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RESEARCH ARTICLE

Strong negative effects of simulated heat waves in a tropical butterfly

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ABSTRACT

Climate change poses a significant challenge to all natural systems on Earth. Especially increases in extreme weather events such as heat waves have the potential to strongly affect biodiversity, though their effects are poorly understood because of a lack of empirical data. Therefore, we here explore the sensitivity of a tropical ectotherm, which are in general believed to have a low warming tolerance, to experimentally simulated climate change using ecologically realistic diurnal temperature cycles. Increasing the mean temperature permanently by 3°C had mostly minor effects on developmental traits in the butterfly *Bicyclus anynana*. Simulated heat waves (strongly elevated temperatures for some time though retaining the same overall temperature mean), in contrast, caused strong negative effects by prolonging development time (by up to 10%) and reducing body mass (−21%), especially when combined with reduced relative humidity. Detrimental effects were carried over into the adult stage, diminishing subsequent performance. Most strikingly, higher temperatures suppressed adult immune function (haemocytes: −54%, lysozyme activity: −32%), which may potentially change the way species interact with antagonists. Heat waves thus reduced fitness parameters by 10–25% for development time and body mass and by up to 54% for immune parameters even in this plastic and widespread butterfly, exemplifying the potentially dramatic impact of extreme weather events on biodiversity.

KEY WORDS: Climate warming, Extreme weather event, Global change, Immune function, Tropical ectotherm, Warming tolerance

INTRODUCTION

Climate change is predicted to raise global surface temperature, change the distribution and magnitude of precipitation, and increase extreme weather events such as heat waves (Diffenbaugh et al., 2005; Coumou and Rahmstorf, 2012; Hansen et al., 2012). We here define 'heat wave' according to the World Meteorological Organization (WMO) as a period of more than five consecutive days during which the daily maximum temperature exceeds the average maximum temperature by at least 5°C. Climate change already affects biological systems, driving phenological, range and abundance shifts as well as extinctions (Parmesan and Yohe, 2003; Thomas et al., 2004; Parmesan, 2006; Chown et al., 2010). Although the majority of changes reported thus far are relatively mild, identifying which species are most at risk from climate change is a major scientific challenge in the face of the anticipated future changes in climate (Deutsch et al., 2008; Huey et al., 2009; Chown et al., 2010; Diamond et al., 2012). In this context, increasing frequencies of heat waves may play a pivotal role (Easterling et al., 2000; Angilletta, 2009; Coumou and Rahmstorf, 2012; Sentis et al., 2013). While slightly increased mean temperatures per se are unlikely to exert strong negative effects on biodiversity, the high temperatures associated with heat waves may exceed the physiological tolerances of an array of species, depending on their respective sensitivity to heat (Huey et al., 2009; Chown et al., 2010). This may pose significant challenges as indeed evidence suggests that the frequency and duration of heat waves has changed already (Coumou and Rahmstorf, 2012).

Tropical ectotherms are believed to be particularly sensitive to climate change, because upper thermal limits are relatively constant across species (Deutsch et al., 2008; Huey et al., 2009; Chown et al., 2010; Overgaard et al., 2011; Diamond et al., 2012; Kellermann et al., 2012). As these organisms already live close to their upper thermal limits, they are expected to have a low warming tolerance (Deutsch et al., 2008; Tewksbury et al., 2008; Diamond et al., 2012). However, such generalizations may be complicated by, for example, taxon-specific differences and variation in plastic capacities, and have therefore been challenged, warranting more empirical tests (Calosi et al., 2008; Clusella-Trullas et al., 2011; Kellermann et al., 2012). Providing more experimental data on the impacts of heat waves seems particularly important because of the elusive nature of such events, strongly limiting the possibility of inferring their impact based on field observations.

Against this background we here explore the sensitivity of a tropical ectotherm, the butterfly *Bicyclus anynana* (Butler 1879), to experimentally simulated heat waves using ecologically realistic diurnal temperature cycles. This species is eminently suitable for such a study as it inhabits environments with alternating warm–wet and cool–dry seasons, and shows strong temperature-mediated plasticity in an array of traits such that it might be relatively well equipped to buffer detrimental effects of increasing temperatures (Fischer et al., 2003; Fischer et al., 2010; Franke et al., 2012). This fruit-feeding butterfly is distributed from southern Africa to Ethiopia (Larsen, 1991). *Bicyclus anynana* exhibits two seasonal morphs as an adaptation to its wet–dry seasonal environments and the associated changes in resting background and predation (Lyytinen et al., 2004). During the colder dry season (ca. 18°C, May–November) the species has rather uniform wing patterns and small eyespots, while it exhibits large eyespots and bright bands on both wings in the warmer wet season (ca. 23°C, December–April) (Larsen, 1991; Lyytinen et al., 2004). During the dry season, reproduction ceases and butterflies do not mate before the onset of the next wet season (Lyytinen et al., 2004).

Our target traits include developmental traits (e.g. growth rate, body mass) and an array of physiological adult traits reflecting condition, in order to get a handle on both immediate and longer-term effects of environmental stress (Kleynhans et al., 2014). Assessing the impact of anticipated climate change on associated physiological processes is obviously crucial, but has not yet received

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List of abbreviations

ADH	alcohol dehydrogenase
HSD	honestly significant different
OD	optical density
PBS	phosphate buffered saline
T1–T4	treatment groups 1 to 4
WMO	World Meteorological Organization

much attention (e.g. Chown et al., 2010; Andrew et al., 2013). We therefore include physiological traits such as protein and fat content and immune function (haemocyte numbers, lysozyme activity). Specifically, we explore: (1) the extent to which heat waves, including strongly increased temperatures, are more detrimental than permanently but moderately increased temperatures, despite having the same temperature mean; and (2) whether applying additionally low relative humidity will exaggerate detrimental effects. The latter will further increase realism as in nature heat waves will typically involve low levels of relative humidity (Kleynhans et al., 2014). We used four treatments, including one control treatment (T1) mimicking the conditions currently found at the origin of our stock population. The other three treatments all had a 3°C-increased mean temperature but differed in temperature variance and relative humidity. In contrast to T2 (increase in mean by 3°C only), treatments T3 and T4 both used a change between control conditions and a heat wave, and relative humidity was additionally lowered in T4 (see Table 1 for details). To simulate a heat wave, we used a temperature increment of 6°C for 11 days.

RESULTS

Developmental traits and body mass

All seven developmental traits measured differed significantly across treatment groups, with treatment group T2 having the shortest larval and pupal times (Tables 2, 3). Group T2 also had the highest larval growth rate, though this difference was not significant compared with groups T1 and T3. In contrast to T2, both groups exposed to heat waves (T3, T4) could not benefit to the same extent from their higher mean temperature, as they developed slower than T2 (except for growth rate in T3). Larval time was even longest in group T4 (Fig. 1). Pupal, adult body, thorax and abdomen mass were all highest in control (T1) and lowest in T4 animals, showing very similar overall patterns. All above traits (except pupal time) also differed significantly among sexes (Table 2). Males showed a shorter larval development time (19.6±0.2 days compared with 21.3±0.2 days) facilitated by higher growth rates (0.25±0.01 mg day^{-1} compared with 0.24±0.01 mg day^{-1}) than females, while females reached higher pupal (160.2±1.2 mg compared with 130.1±1.3 mg; Fig. 1), adult (52.4±0.6 mg compared with 35.3±0.6 mg), thorax (18.3±0.3 mg compared with 14.2±0.3 mg) and abdomen masses (23.3±0.5 mg compared with 12.5±0.5 mg). Significant treatment group by sex interactions were present for pupal time and abdomen mass only, indicating that (1) pupal development was longer in males than in females in groups T1 and T2 but vice versa in the groups exposed to a heat wave (T3 and T4; significantly different only in T1, Tukey's HSD after ANOVA; Fig. 1B), and (2) females showed a linear decrease in abdomen mass from T1 to T4 while males showed no significant differences at all (Tukey's HSD after ANOVA; Fig. 1D).

Body composition and immune function

Out of the physiological traits measured, fat content, haemocyte numbers and lysozyme activity differed significantly among treatment groups, while protein and alcohol dehydrogenase (ADH) activity did not (Table 2). Group T1 showed the highest fat content, haemocyte numbers and lysozyme activity (Table 3). Thus, higher temperatures reduced lysozyme activity and fat content. Compared with males, females had significantly higher haemocyte numbers (137.6±8.1 versus 81.3±5.2; Fig. 1), ADH activity (0.78±0.02 versus 0.42±0.02 mean optical density mg^{-1}) and fat content (1.21±0.07 versus 1.16±0.10 mg). Significant treatment group by sex interactions were found for haemocyte number, and lysozyme and ADH activity. Regarding haemocyte number, females (T4<T1=T2=T3) showed a much stronger response to treatments than males, in which no significant reduction was found (Tukey's HSD after ANCOVA; Fig. 1F). Sexes also seemed to respond differently to treatments in lysozyme activity (Fig. 1G), but activity was significantly higher in males than in females in group T3 only (Tukey's HSD after ANCOVA). Finally, ADH activity did not significantly respond to treatments in females, while it was significantly reduced in groups T2 and T4 in males (Tukey's HSD after ANCOVA; Fig. 1H).

DISCUSSION

Our experimental manipulations showed significant effects of thermal treatments on nearly all traits investigated (except for protein content and ADH activity, for which a main effect of treatment group is lacking), although absolute differences in mean temperature were small (3°C). Exclusively increasing the mean temperature speeded up development but decreased body mass, haemocyte numbers, lysozyme activity and fat content (comparison of group T1 versus T2). Applying a 3°C temperature increase as a combination of control and heat wave conditions increased development times to match controls and further reduced body mass despite similar temperature means (group T2 versus T3), indicating negative effects of heat waves. Thus, applying 33°C for 6 h per day over 11 consecutive days seems to have exceeded the optimal temperature range of *B. anynana*, causing stress and therefore

Table 1. Temperature and humidity conditions for the four treatments used

Group	T1	T2	T3		T4	
Mean temperature (°C)	24	27	27		27	
Heat wave	No	No	Non-HW	HW	Non-HW	HW
Time of day (h)	Temperature (°C)					
08:00–14:00	24	27	24	30	24	30
14:00–20:00	27	30	27	33	27	33
20:00–02:00	24	27	24	30	24	30
02:00–08:00	21	24	21	27	21	27
Humidity (%)	80	80	80	80	80	50

For treatment groups T3 and T4, conditions are presented separately for control and heat wave (HW) periods. The respective groups experienced control conditions on days 1–7 (after allocation to treatments; cf. T1), a heat wave on days 8–18, and again control conditions from day 18 onwards.

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Table 2. ANOVA and ANCOVA results for the effects of treatment group and sex on various traits in the butterfly *Bicyclus anynana*

Trait/source	MS	d.f.	*F*	*P*
Larval time				
Group	0.075	3	29.4	**<0.001**
Sex	0.129	1	50.8	**<0.001**
Group × Sex	0.003	3	1.3	0.283
Error	0.003	382		
Growth rate				
Group	0.036	3	45.6	**<0.001**
Sex	0.011	1	13.6	**<0.000**
Group × Sex	0.001	3	1.0	0.378
Error	0.001	382		
Pupal time				
Group	52.8	3	104.8	**<0.001**
Sex	0.6	1	1.2	0.280
Group × Sex	2.4	3	4.8	**0.003**
Error	0.5	382		
Pupal mass				
Group	0.255	3	91.3	**<0.001**
Sex	0.799	1	2864.0	**<0.001**
Group × Sex	0.001	3	0.3	0.833
Error	0.003	382		
Body mass				
Group	2062.1	3	27 .8	**<0.001**
Sex	28,155.1	1	378.9	**<0.001**
Group × Sex	39.0	3	0.5	0.665
Error	74.3	382		
Thorax mass				
Group	6.1×10^{-4}	3	21.2	**<0.001**
Sex	1.1×10^{-3}	1	107.9	**<0.001**
Group × Sex	3.2×10^{-5}	3	1.1	0.355
Error	2.3×10^{-3}	233		
Abdomen mass				
Group	9.4×10^{-4}	3	11.9	**<0.001**
Sex	6.9×10^{-3}	1	264.6	**<0.001**
Group × Sex	3.9×10^{-4}	3	4.9	**0.002**
Error	6.1×10^{-3}	233		
Protein content				
Group	1.4×10^{-5}	3	0.7	0.526
Sex	1.3×10^{-5}	1	2.2	0.143
Group × Sex	3.1×10^{-5}	3	1.6	0.188
Error	1.3×10^{-3}	222		
Abdomen fat content				
Group	0.1×10^{-5}	3	3.8	**0.011**
Sex	2.2×10^{-5}	1	58.1	**<0.001**
Group × Sex	$<0.1\times10^{-5}$	3	0.4	0.755
Lipid-free dry mass	2.8×10^{-5}	1	74.4	**<0.001**
Error	0.1×10^{-5}	129		
Haemocyte number				
Group	4.4	3	10.5	**<0.001**
Sex	4.4	1	10.6	**0.001**
Group × Sex	1.2	3	2.9	**0.035**
Thorax	3.0	1	7.1	**0.008**
Error	0.4	222		
Lysozyme activity				
Group	0.105	3	7.3	**<0.001**
Sex	0.030	1	2.1	0.147
Group × Sex	0.059	3	4.1	**0.007**
Abdomen	0.014	1	1.0	0.325
Error	0.014	185		
Alcohol dehydrogenase				
Group	0.1	3	2.4	0.069
Sex	1.3	1	39.9	**<0.001**
Group × Sex	0.3	3	8.9	**<0.001**
Abdomen	1.1	1	32.7	**<0.001**
Error	0.1	230		

Covariates were added as appropriate. Significant *P*-values are given in bold.

diminished performance. Additionally using reduced relative humidity (group T3 versus T4) exaggerated negative effects on developmental traits and body mass, and additionally diminished immune function.

As higher temperatures generally accelerate ecthotherm growth (Atkinson, 1994; Karl and Fischer, 2008; Angilletta, 2009), the faster growth of group T2 relative to control levels (T1) was expected. However, it is remarkable that both groups exposed to heat waves (T3 and T4) did not benefit from their higher mean temperature. In particular, group T4, additionally exposed to a lower relative humidity, was negatively affected. The poor performance of this group is probably at least partly caused by negative effects of low humidity on food plant quality, which was deliberately enforced here to increase ecological realism. All body mass measures showed a very similar overall pattern, with control animals being heaviest followed by groups T2, T3 and finally T4. While a reduction in body mass at the higher temperature was expected based on the temperature–size rule (Atkinson, 1994; Partridge and French, 1996; Karl and Fischer, 2008), our results additionally show negative effects of heat waves. Obviously, the high temperatures experienced during the heat wave induced substantial stress, which was probably not yet the case at a temperature increase of 3°C. Decreased body mass is expected to diminish individual fitness in *B. anynana*, as body size is typically positively related to fitness in insects, being most obvious in females because of positive correlations between body mass and fecundity (Honek, 1993: Blanckenhorn, 2000) [for *B. anynana* (Bauerfeind and Fischer, 2005)].

Regarding sexual differences, the observed patterns were mainly expected based on (1) protandry selection, favouring early emergence and thus rapid growth in males, and (2) fecundity selection, causing large body size in females (Wiklund and Fagerström, 1977; Honěk, 1993; Fischer and Fiedler, 2000; Karl and Fischer, 2008). Interestingly, female abdomen mass responded in a linear fashion to the treatments imposed, while males showed no response at all. The former may indicate that females reduce their investment into reproduction with increasing stress level, whereas males may not have this option owing to a generally much lower abdomen mass. We have no explanation for pupal time being slightly longer or shorter in males compared with females depending on the specific treatment. However, as differences were generally small and significant in one out of four comparisons only, we believe that this pattern is not of biological relevance.

Regarding adult traits, group T1 showed the highest relative fat content, haemocyte numbers and lysozyme activity, indicating a particularly good performance under control conditions. Higher temperatures, in contrast, substantially reduced haemocyte numbers (see Karl et al., 2011), being particularly low in group T4, lysozyme activity and fat content. These results indicate that the stress imposed by increased temperatures may substantially reduce immune competence and storage reserves in *B. anynana* (see Sørensen et al., 2003; Karl et al., 2011). The response in haemocytes was much more pronounced in females than in males, indicating that the former are not able to maintain their typically high investment into immune competence when exposed to stress.

Females had higher haemocyte numbers, ADH activity and fat content than males. The latter has been often found in insects, reflecting storage reserves for egg production (e.g. Lease and Wolf, 2011). An enhanced energy allocation to immune function in females might be in turn related to females favouring longer lifespans, being positively related to realised fecundity in *B. anynana* (Franke and Fischer, 2013). Finally, the females' higher ADH activity might be explained by higher metabolic rates to fuel

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Table 3. Effects of thermal treatment on developmental and adult traits in *Bicyclus anynana*

Trait	T1	T2	T3	T4
Larval time (days)	20.3±0.3^{a}	19.1±0.3^{b}	20.1±0.3^{a}	22.4±0.3^{c}
Growth rate (mg day^{-1})	0.25±0.01^{a,b}	0.26±0.01^{a}	0.25±0.01^{a,b}	0.22±0.01^{b}
Pupal time (days)	8.7±0.1^{a}	7.1±0.1^{b}	7.8±0.1^{c}	8.6±0.1^{a}
Pupal mass (mg)	164.4±1.8^{a}	149.4±1.8^{b}	143.0±1.8^{c}	124.3±1.8^{d}
Body mass (mg)	50.2±0.9^{a}	44.0±0.9^{b}	42.5±0.9^{b,c}	39.7±0.9^{c}
Thorax mass (mg)	18.7±0.4^{a}	16.6±0.4^{b}	15.3±0.4^{b,c}	14.5±0.4^{c}
Abdomen mass (mg)	21.0±0.7^{a}	18.3±0.7^{b}	16.6±0.7^{b,c}	15.8±0.7^{c}
Abdomen fat content (mg)	1.55±0.13^{a}	1.07±0.14^{b}	0.96±0.14^{b}	1.16±0.14^{b}
Haemocytes (*n*)	135.4±12.2^{a}	113.6±7.2^{b}	117.6±10.6^{b}	61.7±6.6^{c}
Lysozyme activity (mean OD ml^{-1})	0.28±0.02^{a}	0.18±0.02^{c}	0.23±0.02^{b}	0.19±0.02^{c}
ADH activity (mean OD ml^{-1})	0.69±0.02^{a}	0.58±0.02^{a}	0.60±0.02^{a}	0.54±0.02^{a}

Given are means ± 1 s.e.m. per treatment group (T1–T4). Different letters within rows indicate significant differences between treatment groups (Tukey's HSD). Group T1 represents control conditions (mean temperature 24°C), T2 increased temperatures (27°C), T3 increased temperatures combined with a heat wave, and T4 increased temperatures combined with a heat wave and reduced relative humidity. ADH, alcohol dehydrogenase; OD, optical density.

reproduction. A higher ADH activity may result in an increased catalytic activity in oxidizing alcohol, but probably also serves other functions (Middleton and Kacser, 1983). The ADH locus has been shown to affect several traits including egg hatchability, survival rate and development time (Cosmides et al., 1997).

Though clearly documenting detrimental effects of increased temperatures and heat waves, our study has of course some limitations that are typical of experimental laboratory studies being based on artificial settings. However, we did try to mimic natural conditions at least to some extent using ecologically realistic diurnal temperature cycles. Nevertheless, it may be difficult to extrapolate our results to natural conditions as we (1) have not considered potential changes in biotic interactions (Sentis et al., 2013), (2) did not quantitatively assess changes in host plant quality due to heat stress and only included low relative humidity as an additional factor (Bauerfeind and Fischer, 2013a), (3) did not consider effects of different host plants (Bauerfeind and Fischer, 2013b), and (4) did not apply temperature variation in the adult stage. Note, though, that heat stress should be more problematic in the less mobile juvenile stages in butterflies.

In summary, using ecologically realistic temperature scenarios, our results show that both increased temperatures in general and heat waves elicited negative effects on developmental traits in this common tropical butterfly. Negative effects were greatly exaggerated through heat waves, particularly if combined with low humidity. Importantly, diminished condition was carried over into the adult stage, as evidenced by a strongly reduced immune function and partly reduced storage reserves. Our findings suggest that increases in mean temperature alone will negatively affect performance in this tropical butterfly. However, the more frequent occurrence of heat waves and thus periods of strongly elevated temperatures, which is another component of climate change, may pose much more significant challenges to survival, potentially even affecting such widespread species. Our results furthermore highlight that synergistic effects of different stressors (high temperature and drought) will likely exaggerate deleterious consequences. In particular, immune function seems to be compromised by such conditions, warranting further investigation, as this may profoundly affect the way species interact with diseases (Martin et al., 2010).

MATERIALS AND METHODS

Study organism

A stock population of *B. anynana* was founded at Greifswald University, Germany, in 2007 from several hundred individuals derived from a well-established stock population at Leiden University, The Netherlands. The latter was founded in 1988 from over 80 gravid females collected at a single locality in Nkhata Bay, Malawi. To maintain high levels of heterozygosity, several hundred adults are used per generation to produce the subsequent generation (Van't Hof et al., 2005). Eggs for this experiment were collected from >500 Greifswald stock females within 24 h.

Experimental design

Eggs and young larvae were reared until day 7 of larval development in large population cages on young maize plants under controlled environmental conditions (27°C, 70% relative humidity, 12 h:12 h light:dark cycle). These conditions are routinely used in our laboratory and reflect the daily highs experienced by the butterflies during the wet season in the field. On day 7 after hatching (i.e. at the beginning of third out of seven instars), larvae were transferred individually to translucent plastic boxes (250 ml) lined with moist tissue, and were randomly divided among four treatment groups (n=110 each). We used four treatments involving ecologically realistic, diurnal temperature cycles, with one control treatment (T1) mimicking the conditions currently found at Lake Malawi, the origin of our stock population, during the favourable wet season (National Geographic, 2011). The other three treatments (T2, T3 and T4) all had all a 3°C-increased mean temperature. However, they differed in both temperature variance and relative humidity as detailed below (see Table 1 for details). In treatment T2, we increased the temperature by 3°C throughout as compared with T1. Treatments T3 and T4 included, in contrast to T2, a heat wave with 6°C-increased temperatures for 11 days, which alternated with control (i.e. T1) conditions, thus also resulting in an overall increase by ca. 3°C in mean temperature. In T4, relative humidity was additionally lowered to 50% compared with 80% in the other treatments. The heat wave conditions used here are based on the concurrent WMO definition (i.e. a period of more than five consecutive days during which the daily maximum temperature exceeds the average maximum temperature by at least 5°C). Note that the animals in groups T3 and T4 have spent on average a bit more time at control as compared with heat wave conditions (because they needed longer than expected until pupation), lowering the effective temperature means, calculated across all individuals within a given treatment, to 26.1°C (T3) and 25.9°C (T4), respectively. We calculated the above effective temperature means using the time in days spent at control and heat wave conditions for each individual. Each treatment group was reared in a single temperature-, light- and humidity-controlled climate cabinet (Sanyo MLR-351H, Tokyo, Japan). The light cycle was 12 h:12 h light:dark throughout.

All larvae were supplied daily with fresh cuttings of maize for larval feeding, which were replaced as necessary. The position of the plastic boxes within the cabinets was randomized daily to even out potential slight temperature differences. We determined larval time, pupal time and pupal mass (1 day after pupation; Sartorius LE225D, Göttingen, Germany) for all individuals. Larval growth rate was calculated as the natural logarithm of pupal mass divided by larval time. Resulting butterflies were stored on the day following adult eclosion at −80°C for later analyses of adult traits.

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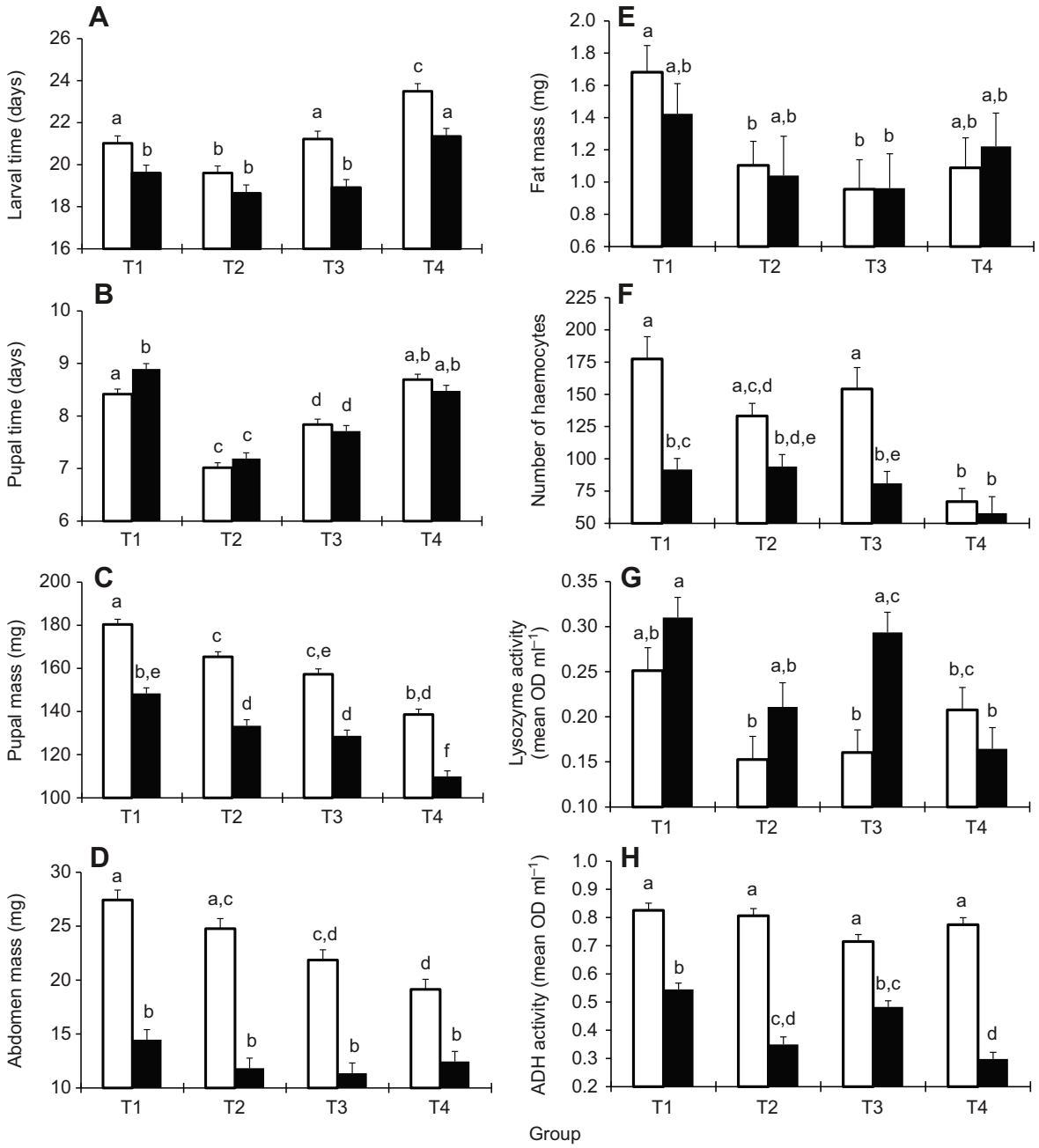

Fig. 1. Effects of thermal treatment and sex on developmental and adult traits in *Bicyclus anynana*. Given are means + 1 s.e.m. for (A) larval time, (B) pupal time, (C) pupal mass, (D) abdomen mass, (E) abdomen fat content, (F) number of haemocytes, (G) lysozyme activity and (H) alcohol dehydrogenase activity. Out of the traits investigated (see Table 2), we did not include: larval growth rate, because of very similar patterns compared with larval time; body and thorax mass, because of very similar patterns compared with pupal mass; or protein content, which was not significantly affected by any factor. Group T1 represents control conditions (mean temperature 24°C), T2 increased temperatures (27°C), T3 increased temperatures combined with heat waves, and T4 increased temperatures combined with heat waves and reduced relative humidity. Different letters above bars indicate significant group differences. Open bars: females; black bars: males. Group sample sizes range between 22 and 60. OD, optical density.

Laboratory analyses

Frozen butterflies were used to measure body mass, thorax mass, abdomen mass, (thorax) protein content, (abdomen) fat content, (thorax) haemocyte numbers, (abdomen) lysozyme activity and (abdomen) ADH activity. Haemocyte numbers and lysozyme activity resemble important components of the insect immune system (Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003). Constitutive haemocyte load is correlated with an increased resistance against parasitoids, at least in fruit flies (Kacsoh and Schlenke, 2012). Lysozymes are a class of enzymes that lyse the cell walls of certain Gram-positive bacteria and inactivate certain viruses (Ibrahim et al., 2001). ADH is a metabolic enzyme that catalyses the reaction of alcohols to the corresponding aldehydes and back, and is important for *B. anynana* as it feeds on fermenting fruit (Larsen, 1991). Butterflies were first weighed with an accuracy of 0.01 mg (Sartorius LE225D). Afterwards wings, legs and heads were removed, and the thorax and abdomen were separated. Both of the latter were weighed after separation. Thoraces were used for scoring protein content and haemocyte numbers (n=51–60 per treatment group), and abdomens for measuring fat content (n=31–39), and lysozyme and ADH activity (n=60).

Thoraxes were perfused with 0.3 ml cacodylate buffer (0.01 mol l^{-1} sodium cacodylatetrihydrate, 0.005 mol l^{-1} $CaCl_2$; syringe: Sterican 20, 0.40×20 mm,

Braun, Melsungen, Germany) to obtain haemolymph extract. To determine haemocyte numbers, 10 µl haemolymph extract was transferred per individual to one well of a multiwell slide. Per well, 2.5 µl ethidium bromide [1:25 in phosphate buffered saline (PBS); 11.9 mmol l^{-1} $Na_2HPO_4 \cdot 2H_2O$, 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, pH 7.4; 1% ethidium bromide] was added as a fluorescent stain. Haemocyte counts were obtained using a digital camera connected to a Nikon Eclipse 90i fluorescence microscope (magnification ×40, Tokyo, Japan) and the program NIS Elements. A screen was superimposed and we took four random squares (1000×1000 µm each) per individual to count haemocytes (camera Nikon DS-U2-Ri1, Tokyo, Japan). We used the sum of haemocytes for further analyses.

Protein content was quantified using the Bio-Rad protein assay, which is based on the Bradford method (Bradford, 1976). One microlitre of the hemolymph extract was diluted in 160 µl distilled water, and then 40 µl Bio-Rad solution was added. The absorbance was read at 595 nm and 30°C after 10 min of incubation with an Absorbance Microplate Reader (BioTekELx 808, Bad Friedrichshall, Germany). Four replicates were measured per individual. Equal numbers per treatment group were always run together on one micro-well plate. A standard curve was constructed with bovine serum albumin in cacodylate buffer, using a concentration series (0 to 2 mg ml^{-1}). Thereby we obtained a standard equation to calculate protein content. The mean value of the four measurements per individual was used for further analyses.

To determine lysozyme-like activity, we followed the protocol of Drayton and Jennions (Drayton and Jennions, 2011). Abdomens were homogenized in 50 µl PBS (pH 7.4) and afterwards centrifuged (15,366 ***g***, 10 min, 4°C). The wells of 96-well plates were loaded with 20 µl supernatant and 80 µl *Micrococcus luteus* solution (3 mg ml^{-1} in PBS) per individual as well as blank samples (20 µl PBS and 80 µl *Micrococcus luteus* solution). Again, equal numbers per treatment group were always run together on one micro-well plate. We measured the optical density (OD) of the above solutions at 490 nm and 30°C for 5 h (BioTekELx 808). The change in OD was determined by subtracting the final from the initial value. The mean of the blank values was subtracted from the resulting ODs obtained on a specific plate.

For measuring ADH activity, 10 µl of the above supernatant was added to 190 µl reaction solution per individual (0.15 mol l^{-1} Tris-HCl, 30 mmol l^{-1} isopropanol, 3 mmol l^{-1} NAD^+, pH 8.5). The resulting solutions were loaded to the wells of 96-well plates, using equal numbers per treatment group on each plate and eight corresponding blanks. The increase in absorbance was measured at 340 nm and 30°C for 10 min (BioTekELx 808). To calculate ADH activity, the slope of the reaction between the second and sixth minute was used. The mean blank value was subtracted from each measurement, and the mean of two replicate measurements per individual was used for further analyses.

To score fat content, we followed the protocol of Fischer et al. (Fischer et al., 2003). Abdomens were dried for 48 h at 60°C in open 1.5 ml tubes. Afterwards, abdomen dry masses were scored, and the abdomens were transferred to 4 ml glass tubes. Tubes were filled with 2 ml acetone, sealed, and then placed on a shaker (IKA KS 260 basic, 100 min^{-1}) for 48 h. After 2 days, solutions were renewed and samples were once again placed on a shaker for 48 h. Then, solutions were removed and abdomens were dried again for 48 h at 60°C and afterwards weighed. Fat content was calculated by subtracting the fat-free dry mass from the initial dry mass.

Statistical analyses

All data were analyzed with ANOVAs or ANCOVAs using treatment group (T1–T4) and sex as fixed factors. Covariates were added as appropriate (see Table 2 for details). Larval time and pupal mass were ln-transformed prior to analyses to meet ANOVA requirements. Throughout we used Tukey's honestly significant difference (HSD) test for pair-wise comparisons, and means are given ±1 s.e.m. All statistical tests were performed with STATISTICA 8.0.

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.F. conceived of the study and its design, M.K. and E.R. executed the experiments and performed the laboratory experiments. All authors interpreted the findings being published, and drafted and revised the article.

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References

Andrew, N. R., Hart, R. A., Jung, M. P., Hemmings, Z. and Terblanche, J. S. (2013). Can temperate insects take the heat? A case study of the physiological and behavioural responses in a common ant, *Iridomyrmex purpureus* (Formicidae), with potential climate change. *J. Insect Physiol.* **59**, 870-880.

Angilletta, M. J., Jr (2009). *Thermal Adaptation. A Theoretical and Empirical Synthesis*. New York, NY: Oxford University Press.

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

Bauerfeind, S. S. and Fischer, K. (2005). Effects of adult-derived carbohydrates, amino acids and micronutrients on female reproduction in a fruit-feeding butterfly. *J. Insect Physiol.* **51**, 545-554.

Bauerfeind, S. S. and Fischer, K. (2013a). Increased temperature reduces herbivore host-plant quality. *Glob. Chang. Biol.* **19**, 3272-3282.

Bauerfeind, S. S. and Fischer, K. (2013b). Targeting the right trait: the relative suitability of a host plant depends on the herbivore trait considered and ambient temperature. *Basic Appl. Ecol.* **14**, 555-564.

Blanckenhorn, W. U. (2000). The evolution of body size: what keeps organisms small? *Q. Rev. Biol.* **75**, 385-407.

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

Calosi, P., Bilton, D. T. and Spicer, J. I. (2008). Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biol. Lett.* **4**, 99-102.

Chown, S. L., Hoffmann, A. A., Kristensen, T. N., Angelletta, M. J., Stenseth, N. C. and Pertoldi, C. (2010). Adapting to climate change: a perspective from evolutionary physiology. *Clim. Res.* **43**, 3-15.

Clusella-Trullas, S., Blackburn, T. M. and Chown, S. L. (2011). Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. *Am. Nat.* **177**, 738-751.

Cosmides, N., Loukas, M. and Zouros, E. (1997). Differences in fitness components among alcohol dehydrogenase genotypes of the olive fruit fly (Diptera: Tephritidae) under artificial rearing. *Ann. Entomol. Soc. Am.* **90**, 363-371.

Coumou, D. and Rahmstorf, S. (2012). A decade of weather extremes. *Nature Clim. Change* **2**, 491-496.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. and Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci. USA* **105**, 6668-6672.

Diamond, S. E., Nichols, L. M., McCoy, N., Hirsch, C., Pelini, S. L., Sanders, N. J., Ellison, A. M., Gotelli, N. J. and Dunn, R. R. (2012). A physiological trait-based approach to predicting the responses of species to experimental climate warming. *Ecology* **93**, 2305-2312.

Diffenbaugh, N. S., Pal, J. S., Trapp, R. J. and Giorgi, F. (2005). Fine-scale processes regulate the response of extreme events to global climate change. *Proc. Natl. Acad. Sci. USA* **102**, 15774-15778.

Drayton, J. M. and Jennions, M. D. (2011). Inbreeding and measures of immune function in the cricket *Teleogryllus commodus*. *Behav. Ecol.* **22**, 486-492.

Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R. and Mearns, L. O. (2000). Climate extremes: observations, modeling, and impacts. *Science* **289**, 2068-2074.

Fischer, K. and Fiedler, K. (2000). Sex-related differences in reaction norms in the butterfly *Lycaena tityrus* (Lepidoptera: Lycaenidae). *Oikos* **90**, 372-380.

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

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**, e15284.

Franke, K. and Fischer, K. (2013). Effects of inbreeding and temperature stress on life history and immune function in a butterfly. *J. Evol. Biol.* **26**, 517-528.

Franke, K., Dierks, A. and Fischer, K. (2012). Directional selection on cold tolerance does not constrain plastic capacity in a butterfly. *BMC Evol. Biol.* **12**, 235.

Hansen, J., Sato, M. and Ruedy, R. (2012). Perception of climate change. *Proc. Natl. Acad. Sci. USA* **109**, E2415-E2423.

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

Huey, R. B., Deutsch, C. A., Tewksbury, J. J., Vitt, L. J., Hertz, P. E., Alvarez Pérez, H. J. and Garland, T., Jr (2009). Why tropical forest lizards are vulnerable to climate warming. *Proc. Biol. Sci.* **276**, 1939-1948.

Ibrahim, H. R., Matsuzaki, T. and Aoki, T. (2001). Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. *FEBS Lett.* **506**, 27-32.

Kacsoh, B. Z. and Schlenke, T. A. (2012). High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. *PLoS ONE* **7**, e34721.

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

Karl, I., Stoks, R., De Block, M., Janowitz, S. A. and Fischer, K. (2011). Temperature extremes and butterfly fitness: conflicting evidence from life history and immune function. *Glob. Chang. Biol.* **17**, 676-687.
Kellermann, V., Loeschcke, V., Hoffmann, A. A., Kristensen, T. N., Fløjgaard, C., David, J. R., Svenning, J.-C. and Overgaard, J. (2012). Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species. *Evolution* **66**, 3377-3389.
Kleynhans, E., Clusella-Trullas, S. and Terblanche, J. S. (2014). Impacts of environmental variability on desiccation rate, plastic responses and population dynamics of *Glossina pallidipes*. *J. Evol. Biol.* **27**, 337-348.
Larsen, T. B. (1991). *The Butterflies of Kenya*. New York, NY: Oxford University Press.
Lease, H. M. and Wolf, B. O. (2011). Lipid content of terrestrial arthropods in relation to body size, phylogeny, ontogeny and sex. *Physiol. Entomol.* **36**, 29-38.
Lyytinen, A., Brakefield, P. M., Lindström, L. and Mappes, J. (2004). Does predation maintain eyespot plasticity in *Bicyclus anynana*? *Proc. Biol. Sci.* **271**, 279-283.
Martin, L. B., Hopkins, W. A., Mydlarz, L. D. and Rohr, J. R. (2010). The effects of anthropogenic global changes on immune functions and disease resistance. *Ann. N. Y. Acad. Sci.* **1195**, 129-148.
Middleton, R. J. and Kacser, H. (1983). Enzyme variation, metabolic flux and fitness: alcohol dehydrogenase in *Drosophila melanogaster*. *Genetics* **105**, 633-650.
National Geographic (2011). *Atlas of the World*, 9th edn. Washington, DC: National Geographic.
Overgaard, J., Kristensen, T. N., Mitchell, K. A. and Hoffmann, A. A. (2011). Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? *Am. Nat.* **178 Suppl. 1**, S80-S96.
Parmesan, C. (2006). Ecological and evolutionary responses to recent climate change. *Annu. Rev. Ecol. Evol. Syst.* **37**, 637-669.
Parmesan, C. and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature* **421**, 37-42.
Partridge, L. and French, V. (1996). Thermal evolution of ectotherm body size: why get big in the cold? In *Animals and Temperature: Phenotypic and Evolutionary Adaptation* (ed. I. A. Johnston and A. F. Bennett), pp 265-292. Cambridge: Cambridge University Press.
Rolff, J. and Siva-Jothy, M. T. (2003). Invertebrate ecological immunology. *Science* **301**, 472-475.
Schmid-Hempel, P. (2003). Variation in immune defence as a question of evolutionary ecology. *Proc. Biol. Sci.* **270**, 357-366.
Sentis, A., Hemptinne, J. L. and Brodeur, J. (2013). Effects of simulated heat waves on an experimental plant-herbivore-predator food chain. *Glob. Chang. Biol.* **19**, 833-842.
Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* **6**, 1025-1037.
Tewksbury, J. J., Huey, R. B. and Deutsch, C. A. (2008). Ecology. Putting the heat on tropical animals. *Science* **320**, 1296-1297.
Thomas, C. D., Cameron, A., Green, R. E., Bakkenes, M., Beaumont, L. J., Collingham, Y. C., Erasmus, B. F., De Siqueira, M. F., Grainger, A., Hannah, L. et al. (2004). Extinction risk from climate change. *Nature* **427**, 145-148.
Van't Hof, A. E., Zwaan, B. J., Saccheri, I. J., Daly, D., Bot, A. N. M. and Brakefield, P. M. (2005). Characterization of 28 microsatellite loci for the butterfly Bicyclus anynana. *Mol. Ecol. Notes* **5**, 169-172.
Wiklund, C. and Fagerström, T. (1977). Why do males emerge before females? A hypothesis to explain the incidence of protandry in butterflies. *Oecologia* **31**, 153-158.