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RESPONSE TO SELECTION ON COLD TOLERANCE IS CONSTRAINED BY INBREEDING

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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

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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

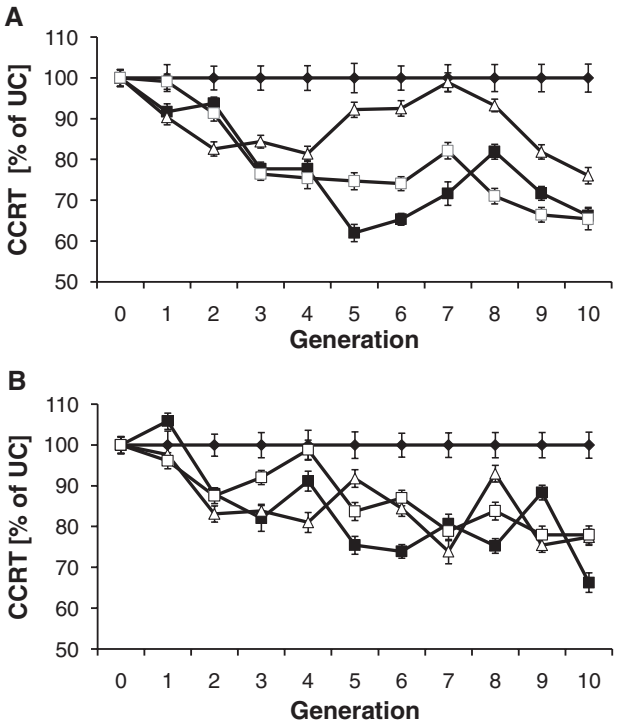

Figure 1. Response to selection on reduced chill-coma recovery time (CCRT) in *Bicyclus anynana* over 10 generations. Note that unselected control lines were set to 100%, and that the data for all other lines are presented relative to the unselected controls. Population means (±SE) are given for each 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**

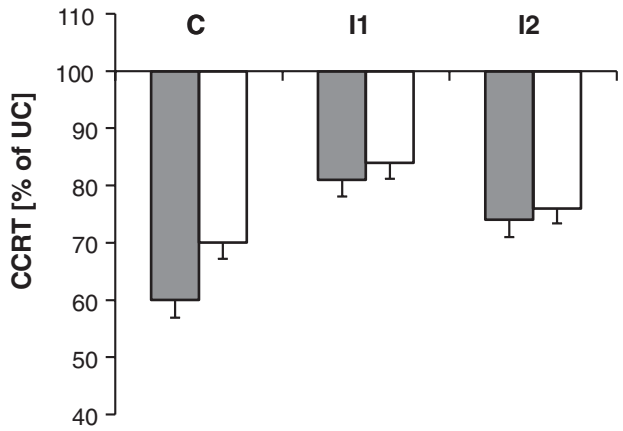

Figure 2. Least square means (−1 SE) for chill-coma recovery time (CCRT, relative to unselected controls set to 100%) in *Bicyclus anynana* across different inbreeding levels (C: control; I1: one full-sibling mating; I2: two full-sibling matings) and replicate lines (gray bars: replicate 1; white bars: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

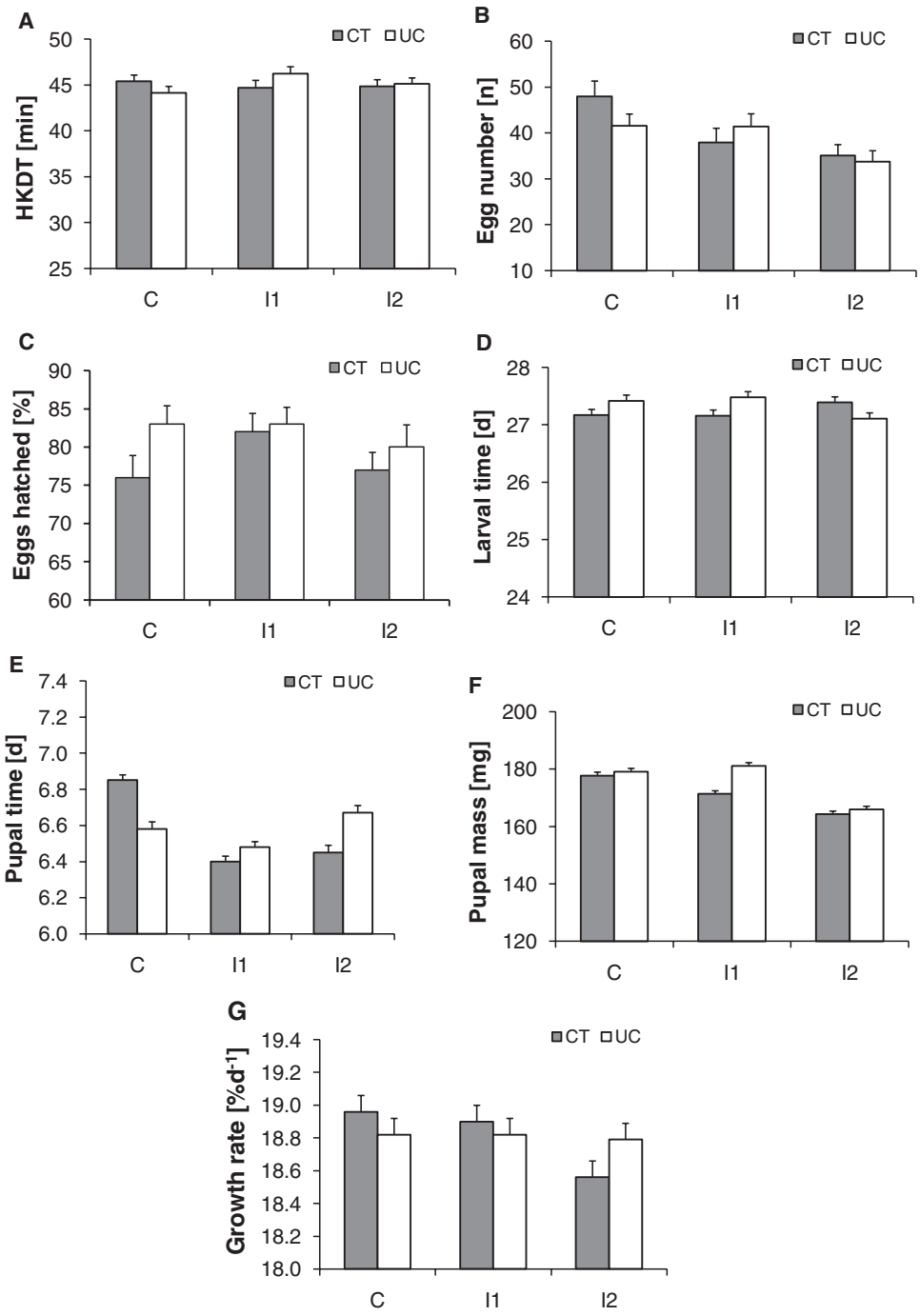

Figure 3. Least square means (+1 SE) for heat knock-down time (HKDT; A), egg number (B), egg hatching success (C), larval time (D), pupal time (E), pupal mass (F), and larval growth rate (G) in relation to inbreeding level (C: outbred control; I1: one full-sibling mating; I2: two full-sibling matings)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

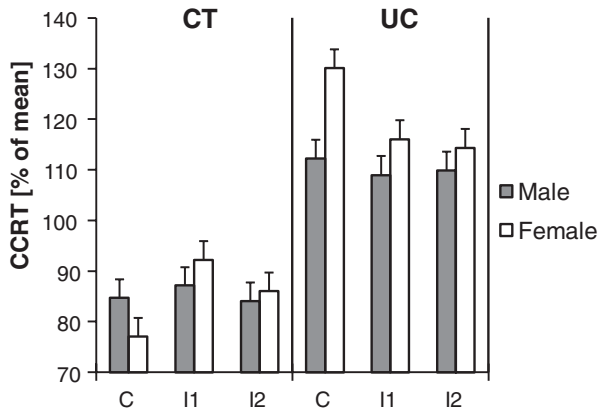

Figure 4. Least square means (+1 SE) for chill-coma recovery times (CCRT) across different inbreeding levels (C: outbred control; I1: one full-sibling mating, I2: two full-sibling matings), sexes, and selection regimes (CT: lines selected for increased cold tolerance; UC: unselected control lines) in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

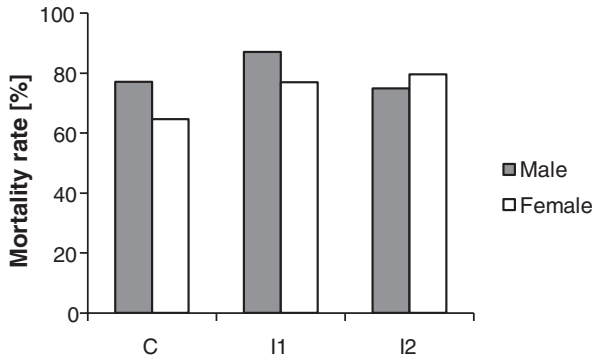

Figure 5. Mortality rates after heat exposure across different inbreeding levels (C: outbred control; I1: one full-sibling mating; I2: two full-sibling mating) and sexes in *Bicyclus anynana*.

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

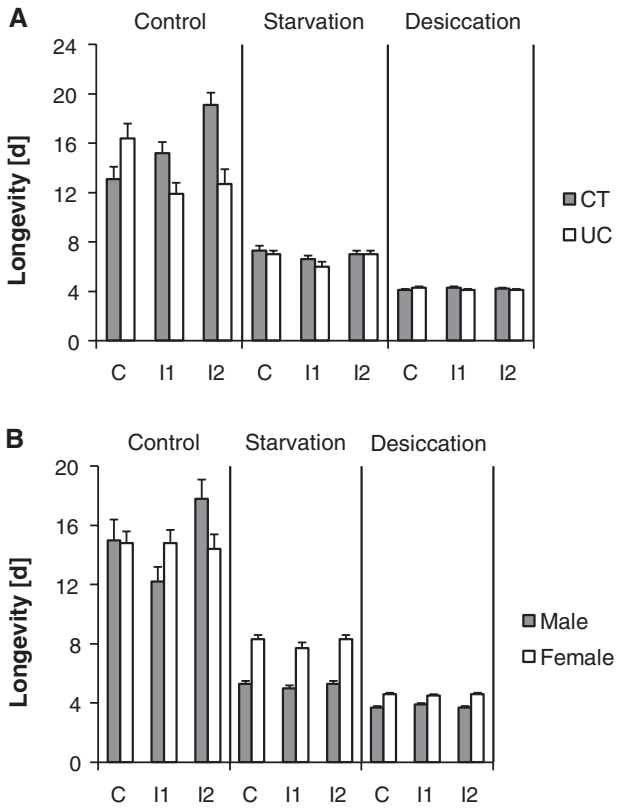

Figure 6. Means (+1 SE) for longevity across selection regimes (CT: lines selected for increased cold tolerance; UC: unselected control lines), feeding regimes (control: banana-fed; starvation: water-fed; desiccation: neither food nor water) and different inbreeding levels (C: control, I1: one full-sibling 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.

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