

# Heat resistance throughout ontogeny: body size constrains thermal tolerance

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## Abstract

Heat tolerance is a trait of paramount ecological importance and may **determine a species' ability to cope with ongoing climate change**. Although critical thermal limits have consequently **received substantial attention in recent years**, their potential variation throughout ontogeny remained largely neglected. We investigate whether such neglect may bias conclusions regarding a species' sensitivity to climate change. Using a tropical butterfly, we found that developmental stages clearly differed in heat tolerance. It was highest in pupae followed by larvae, adults and finally eggs and hatchlings. Strikingly, most of the variation found in thermal tolerance was explained by differences in body mass, which may thus impose a severe constraint on adaptive variation in stress tolerance. Furthermore, temperature acclimation was beneficial by increasing heat knock-down time and therefore immediate survival under heat stress, but it affected reproduction negatively. Extreme temperatures strongly reduced survival and subsequent reproductive success even in our highly plastic model organism, exemplifying the potentially dramatic impact of extreme weather events on biodiversity. We argue that predictions regarding a species' fate under changing environmental conditions should consider variation in thermal tolerance throughout ontogeny, variation in body mass and acclimation responses as important predictors of stress tolerance.

**Keywords:** acclimation, Bogert effect, climate change, constraint, development, heat stress resistance, reproduction, tropical butterfly

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## Introduction

Temperature is a key ecological factor evidenced by strong effects of ambient temperature on species distributions (Sunday *et al.*, 2011; Araújo *et al.*, 2013). In particular, stressfully high temperatures may easily decrease individual fitness and ultimately cause death (Le Moullac & Haffner, 2000; Overgaard & Sørensen, 2008). Although several studies indicated genetically based differences in heat tolerance suggesting evolutionary potential (Blackburn *et al.*, 2014; Geerts *et al.*, 2015; Hangartner & Hoffmann, 2015), variation in upper thermal limits is strikingly reduced as compared with lower thermal limits, presumably due to physiological constraints such as protein denaturation (Feder & Hofmann, 1999; Robertson, 2004; Bowler & Terblanche, 2008). Given the prominent fitness implications of heat stress, upper critical thermal limits have recently received substantial attention (Sunday *et al.*, 2012; Kaspari *et al.*, 2015; Pincebourde & Casas, 2015). This interest is driven by both basic considerations and increasing concerns about potential effects of current climate change on extant biodiversity (Thuiller *et al.*, 2004; Loarie *et al.*, 2009; Pereira *et al.*, 2010; Thompson *et al.*, 2013).

Ongoing anthropogenic climate change has already resulted in an increase of the Earth's mean surface temperature and the frequency of extreme weather events such as heat waves (Easterling *et al.*, 2000; Meehl & Tebaldi, 2004; McKechnie & Wolf, 2010; Hansen *et al.*, 2012; Field *et al.*, 2014). These changes resulted in range shifts, abundance and phenological changes (Parmesan & Yohe, 2003; Thomas *et al.*, 2004; Parmesan, 2006; Chown *et al.*, 2010; Thomas, 2010; Chen *et al.*, 2011; Sunday *et al.*, 2012). Notably, climate change is predicted to accelerate in future decades likely posing a severe threat to biodiversity (Novacek & Cleland, 2001; Thuiller *et al.*, 2005; Keith *et al.*, 2014; Mantyka-Pringle *et al.*, 2015; Stanton *et al.*, 2015). In particular, concomitant increases in temperature extremes may strongly affect biodiversity in the future (Clusella-Trullas *et al.*, 2011; Hoffmann *et al.*, 2013). For instance, species distributions are more closely related to extreme rather than mean temperatures (Zimmermann *et al.*, 2009; Kellermann *et al.*, 2012), underlining the ecological importance of critical thermal limits. Tropical ectotherms may be particularly sensitive to such changes, as they live already close to their upper thermal limits while temperate-zone species may even benefit (Deutsch *et al.*, 2008; Kingsolver, 2009; Kellermann *et al.*, 2012; Fischer *et al.*, 2014; but Vasseur *et al.*, 2014).

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A potentially crucial aspect of thermal biology has hitherto received surprisingly little attention, namely variation in thermal limits throughout ontogeny (Bowler & Terblanche, 2008; Kingsolver *et al.*, 2011; Chiu *et al.*, 2015; Pincebourde & Casas, 2015). In particular, in arthropods, our view on organismal biology is still too often focussed on the typically more conspicuous adult stage (Kingsolver, 2009; Kingsolver *et al.*, 2011; Radchuk *et al.*, 2013). The concomitant neglect of other developmental stages may result in false predictions regarding the fate of species facing environmental change, if variation throughout ontogeny is substantial and if the adult stage is not the most sensitive one (Krebs & Loeschcke, 1995; van der Have, 2002; Radchuk *et al.*, 2013; Levy *et al.*, 2015). In insects, for instance, developmental stages vary in size, morphology, physiology and behaviour which may well affect thermal limits (Krebs & Loeschcke, 1995; Bowler & Terblanche, 2008; Kingsolver *et al.*, 2011). Variation in body size may also show complex interactions with ambient and leaf temperature (Woods, 2013). In this context, the Bogert effect predicts that immobile stages should be more temperature-tolerant than mobile ones, as they lack the ability of behavioural thermoregulation (Huey *et al.*, 2003), although this hypothesis has been challenged (Marais *et al.*, 2009; Mitchell *et al.*, 2013). In any case, predicting responses to climate change obviously requires the consideration of heat stress on survival and reproduction throughout development (Bowler & Terblanche, 2008; Kingsolver *et al.*, 2011).

We here investigate heat tolerance in the tropical butterfly *Bicyclus anynana* (Butler 1897) throughout development to test for the occurrence and extent of stage-specific variation. Based on the Bogert effect, it might be predicted that immobile stages (eggs, pupae) are most heat-tolerant, although we assume that body mass, often being positively correlated with higher heat tolerance (Sibly & Atkinson, 1994; Blanckenhorn, 2000; Gibbs, 2002; Kingsolver & Huey, 2008; Terblanche *et al.*, 2011; Mitchell *et al.*, 2013; Nielsen & Papaj, 2015), will have the strongest impact. We further test whether egg-hatching success in relation to temperature is affected by egg handling, that is whether it differs among eggs being (i) removed from maize leaves, (ii) left on cut-off leaves, (iii) left on turgorescent leaves or (iv) left on intact plants. This way we examine whether results obtained in artificial laboratory settings are likely to resemble situations that are more natural. Finally, we test for a negative impact of thermal acclimation, resulting in increased heat tolerance, on reproduction, as such plastic responses are predicted to involve costs (DeWitt *et al.*, 1998; Wilson & Franklin, 2002; Murren *et al.*, 2015).

## Materials and methods

### Study organism

*Bicyclus anynana* is a tropical, fruit-feeding butterfly ranging from southern Africa to Ethiopia (Larsen, 1991). As an adaptation to alternate wet-dry seasonal environments and the associated changes in ground vegetation used for resting, this species exhibits striking phenotypic plasticity (two seasonal morphs; Lyytinen *et al.*, 2004). Reproduction is confined to the favourable wet season during which oviposition plants are abundantly available (Brakefield & Reitsma, 1991; Brakefield, 1997). A laboratory stock population was established at Greifswald University, Germany, in 2008, from several hundred eggs derived from a well-established stock population at Leiden University, the Netherlands. The latter population was founded in 1988 from 80 gravid females caught at a single locality in Nkhata Bay, Malawi. Several hundred adults are used per generation to produce the subsequent generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof *et al.*, 2005).

### Experimental design

To investigate effects of heat exposure on mortality and reproduction of *B. anynana*, we performed four separate experiments. All animals were reared at 27 °C, 70% relative humidity and a photoperiod of L12:D12 within a single temperature-, light- and humidity-controlled climate chamber. Larvae were fed on young, potted maize plants *ad libitum* in population cages.

**Experiment 1.** In the first experiment, we investigated mortality rates in four different life stages over a broad temperature range (20–40 °C) to get handle on the relevant temperature range in terms of critical thermal limits. We hypothesized that increasing temperature will initially not or only weakly increase mortality rates, followed by a steep increase when approaching 40 °C. We scored mortality rates of eggs, hatchlings, small (2nd instar) and intermediate larvae (3rd/4th instar) randomly collected from the stock population. For testing, individuals were placed, separated by developmental stage, into petri dishes in groups of ca. 20 (range 10–31) using 8–47 replicate dishes per stage and temperature ( $n = 9834$  individuals). All animals were exposed for 24 h to either 20, 24, 28, 32, 36 or 40 °C using climate cabinets (Sanyo MLR-351H; Bad Nenndorf, Germany). These temperatures were used to cover the range of temperatures butterflies will experience in its natural habitat, although exposure times in nature will be evidently shorter than 24 h. We used such a period here for practical reasons, that is for maximizing the chances to detect differences among the temperature treatments used. After exposure, all individuals were back-transferred to control conditions (27 °C). Mortality rates were scored 24 h later except for eggs, for which mortality was determined as the percentage of eggs that did not hatch. We used the percentage of dead individuals per dish for further analyses.

**Experiment 2.** Experiment 1 indicated a steep increase in mortality rates between 36 and 40 °C, with little variation between 20 and 32 °C. To estimate stage-specific critical thermal limits, we therefore focused here exclusively on the temperature range between 36 and 40 °C. We here also included later developmental stages to assess critical thermal limits throughout ontogeny, resulting in seven stages in total: eggs, hatchlings (1st instar), small larvae (2nd instar), intermediate larvae (3rd/4th instar), large larvae (5th instar; larval stages according to Bear *et al.*, 2010), pupae (one day after pupation) and adults (one day after eclosion). We hypothesize that the pupal stage will be the most heat-tolerant one, based on its large mass and the Bogert effect.

To keep the size of the experiment manageable, it was divided into three consecutive parts. In the first part of the experiment, we investigated eggs and hatchlings, in the second, the three larval stages, and in the third, pupae and adults. For each part of the experiment, virgin females and males were randomly selected from the stock population and set up in cylindrical hanging cages (30 × 39 cm) for mating. Mating couples were placed individually into translucent 1-l plastic pots. After mating, males were removed and females were provided with a leaf of maize for egg laying and with moist banana for feeding. We used a split-brood design, that is the offspring produced by individual females were randomly divided among five heat treatments entailing exposure for 24 h to either 36, 37, 38, 39 or 40 °C using climate cabinets (Sanyo MLR-351H). After heat exposure, all individuals were back-transferred to control conditions. Mortality rates were scored 24 h later except for eggs and pupae, for which it was tested whether they hatched or eclosed successfully. For scoring mortality rates, we used at least five (up to 47) eggs or hatchlings per female and temperature, which were collected from the egg-laying pots and placed family-wise into petri dishes (eggs:  $n = 16$  females, in total 1647 eggs; hatchlings:  $n = 58$  females, in total 4939 hatchlings). We used the percentage of dead individuals per dish for further analyses. For later developmental stages, the eggs produced by individual females were transferred to elongated, sleeve-like gauze cages containing a young maize plant. Each cage represented one full-sib family. Maize plants were replaced as necessary and the density per cage was culled to a maximum of 40 larvae. Larvae from the respective instars were collected from these cages and tested individually in translucent plastic boxes (250 ml) containing a fresh maize leaf for feeding. We used 3–28 larvae per female and temperature (2nd instar:  $n = 33$  females, in total 786 larvae; 3rd/4th instar:  $n = 25$  females, in total 632 larvae; 5th instar:  $n = 41$  females, in total 1790 larvae). Pupae and adults were tested individually in plastic pots (125 ml) covered with gauze, using 3 to 10 individuals per female and temperature (pupae:  $n = 66$  females, in total 1244 pupae; adults:  $n = 51$  females, in total 1149 adults). Adults were provided moist cotton for drinking during heat exposure.

**Experiment 3.** Here, we tested the impact of different micro-climatic conditions on egg mortality to rule out that results are biased using artificial settings. We therefore investigated egg

mortality in relation to temperature and the following four treatment groups: (i) eggs were removed from maize leaves and placed into petri dishes (control,  $n = 116$  dishes), (ii) eggs were left on leaves, which were cut off and placed into small glass vials filled with water ( $n = 111$  leaves), (iii) eggs were left on leaves, which were cut off and placed into small glass vials without water ( $n = 111$ ), and (iv) eggs were left on leaves, which were not cut off but remained untouched on small plants ( $n = 147$ ). All eggs used derived from the stock population, by placing small maize plants into stock population cages. Group sample sizes per dish or maize leave ranged between six and 363. We scored egg mortality as the percentage of eggs that did not hatch after exposure for 24 h to either 27, 34 or 38 °C using climate cabinets (Sanyo MLR-351H). After exposure, all groups were back-transferred to control conditions to score egg mortality. Additionally, we scored the proportion of eggs that fell from leaves during heat exposure, as heat may interfere with the glue used to attach eggs to host plants.

**Experiment 4.** Here, we tested for effects of temperature acclimation and heat exposure on heat knock-down time and reproduction. Thus, while experiments 1–3 focussed on heat-induced mortality and critical thermal limits, we here investigated plastic responses in heat tolerance, and whether females exposed to severe heat stress are subsequently still able to successfully reproduce. We hypothesize that higher acclimation temperatures confer increased subsequent heat tolerance, and that heat stress affects reproduction negatively. We randomly collected virgin females and males from the stock population and kept the individuals separated by sex. Two days after eclosion, animals were randomly divided among five thermal treatment groups per sex, being acclimated for 48 h to 20, 23, 27, 31 or 34 °C (70% relative humidity, L12:D12; Sanyo MLR-351H). A period of two days is sufficient to induce pronounced physiological responses to the above temperatures (Geister & Fischer, 2007; Fischer *et al.*, 2010). After acclimation, butterflies were randomly allocated to heat stress at either 43, 45 or 47 °C. Butterflies were exposed to heat group-wise in translucent 1-l plastic pots covered with gauze, using 10 butterflies each ( $n = 148$ –175 groups per acclimation by heat treatment group). We scored heat knock-down time as the point in time when 50% (= 5) of the butterflies had been physically knocked down. At this time point, the pots containing the butterflies were immediately back-transferred to control conditions. After one day for recovery, the males and females having experienced the same acclimation and heat stress conditions were set up for mating within translucent 1-l plastic pots covered with gauze for two days. Afterwards, females were set up individually for egg laying in 1-l plastic pots containing a maize leaf as oviposition substrate. We counted egg numbers per female for the following six days, during which the majority of eggs is laid, and additionally scored whether eggs were viable or not. Note that, for the latter, we only scored whether at least one egg hatched to test whether females are able to successfully mate and reproduce after severe heat stress. Throughout, all butterflies were fed with moist banana.

### Statistical analyses

In experiment 1, we analysed mortality rates (% dead individuals) of eggs, hatchlings, small and intermediate larvae using generalized linear models (GLMs; normal distribution and log-link function) with temperature as fixed effect. For larvae, larval instar was included as additional fixed effect. In experiment 2, mortality rates in per cent were analysed using generalized linear mixed models (GLMMs; normal distribution and log-link function) with temperature and developmental stage as fixed and female as random effect. For the data sets obtained from experiments 1 and 2, we additionally performed MANOVAS, using temperature as continuous variable and different life stages as dependent variables. In experiment 3, egg mortality in per cent and egg drop rate were analysed using GLMs with plant treatment and temperature as fixed effects. In experiment 4, heat knock-down times were analysed using a GLM with acclimatization temperature, heat treatment, and sex as fixed effects. Egg numbers were analysed using a GLM with acclimatization temperature and heat treatments as fixed effects. Egg viability, finally, was analysed using a nominal logistic regression on binary data (yes/no) with acclimatization temperature and heat treatment as fixed factors. Data were analysed using STATISTICA 8.0 (StatSoft, Tulsa, OK, USA) or JMP 7.0.1 (SAS institute, Cary, NC, USA). Pair-wise comparisons after GLMs were performed employing Tukey's HSD. Throughout the text, means are given  $\pm 1$  SE. For model diagnostic plots see Appendix S1. To back up our results, we also performed nonparametric Kruskal–Wallis tests for all significant main effects, which confirmed all results with exception of the significant differences between larval stages in experiment 2 (Table 2b;  $P = 0.10$ ).

## Results

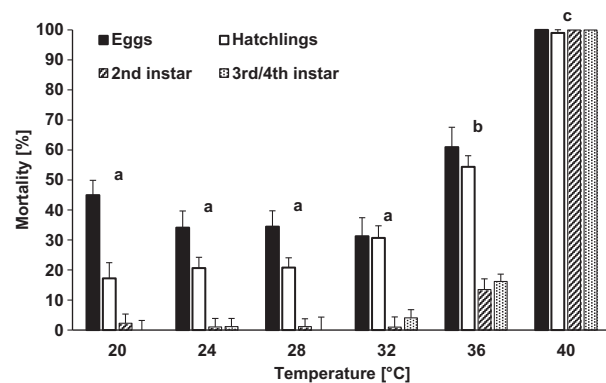
### Mortality rates

**Experiment 1.** Average mortality rates across temperatures were  $50.9 \pm 6.2\%$  for eggs,  $40.5 \pm 4.0\%$  for hatchlings,  $19.8 \pm 3.0\%$  for 2nd and  $20.2 \pm 3.1\%$  for 3rd/4th instar larvae. Regarding the effects of temperature, we found a qualitatively identical pattern across all four developmental stages investigated. While exposure for 24 h to temperatures between 20 and 32 °C did not cause significant variation, mortality rates increased significantly at 36 °C and especially at 40 °C (Table 1; Fig. 1). The different larval instars did not respond differently to increasing temperatures. The strong temperature impact is also confirmed by MANOVA results ( $F_{4,59} = 23.2$ ,  $P < 0.0001$ ; see also Appendix S2).

**Experiment 2.** Mortality rates between 36 and 40 °C. Overall, egg mortality ( $69.0 \pm 4.3\%$ ) was significantly higher than hatchling mortality ( $55.7 \pm 2.3\%$ ) within the temperature range of 36–40 °C (Table 2a, Fig. 2a). Mortality rates generally increased significantly with increasing temperature, which was more pronounced

**Table 1** Results of generalized linear models for the effects of temperature (and larval instar, 2nd vs. 3rd/4th instar, for c) on egg (a), hatchling (b) and larval (c) mortality rates in *Bicyclus anynana*. Significant  $P$ -values are given in bold

	MQ	df	$F$	$P$
(a) Eggs ( $r^2 = 0.298$ )				
Temperature	9757	5	11.6	<b>&lt;0.001</b>
Error	838	137		
(b) Hatchlings ( $r^2 = 0.773$ )				
Temperature	15506	5	1313.3	<b>&lt;0.001</b>
Error	245	93		
(c) Larvae ( $r^2 = 0.906$ )				
Temperature	55708	5	371.9	<b>&lt;0.001</b>
Instar	8	1	0.1	0.816
Temperature $\times$ Instar	34	5	0.2	0.951
Error	150	195		



**Fig. 1** Mortality rates for eggs (black bars), hatchlings (open bars), small larvae (2nd instar, hatched bars) and intermediate larvae (3rd/4th instar, dotted bars) of *Bicyclus anynana* after 24-h exposure to either 20, 24, 28, 32, 36 or 40 °C. Given are group means  $\pm 1$  SE. Missing error bars indicate 100% mortality and therefore no variation. Sample sizes range between 8 and 47 groups with 10–31 individuals each. In total, 4844 eggs, 3012 hatchlings, 1315 2nd instar larvae and 663 3rd/4th instar larvae were used. Different lower-case letters above bars (a, b, c) indicate significant differences between temperature groups across life stages, with mortality being significantly higher at 40 °C than at all other temperatures, and significantly higher at 36 °C than at 20 to 32 °C. Mortality rates were significantly higher at 40 °C than at any other temperature and significantly higher at 36 °C than at 20–32 °C. Note that patterns were identical across developmental stages.

in hatchlings compared with eggs (significant interaction) owing to the hatchlings' initially lower mortality. While egg mortality raised from  $51.7 \pm 4.3\%$  at 36 °C to  $97.5 \pm 4.3\%$  at 40 °C, hatchling mortality raised from  $23.5 \pm 2.2\%$  at 36 °C to  $100.0 \pm 0\%$  at 40 °C. Regarding the three larval stages, mortality rates increased significantly with increasing

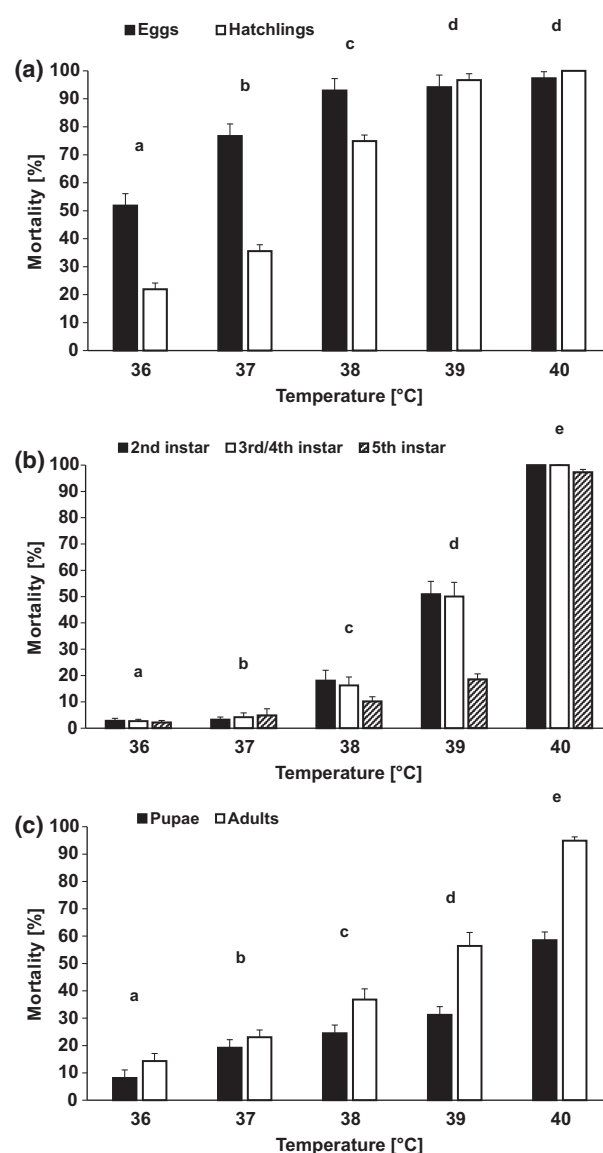


**Table 2** Results of a generalized linear mixed models for the effects of temperature, developmental stage and female on mortality rates of eggs and hatchlings (a), three larval stages (b) and pupae and adults (c) of *Bicyclus anynana*. Female was considered a random effect throughout. Significant *P*-values are given in bold

	MQ	df	<i>F</i>	<i>P</i>
(a) Eggs/Hatchlings ( $r^2 = 0.858$ )				
Temperature	34 847	4	176.5	<0.001
Stage	13 114	1	66.4	<0.001
Temperature $\times$ Stage	4981	4	25.2	<0.001
Female	365	58	1.9	<0.001
Error	198	277		
(b) Larval stages ( $r^2 = 0.882$ )				
Temperature	159 084	4	783.8	<0.001
Stage	3923	2	19.3	<0.001
Temperature $\times$ Stage	2413	8	11.9	<0.001
Female	232	57	1.1	0.230
Error	203	465		
(c) Pupae/Adults ( $r^2 = 0.615$ )				
Temperature	72 130	4	139.5	<0.001
Stage	37 781	1	73.1	<0.001
Temperature $\times$ Stage	5011	4	9.7	<0.001
Female	1143	71	2.2	<0.001
Error	517	499		

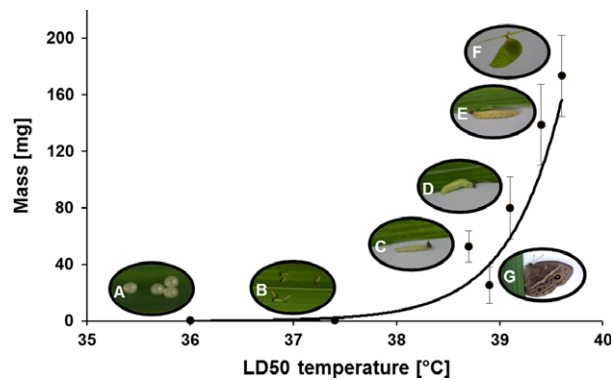
temperature as above, ranging between  $1.7 \pm 0.9\%$  at  $36^\circ\text{C}$  and  $99.3 \pm 0.4\%$  at  $40^\circ\text{C}$  (Table 2b, Fig. 2b). Smaller larvae showed a significant higher mortality rate compared with large larvae (2nd instar:  $35.0 \pm 1.1\%$ ; 3rd/4th instar:  $34.6 \pm 1.3\%$ ; 5th instar:  $26.6 \pm 1.0\%$ ), with differences being obvious at 38 and  $39^\circ\text{C}$  only (significant interaction). Also in pupae and adults mortality rates increased significantly with increasing temperature (Table 2c, Fig. 2c). Overall, mortality was significantly higher in adults ( $45.6 \pm 3.1\%$ ) than in pupae ( $27.7 \pm 2.7\%$ ). The absolute difference between the two stages generally increased with increasing temperature (significant interaction), being 6.0% at  $36^\circ\text{C}$  and 33.9% at  $40^\circ\text{C}$ . Except from larval stages, heat tolerance differed significantly among females. The strong temperature impact is also confirmed by MANOVA results ( $F_{7,75} = 118.2$ ,  $P < 0.0001$ ; see also Appendix S2).

We calculated the LD50 temperature (i.e. the temperature at which 50% of all individuals died) for each developmental stage based on the data given in Fig. 2, equalling  $39.6^\circ\text{C}$  for pupae. Pupae are followed by large (LD50:  $39.4^\circ\text{C}$ ), intermediate (LD50:  $39.1^\circ\text{C}$ ) and small larvae (LD50:  $38.9^\circ\text{C}$ ), adults (LD50:  $38.7^\circ\text{C}$ ), hatchlings (LD50:  $37.4^\circ\text{C}$ ) and finally eggs (LD50:  $36.0^\circ\text{C}$ ). LD50 values were strongly correlated with body mass, with higher mass conferring increased heat tolerance (Fig. 3).



**Fig. 2** Mortality rates for groups of eggs (black bars) and hatchlings (open bars, a), small (2nd instar, black bars), intermediate (3rd/4th instar, open bars) and large larvae (5th instar, hatched bars, b), and pupae (black bars) and adults (open bars, c) of *Bicyclus anynana* after 24 h exposure to either 36, 37, 38, 39 or  $40^\circ\text{C}$ . Given are female means  $\pm 1$  SE. Missing error bars indicate 100% mortality and therefore no variation. Sample sizes for eggs and hatchlings range between 16 and 58 females with 5–47 individuals each, and for larvae, pupae and adults between 25 and 66 females with 3–28 offspring each. In total, 1647 eggs, 4939 hatchlings, 786 2nd instar larvae, 362 3rd/4th instar larvae, 1790 5th instar larvae, 1244 pupae and 1149 adults were used. Different lower-case letters above bars indicate significant differences between temperature groups.

**Experiment 3.** Egg mortality in relation to temperature and handling. As above, mortality rates increased significantly with increasing temperatures, being on



**Fig. 3** Exponential regression of body mass against LD50, that is the temperature at which 50% of all individuals died, throughout the development of the tropical butterfly *Bicyclus anynana* ( $r^2 = 0.956$ ). Mass data are based on (Fischer & Fiedler, 2002) for eggs and hatchlings, (Fischer *et al.*, 2014) for pupae and adults, and on measurements during the current experiment for the three larval stages. All animals were reared at 27 °C, 70% relative humidity and a photoperiod of L12:D12. A: eggs; B: hatchlings; C: small larvae; D: intermediate larvae; E: large larvae; F: pupae; G: adults.

average  $23.1 \pm 1.4\%$  at 27 °C,  $23.5 \pm 1.4\%$  at 34 °C and  $76.8 \pm 1.4\%$  at 38 °C ( $F_{2,473} = 491.2$ ,  $P < 0.001$ ). However, effects of egg handling were not significant as variation across treatments were minimal (min:  $40.2 \pm 1.6\%$ , max:  $41.6 \pm 1.4\%$ ;  $F_{3,473} = 0.2$ ,  $P = 0.918$ ). The proportion of eggs falling from the leaves varied significantly across temperatures ( $F_{2,269} = 5.6$ ,  $P = 0.004$ ), ranging from  $13.8 \pm 1.0\%$  at 38 °C via  $10.5 \pm 1.0\%$  at 34 °C to  $8.9 \pm 1.1\%$  at 27 °C. Again, there were no significant differences among plant treatments ( $F_{2,269} = 0.3$ ,  $P = 0.762$ ).

#### Heat knock-down time, egg number and egg mortality

**Experiment 4.** Heat knock-down time increased significantly with acclimation temperature, on average from  $10.4 \pm 1.5$  min at 20 °C to  $22.1 \pm 1.5$  min at 34 °C (Table 3a, Fig. 4a). This increase was most pronounced at subsequent exposure to 43 °C (significant interaction). In addition to above, increasing temperature during heat exposure significantly decreased heat knock-down time, being longest at 43 °C ( $35.8 \pm 1.1$  min) followed by 45 °C ( $9.3 \pm 1.1$  min) and finally 47 °C ( $6.1 \pm 1.1$  min). Egg numbers decreased significantly with increasing acclimation temperature (Table 3b; Fig. 4b), being highest at 20 °C ( $84.5 \pm 4.7$ ) and lowest at 34 °C ( $53.9 \pm 4.9$ ). Regarding effects of heat exposure, highest egg numbers were found at 45 °C ( $81.7 \pm 3.5$ ) followed by 47 °C ( $67.3 \pm 3.8$ ) and finally 43 °C ( $56.0 \pm 3.9$ ). Patterns for egg mortality were similar to those for egg numbers. Egg mortality increased

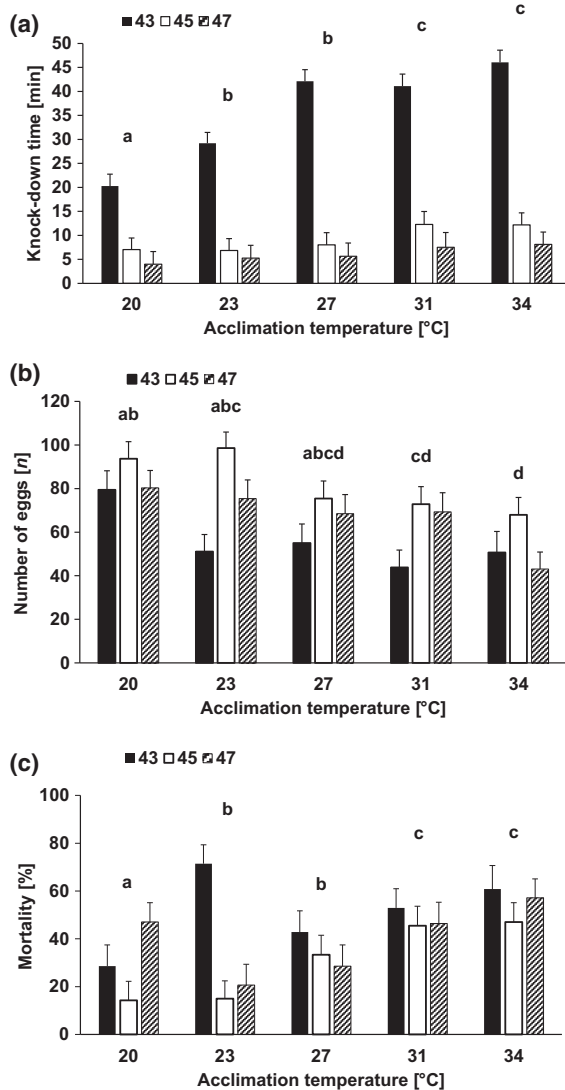
**Table 3** Results of generalized linear models for the effects of acclimation temperature, heat stress temperature and sex on heat knock-down time (time until 50% of the butterflies were knocked down, a), acclimation temperature and heat stress temperature on the number of eggs laid (b) and a nominal logistic regression for the effects of acclimation temperature and heat stress temperature on egg mortality (c) of *Bicyclus anynana*. Significant *P*-values are given in bold

	MQ	df	F	P
(a) Knock-down time ( $r^2 = 0.808$ )				
Acclimation temperature	0.73	4	26.54	<b>&lt;0.001</b>
Heat stress temperature	10.17	2	369.54	<b>&lt;0.001</b>
Sex	<0.01	1	0.02	0.884
Acclimation × Heat	0.06	8	2.04	<b>0.044</b>
Acclimation × Sex	0.03	4	1.12	0.349
Heat × Sex	0.01	2	0.42	0.659
Acclimation × Heat × Sex	0.01	8	0.48	0.868
Error	0.03	215		
(b) Egg number ( $r^2 = 0.121$ )				
Acclimation temperature	13 063	4	6.1	<b>&lt;0.001</b>
Heat stress temperature	26 464	2	12.3	<b>&lt;0.001</b>
Acclimation × Heat	2559	8	1.2	0.303
Error	2149	462		
(c) Egg mortality ( $r^2 = 0.157$ )				
Acclimation temperature		4	18.2	<b>0.001</b>
Heat stress temperature		2	14.7	<b>0.001</b>
Acclimation × Heat		8	23.8	<b>0.003</b>

significantly with increasing acclimation temperature from 29.9% (20 °C) to 54.4% (34 °C) and was higher for exposure to 43 °C (52.0%) than to 47 °C (40.9%) or 45 °C (30.3%; Table 3c, Fig. 4c). The significant acclimation by heat stress temperature interaction indicates variation in the responses to heat stress across acclimation temperature groups. In general, effects of stress temperature were more pronounced after acclimation to 20 and 23 °C. Pearson correlations based on the group means given in Fig. 4 showed negative correlations between heat knock-down time and egg number ( $r = -0.64$ ,  $n = 15$ ,  $P = 0.009$ ) as well as hatching success ( $r = -0.53$ ,  $n = 15$ ,  $P = 0.041$ ).

#### Discussion

Our experiments revealed, as expected, that mortality rates generally increased with increasingly stressful temperatures (Tewksbury *et al.*, 2008; Andrew *et al.*, 2013; Rukke *et al.*, 2015). This well-documented relationship is explained by, for instance, dehydration impairing evaporative cooling, denaturation of proteins, disruption of membrane structure and function, and interactions with oxygen supply (Klose & Robertson, 2004; Chown & Terblanche, 2006; Potter *et al.*,



**Fig. 4** Heat knock-down time (time until 50% of the butterflies were knocked down, a), number of eggs laid (b) and egg mortality (c) of *Bicyclus anynana* in relation to acclimation (20, 23, 27, 31 and 34 °C) and heat stress (43 °C: black bars, 45 °C: open bars, 47 °C: hatched bars) temperature. Given are means + 1 SE. Sample sizes range between 43 and 54 groups of 10 individuals each per acclimation temperature for heat knock-down time and between 89 and 104 females per acclimation temperature for egg number and egg mortality. Different lower-case letters above bars indicate significant differences between acclimation temperature groups.

2009). Our findings indicate that, for the tropical butterfly *B. anynana*, the upper critical thermal limit is reached between 36 and 40 °C when using a 24-h exposure. After 24 h at 40 °C, almost all individuals died except for those in the pupal stage. In contrast, the species seems to be well equipped to bear temperatures below 36 °C for 24 h, as no impact on mortality rates

could be detected between 20 and 32 °C. In other studies, even temperatures >40 °C have been occasionally used when investigating thermal tolerance, but in these cases, exposure time was typically much shorter than 24 h (Bowler & Terblanche, 2008; Ju *et al.*, 2014; Nandi & Chakraborty, 2015). Thus, estimating upper thermal limits is prone to biases caused by the experimental protocol used and the particular trait investigated (Chown & Terblanche, 2006; Potter *et al.*, 2009; Rezende *et al.*, 2014), also because heat damage is the result of both absolute temperature and exposure time. This has been, among others, illustrated in lizards, in which embryos survived thermal cycles that reached 42 °C, while they were not able to survive a constant temperature of 36 °C (Angilletta *et al.*, 2000; Levy *et al.*, 2015). We do not know to what extent the use of fluctuating temperatures may have affected our results, but assume that upper thermal limits would have been even higher. This, however, may not affect the below comparisons across developmental stages (Fischer *et al.*, 2011). However, despite using rather long exposure times to constant temperatures, our results are in broad agreement with findings on other species, indicating that upper thermal limits are in general close to 40 °C in most terrestrial animals, with very little variation across species (Heinrich, 1981; Bowler & Terblanche, 2008; Sunday *et al.*, 2011).

Given that upper thermal limits vary very little even across species, it is surprising that heat tolerance varied substantially throughout development in *B. anynana*. Overall, pupae turned out to be most heat-tolerant. We suspect that these differences, amounting to nearly 4 °C in total, are ecologically highly relevant. Still too often, our view on insect life histories is dominated by a single stage, mainly the adult one, only (Kingsolver, 2009; Kingsolver *et al.*, 2011; Radchuk *et al.*, 2013; Chiu *et al.*, 2015). Our data clearly indicate that eggs and hatchlings are very sensitive to heat while others such as pupae are less susceptible. Some other studies also showed variation in heat resistance throughout ontogeny, indicating large differences with respect to the most heat-tolerant developmental stage ranging from early via intermediate up to late ones (Krebs & Loeschcke, 1995; Abdelghany *et al.*, 2010; Amarasekare & Sifuentes, 2012; Knapp & Nedvěd, 2013).

An important question from an ecological perspective although is whether such results, gained in artificial laboratory settings and using long exposure times, resemble more natural conditions reasonably well. This might be questionable especially for the egg stage, as females deposit their eggs on the leaves of host plants, where they may benefit from plant-mediated changes in microclimate with increased humidity and decreased temperature (Smith, 1978; Potter *et al.*, 2009). Therefore,

we tested whether mortality of detached eggs and those on intact plants differ, which was not the case. These results indicate that, at least under the specific conditions used, egg handling did not bias our results and that we consequently may be able to extrapolate to natural conditions. Additionally, we could show that the proportion of eggs falling down from leaves increased with increasing temperature. This may be caused by an interference of the egg glue with temperature, or alternatively represent an adaptive mechanism to avoid overheating in a life stage lacking behavioural thermoregulation. The latter could be the case if temperatures were lower on the ground as compared to leaves.

The demonstrated variation of heat tolerance across developmental stages could be related to differences in morphology, physiology and/or behaviour (Krebs & Feder, 1998; Spicer & Gaston, 1999; Bowler & Terblanche, 2008). The increased heat tolerance of larvae compared with eggs or hatchlings may be explained by gaining water and energy while feeding, resources of crucial importance for evaporative cooling and fuelling physiological stress responses (Krebs & Feder, 1998; Hofmann & Todgham, 2010). This, however, would not explain why pupae are most heat-tolerant. Also, behavioural thermoregulation may play an important role, with larger larvae having higher behavioural thermoregulation capabilities than eggs or hatchlings (Bowler & Terblanche, 2008). While potentially important, such considerations are probably not relevant in laboratory-based studies with little if any variation in microclimate. Note further that the above hypothesis challenges predictions based on the Bogert effect, according to which less mobile stages should have a higher rather than a lower heat tolerance, precisely because of a lack of alternative behavioural options (Huey *et al.*, 2003; Bowler & Terblanche, 2008). However, our results are also not really in agreement with such predictions. While we found that the immobile pupal stage was very heat-tolerant as would be expected, the same does not apply to eggs being most susceptible. Furthermore, if mobility was the crucial factor, adults should be least tolerant and smaller larvae more tolerant than larger ones rather than vice versa. Other studies also challenged the general applicability of the Bogert effect with regard to developmental stages (Marais *et al.*, 2009; Mitchell *et al.*, 2013), while recent studies did find support within a comparative context across geographical regions (Buckley *et al.*, 2015; Gunderson & Stillman, 2015).

In contrast to the above explanations, variation in stress resistance was in our study closely related to body mass, with higher mass conferring increased heat tolerance Fig. 3; cf. (Bouchebti *et al.*, 2014; Nielsen & Papaj, 2015). Larger individuals have been often found

to be more stress-resistant (Sibly & Atkinson, 1994; Blanckenhorn, 2000; Gibbs, 2002; Kingsolver & Huey, 2008; Chidawanyika & Terblanche, 2011). With respect to heat tolerance, a reduced surface to volume ratio in larger animals reducing water loss might be of crucial importance, as dehydration seems to be a major determinant of survival under heat stress (Addo-Bediako *et al.*, 2001; Chown *et al.*, 2011). Based on the tight correlation between body mass and heat tolerance (explaining 96% of the variation) reported here, we conclude that body mass may comprise a crucial morphological constraint on adaptive responses in heat tolerance. While many studies focus on potential adaptive responses, the role of constraints has in general received much less attention (Gould & Lewontin, 1979; Pigliucci & Kaplan, 2000; Futuyma, 2010; Tammaru *et al.*, 2015). This does not necessarily contradict the existence of significant family effects indicating that heat tolerance has a heritable component that can be exploited by selection. While several studies indicated that heat tolerance is a heritable trait, reported heritabilities are typically fairly low (Krebs & Loeschcke, 1999; Hoffmann *et al.*, 2005; Blackburn *et al.*, 2014; Hangartner & Hoffmann, 2015).

Besides whether an individual does or does not survive an acute heat stress, the survivors' ability to subsequently mate and produce viable offspring is obviously of crucial importance. Our results indicate that this is indeed the case even when using very high temperatures. To address this issue, we here used the time until 50% of individuals were knocked down to make sure that at least a proportion of animals would survive. In fact, nearly all individuals, being knocked down or not, survived our stress treatments, although we did not score exact proportions. Heat knock-down times were, as expected, strongly influenced by stress temperature, being much shorter at 45 and 47 °C as compared with 43 °C. This finding suggests that temperatures above 43 °C can be survived for very short periods only (Chidawanyika & Terblanche, 2011; Terblanche *et al.*, 2011; Condon *et al.*, 2015).

Acclimation, in contrast, had positive effects on heat knock-down times, with individuals acclimated to higher temperatures being able to stand stressful heat for longer periods. Such beneficial acclimation, increasing subsequent heat tolerance, has been demonstrated in several organisms (Fischer *et al.*, 2010; Karl *et al.*, 2014; Scharf *et al.*, 2014) and may be highly relevant under natural conditions by allowing for more time to escape from stressful conditions. On the other hand, higher acclimation temperatures clearly reduced fecundity as well as fertility. Thus, females seem to have sacrificed part of their reproductive potential in order to enhance survival under acute stress, presumably



caused by a resource-allocation trade-off and increased investment into physiological stress responses (Silbermann & Tatar, 2000; Sørensen *et al.*, 2003). Alternatively, the detrimental effects on reproduction may simply reflect the fact that, on average, the individuals acclimated to higher temperatures were exposed to heat stress for longer (Loeschcke & Hoffmann, 2002). Variation in exposure time is also the most obvious explanation for the effects of stress temperature on reproduction. The individuals showing the highest fecundity and fertility were those exposed to 45 °C not 43 °C, which had very short exposure times. The fact that the animals exposed to 47 °C showed a reduced reproductive performance in spite of a short exposure time is likely caused by reaching critical thermal limits even during short exposure at this extreme temperature (Zhang *et al.*, 2013, 2015). However, the important conclusion from these results is that even beneficial plastic responses, enhancing immediate survival rates, may not necessarily increase fitness based on detrimental effects on subsequent reproduction.

Our study provides important insights with regard to critical thermal limits likely to affect future survival in changing environments, although relying on constant temperatures. First, we show pronounced variation throughout development, indicating that a species vulnerability to climate change cannot be assessed using a single or few selected developmental stages only. Second, we show that heat tolerance is likely subject to substantial morphological constraints, potentially limiting adaptive responses. Third, we show that thermal acclimation induces plastic changes in heat tolerance, which also needs to be considered when trying to forecast species-specific responses to climate change. Yet, such plastic responses may reduce subsequent reproductive performance. In summary, our study reveals several caveats worth considering when trying to forecast the response of species to ongoing climate change, in particular stage-specific variation throughout development and morphological constraints. The latter points are probably as or perhaps even more important in species with nonholometabolous life cycles and/or infinite growth.

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## Supporting Information

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

### Appendix S1. Model diagnostic plots.

**Figure S1.** Model diagnostic plots for residuals vs. fitted values and residuals vs. observed values for the mortality rate of eggs and hatchlings (a, b) and larvae (c, d) in experiment 1, mortality rates of eggs and hatchlings (e, f), larvae (g, h) and pupae and adults (i, j) in experiment 2, and for heat-knock down time (k, l), egg number (m, n) and egg mortality (o, p) in experiment 4.

### Appendix S2. MANOVA results.

**Table S1.** Results of a MANOVA for the effects of temperature on mortality rates in eggs, hatchlings, 2nd and 3rd/4th instar larvae in *Bicyclus anynana*. Significant *P*-values are given in bold.

**Table S2.** Results of a MANOVA for the effects of temperature on mortality rates in eggs, hatchlings, 2nd, 3rd and 4th instar larvae, pupae and adults in *Bicyclus anynana*. Significant *P*-values are given in bold.