrast, increased adult cold tolerance tended to decrease cold tolerance during development, especially so during early development. This finding suggests a cost of increased adult cold tolerance, presumably reducing resource availability for offspring provisioning and stress tolerance in other developmental stages. Our results thus highlight that increased performance in a specific trait may not only be traded off against other energy-demanding functions, but also against performance throughout ontogeny, which may substantially affect optimal responses to selection pressures (Marshall & Sinclair 2011; Stoks & De Block 2011).

Acknowledgments

We thank Christin Michalowsky for help with the experiment.

References

Anderson, A.R., Hoffmann, A.A. & McKechnie, S.W. 2005. Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: physiology and life-history traits. *Genet. Res.* 85: 15-22.

Aubret, F. & Shine, R. 2010. Fitness costs may explain the post-colonisation erosion of phenotypic plasticity. *J. Exp. Biol.* 213: 735-739.

Bauerfeind, S.S. & Fischer, K. 2007. Maternal body size as an evolutionary constraint on egg size in a butterfly. *Evolution* 61: 2374-2385.

Bauerfeind, S.S., Perlick, J.E.C. & Fischer, K. 2009. Disentangling environmental effects on adult life span in a butterfly across the metamorphic boundary. *Exp. Gerontol.* 44: 805-811.

Bell, G. & Koufopanou, V. 1986. The cost of reproduction. Oxford University Press, Oxford.

Bertoli, C.I., Scannapieco, A.C., Sambucetti, P. & Norry, F.M. 2010. Direct and correlated responses to chill-coma recovery selection in *Drosophila buzzatii*. *Entomol. Exp. Appl.* 134: 154-159.

Bijlsma, R.R. & Loeschcke, V. 2005. Environmental stress, adaptation and evolution: an overview. *J. Evol. Biol.* 18: 744-749.

Block, W., Erzinclioglu, Y.Z. & Worland, M.R. 1990. Cold resistance in all life stages of two blowfly species (Diptera, Calliphoridae). *Med. Vet. Entomol.* 4: 213-219.

Bourguet, D., Guillemaud, T., Chevillon, C. & Raymond, M. 2004. Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. *Evolution* 58: 128-135.

Brakefield, P.M. 1997. Phenotypic plasticity and fluctuating asymmetry as responses to environmental stress in the butterfly *Bicyclus anynana*. In*: Environmental stress: adaptation and evolution* (R.R. Bijlsma & V. Loeschke, eds), pp. 65–78. Birkhäuser, Basel.

Brakefield, P.M. 2003. Artificial selection and the development of ecologically relevant phenotypes. *Ecology* 84: 1661-1671.

Brakefield, P.M. & Mazzotta, V. 1995. Matching field and laboratory environments: effects of neglecting daily temperature variation on insect reaction norms. *J. Evol. Biol.* 8: 559-573.

Bubliy, O.A. & Loeschcke, V. 2005. Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. *J. Evol. Biol.* 18: 789-803.

Charmantier, A., Perrins, C., McCleery, R. & Sheldon, B.C. 2006. Age-dependent genetic variance in a life-history trait in the mute swan. *Proc. R. Soc. B* 273: 225-232.

Chen, C.-P., Denlinger, D.L. & Lee, R.E. 1987. Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Physiol. Zool.* 60: 297-304.

Cheverud, J.M. 1984. Quantitative genetics and developmental constraints on evolution by selection. *J. Theor. Biol.* 110: 155-171.

Cheverud, J.M., Rutledge, J.J. & Atchley, W.R. 1993. Quantitative genetics of development - genetic correlations among age-specific trait values and the evolution of ontogeny. *Evolution* 37: 895-905.

Czesak, M.E. & Fox, C.W. 2003. Evolutionary ecology of egg size and number in a seed beetle: genetic trade-off differs between environments. *Evolution* 57: 1121-1132.

David, J.R., Araripe, L.O., Chakir, M., Legout, H., Lemos, B., Petavy, G. *et. al.* 2005. Male sterility at extreme temperatures: a significant but neglected phenomenon for understanding *Drosophila* climatic adaptations. *J. Evol. Biol.* 18: 838-846.

Descamps, S., Boutin, S., Berteaux, D. & Gaillard, J.M. 2008. Age-specific variation in survival, reproductive success and offspring quality in red squirrels: evidence of senescence. *Oikos* 117: 1406-1416.

DeWitt, T.J., Sih, A. & Wilson, D.S. 1998. Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* 13: 77-81.

Dierks, A., Hoffmann, B., Bauerfeind, S.S. & Fischer, K. 2012a. Effects of inbreeding on life history and thermal performance in the tropical butterfly *Bicyclus anynana. Popul. Ecol*. 54: 83-90.

Dierks, A., Baumann, B. & Fischer, K. 2012b. Response to selection on cold tolerance is constrained by inbreeding. Evolution. DOI: 10.1111/j.1558-5646.2012.01604.x.

Duncan, A.B., Fellous, S. & Kaltz, O. 2011. Reverse evolution: selection against costly resistance in disease-free microcosm populations of *Paramecium caudatum*. *Evolution* 65: 3462-3474.

Fischer, K., Bot, A.N.M., Brakefield, P.M. & Zwaan B.J. 2006. Do mothers producing large offspring have to sacrifice fecundity? *J. Evol. Biol.* 19: 380-391.

Fischer, K., Dierks, A., Franke, K., Geister, T.L., Liszka, M., Winter, S. & Pflicke, C. 2010. Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. *PLoS ONE* 5: e15284.

Frankham, R. 2005. Genetics and extinction. *Biol. Conserv.* 126: 131-140.

Frankham, R. & Kingsolver, J.G. 2004. Response to environmental change: adaptation or extinction. In: *Evolutionary Conservation Biology* (R. Ferrier, U. Dieckman & D. Couvet, eds), pp. 85-100. Cambridge University Press, Cambridge.

Frankham, R., Gilligan, D.M., Morris, D. & Briscoe, D.A. 2001. Inbreeding and extinction: effects of purging. *Conserv. Genet*. 2: 279-284.

Fowler, K. & Whitlock, M.C. 1999. The distribution of phenotypic variance with inbreeding. *Evolution* 53: 1143-1156.

Geister T.L. & Fischer, K. 2007. Testing the beneficial acclimation hypothesis: temperature effects on mating success in a butterfly. *Behav. Ecol*. 18: 658-664.

Gilchrist G.W., Huey R.B. & Partridge, L. 1997. Thermal sensitivity of *Drosophila melanogaster:* evolutionary responses of adults and eggs to laboratory natural selection at different temperatures. *Physiol. Zool.* 70: 403-414.

Hoffmann, A.A., Anderson, A. & Hallas, R. 2002. Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecol. Lett.* 5: 614-618.

Hoffmann, A.A., Hallas, R., Anderson A.R. & Telonis-Scott, M. 2005. Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in *Drosophila melanogaster*. *J. Evol. Biol.* 18: 804-810.

Jensen, D., Overgaard, J. & Sørensen, J.G. 2007. The influence of developmental stage on cold shock resistance and ability to cold-harden in *Drosophila melanogaster*. *J. Insect Physiol.* 53: 179-186.

Krebs R.A. & Loeschcke, V. 1997. Resistance to thermal stress in preadult *Drosophila buzzatii:* variation among populations and changes in relative resistance across life stages. *Biol. J. Linnean Soc.* 56: 517-531.

Kristensen, T.N., Dahlgaard, J. & Loeschcke, V. 2003. Effects of inbreeding and environmental stress on fitness – using *Drosophila buzzatii* as a model organism. *Conserv. Genet*. 4: 453-465.

Kristensen, T.N., Knudsen, M.R. & Loeschcke, V. 2011. Slow inbred lines of *Drosophila melanogaster* express as much inbreeding depression as fast inbred lines under semi-natural conditions. *Genetica* 139: 441-451.

Lalouette, L., Vernon, P., Amat, H. & Renault, D. 2010 Ageing and thermal performance in the sub-Antarctic wingless fly *Anatalanta aptera* (Diptera: Shaeoceridae): older is better. *Biol. Lett.* 6: 346-349.

Larsen, T.B. 1991. The butterflies of Kenya. Oxford Univ. Press, Oxford.

Loeschcke V. & Krebs, R.A. 1996. Selection for heat-shock resistance in larval and adult *Drosophila buzzatii*: comparing direct and indirect responses on viability and development. *Evolution* 50: 2354-2359.

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

Marshall, K.E. & Sinclair, B.J. 2010. Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster. Proc. R. Soc. B* 277: 963-969.

Meyer, J.N. & Di Giuliuo, R.T. 2003. Heritable adaptation and fitness costs in killifish (*Fundulus beteroclitus*) inhabiting a polluted estuary. *Ecol. Appl.* 13: 490-503.

Mikkelsen, K., Loeschcke, V. & Kristensen, T.N. 2010. Trait specific consequences of fast and slow inbreeding: lessons from captive populations of *Drosophila melanogaster*. *Conserv. Genet.* 11: 497-488.

Mori, N. & Kimura, M.T. 2008. Selection for rapid and slow recovery from chill- and heat-coma in *Drosophila melanogaster*. *Biol. J. Linn. Soc.* 95: 72-80.

Nunney, L. 1996. The response to selection for fast larval development in *Drosophila melanogaster* and its effect on adult weight: an example of a fitness trade-off. *Evolution* 50: 1193-1204.

Pedersen, K.S., Kristensen, T.N. & Loeschcke, V. 2005. Effects of inbreeding and rate of inbreeding in *Drosophila melanogaster* – Hsp70 expression and fitness. *J. Evol. Biol.* 18: 756-762.

Pigliucci, M. 2005. Evolution of phenotypic plasticity: where are we going now? *Trends Ecol. Evol.* 20: 481-486.

Reed, D.H., Briscoe D.A. & Frankham, R. 2002. Inbreeding and extinction: the effect of environmental stress and lineage. *Conserv. Genet.* 3: 301-307.

Relyea, R.A. 2002. Costs of phenotypic plasticity. *Am. Nat.* 159: 272-282.

Roff, D.A. 2002. Life history evolution. Sinauer Associates, Sunderland, Massachusetts.

Roff, D.A. 2007. Contributions of genomics to life-history theory. *Genetics* 8: 116-125.

Roff, D.A. & Fairbairn D.J. 2007. The evolution of trade-offs: where are we? *J. Evol. Biol.* 20: 433-447.

Saccheri, I.J., Brakefield, P.M. & Nichols, R.A. 1996. Severe inbreeding depression and rapid fitness rebound in the butterfly *Bicyclus anynana* (Satyridae). *Evolution* 50: 2000-2013.

Sørensen, J.G., Norry, F.M., Scannapieco, A.S. & Loeschcke, V. 2005. Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. *J. Evol. Biol.* 18: 829-837.

Stearns S.C. 1989. Trade-offs in life-history evolution. *Funct. Ecol.* 3: 259-268.

Stearns, S.C. 1992. *The evolution of life histories.* Oxford University Press, New York.

Stoks, R. & De Block, M. 2011. Rapid growth reduces cold resistance: evidence from latitudinal variation in growth rate, cold resistance and stress proteins. *PLoS ONE* 6: e16935.

Tsutsayeva, A.A. & Sevryukova, L.G. 2001. Effect of cold exposure on survival and stress protein expression of *Drosophila melanogaster* at different development stages. *Cryo Letters* 22: 145-150.

Tucic, N. 1979. Genetic capacity for adaptation to cold resistance at different developmental stages of *Drosophila melanogaster*. *Evolution* 33: 350-358.

Udaka, H., Ueda, C. & Goto, S.G. 2010. Survival rate and expression of heat-shock protein 70 and frost genes after temperature stress in *Drosophila melanogaster* lines that are selected for recovery time from temperature coma. *J. Insect Physiol.* 56: 1889-1894.

Van Oosterhout, C., Zijlstra, W.G., Van Heuven, M.K. & Brakefield, P.M. 2000. Inbreeding depression and genetic load in laboratory metapopulations of the butterfly *Bicyclus anynana*. *Evolution* 54: 218-225.

Van't Hof, A.E., Zwaan, B.J., Saccheri, I.J., Daly, D., Bot A.N.M. & Brakefield, P.M. 2005. Characterization of 28 microsatellite loci for the butterfly *Bicyclus anynana*. *Mol. Ecol. Notes* 5: 169-172.

Watson, M.J.O. & Hoffmann, A.A. 1996. Acclimation, cross-generation effects, and the response to selection for increased cold resistance in *Drosophila*. *Evolution* 50: 1182-1192.

Wright, L.I., Tregenza, T. & Hosken, D.J. 2008. Inbreeding, inbreeding depression and extinction. *Conserv. Genet*. 9: 833-843.

7. Erklärung

7. Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, daß ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Unterschrift des Promovenden