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Cooler butterflies lay larger eggs: developmental plasticity versus acclimation

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We use a full factorial design to investigate the effects of maternal and paternal developmental temperature, as well as female oviposition temperature, on egg size in the butterfly *Bicyclus anynana*. Butterflies were raised at two different temperatures and mated in four possible sex-by-parental-temperature crosses. The mated females were randomly divided between high and low oviposition temperatures. On the first day after assigning the females to different temperatures, only female developmental temperature affected egg size. Females reared at the lower temperature laid larger eggs than those reared at a higher temperature. When eggs were measured again after an acclimation period of 10 days, egg size was principally determined by the prevailing temperature during oviposition, with females ovipositing at a lower temperature laying larger eggs. In contrast to widely used assumptions, the effects of developmental temperature were largely reversible. Male developmental temperature did not affect egg size in either of the measurements. Overall, developmental plasticity and acclimation in the adult stage resulted in very similar patterns of egg size plasticity. Consequently, we argue that the most important question when testing the significance of acclamatory changes is not at which stage a given plasticity is induced, but rather whether plastic responses to environmental change are adaptive or merely physiological constraints.

Keywords: phenotypic plasticity; developmental plasticity; acclimation; offspring size; Lepidoptera

1. INTRODUCTION

Recent developments in evolutionary physiology, in particular the use of more theoretical and hypothesis-driven approaches to evolutionary questions in physiological research, stimulated a renewed and increasing interest in the magnitude and nature of non-genetic effects on an organism's phenotype (e.g. Nylin & Gotthard 1998; Feder *et al.* 2000; Wilson & Franklin 2002; Woods & Harrison 2002). Such environmental, non-genetic effects are relevant not only to functional biologists studying how organisms work, but also to evolutionary biologists studying the dynamics of phenotypic evolution (Crill *et al.* 1996).

Traditionally, it has been assumed that all acclimation changes to the phenotype enhance the performance of an individual organism in the environment in which those changes were induced (beneficial acclimation hypothesis; Wilson & Franklin 2002). This long-held, intuitive assumption has recently received a considerable amount of experimental analysis, predominantly by examining the acclamatory responses of ectotherms to temperature (Leroi *et al.* 1994; Bennett & Lenski 1997; Huey *et al.* 1999; Gibert *et al.* 2001). Perhaps surprisingly, all such analyses of the beneficial acclimation hypothesis to date have rejected its generality (Wilson & Franklin 2002). However, these studies should be considered tests of the adaptive significance of developmental plasticity (a generally non-reversible cascade of phenotypic changes owing to differences in the developmental environment) rather than of acclimation (a reversible, facultative response to changes in a single environmental variable in the adult stage; Willmer *et al.* 2000; Wilson & Franklin 2002). Thus, when studying environmental effects it may be important to distinguish between different types of phenotypic plasticity, but studies investigating the relative importance of developmental plasticity versus acclimation in the adult stage seem to be almost non-existent (but see Gibert *et al.* 2001).

Here, we adopt a novel approach to investigate temperature-mediated plasticity in a key life-history trait, egg size, in the butterfly *Bicyclus anynana* (Butler 1879). We used a similar experimental protocol to that developed by Crill *et al.* (1996) to explore cross-generational effects on physiological and morphological traits. Butterflies were raised at different temperatures for two generations, mated in the four possible sex-by-parental-temperature crosses, and then the mated females were divided randomly across high and low oviposition temperature. Egg size is a particularly suitable candidate trait for studying plastic responses to temperature. First, it is generally believed to be closely related to fitness (Fox & Czesak 2000; for *B. anynana* see Fischer *et al.* 2003*a*). Second, it is known to respond readily to differences in both developmental and adult temperature (Azevedo *et al.* 1996; Crill *et al.* 1996; Ernsting & Isaaks 1997; Blanckenhorn 2000; Fischer *et al.* 2003*a*).

We used this factorial design specifically to address the following issues.

(i) What is the relative importance of female developmental versus adult temperature?
(ii) Is the developmental response to temperature reversible? and

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(iii) Does male developmental temperature affect the egg size of female partners?

The last question may require some justification. As egg size is generally believed to be determined by the maternal genotype and environment, little attention has been given to potential paternal effects on egg size (Weigensberg *et al.* 1998; Fox & Czesak 2000). However, there are at least two processes by which males could influence egg size or initial offspring size, namely a paternal genetic effect or a paternal environmental effect. Such a genetic effect is not relevant here. However, the quality or quantity of paternal investment could vary among male phenotypes and thus affect the size of the eggs that the females subsequently produce (Weigensberg *et al.* 1998). At least two studies have revealed evidence for such mechanisms (Crill *et al.* 1996; Weigensberg *et al.* 1998). Male insects transfer not only sperm but also a cocktail of substances to the females during mating that can affect female reproduction in many ways (e.g. fecundity, oogenesis, egg deposition, egg hatchability; cf. Boggs 1990; Chapman 2001; Chapman *et al.* 2001; Heifetz *et al.* 2001). The composition and quantity of such 'nuptial gifts' could potentially change with temperature. A male contribution to temperature-mediated plasticity in egg size could be favoured during evolution if components of this plasticity were adaptive, an issue that is beyond the scope of investigation in this contribution. Accumulating evidence, however, suggests that this may indeed be the case in this particular species (Fischer *et al.* 2003*a*,*b*).

2. MATERIAL AND METHODS

(a) *Study organism and experimental population*

Bicyclus anynana is a tropical, fruit-feeding butterfly distributed from southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternative wet–dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997). A laboratory stock population of *B. anynana* was established at Leiden University in 1988 from over 80 gravid females collected at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity (Saccheri & Bruford 1993). Butterflies from the stock population were used for this study.

(b) *Experimental design*

We reared stock population butterflies (population size of founding lines were several hundred butterflies) at either 20 °C or 27 °C for two generations in climate cells with high humidity and a photoperiod of 12 L : 12 D. These temperatures are similar to those at which the larvae of the wet and dry seasonal forms, respectively, develop in the field (Brakefield & Mazzotta 1995). At each temperature, two replicate populations were set up. Larvae were fed on young maize plants, adults on moist banana. Second-generation butterflies were used for this experiment. Following adult eclosion, all females were individually marked. Males and females were kept separately at their respective larval rearing temperature for 2–6 days. Afterwards, the butterflies were set up randomly for mating in the four possible sex-by-parental-temperature crosses at an intermediate temperature of 23 °C for 2 days. Following mating, females were randomly divided between 20 °C or 27 °C for egg laying. They were placed individually in translucent plastic pots (1 l) containing a fresh cutting of maize for ovipositing. This design results in eight treatment groups with different thermal histories (table 1).

Table 1. Overview of experimental protocol.
(*Bicyclus anynana* butterflies were reared for two generations at either 20 °C or 27 °C, afterwards mated in the four possible sex-by-parental-temperature crosses, and then exposed to either 20 °C or 27 °C for egg laying. M, male; F, female.)

group	sex-by-parental-temperature crosses	oviposition temperature (°C)
1	20 °C M × 20 °C F	20
2	20 °C M × 20 °C F	27
3	27 °C M × 27 °C F	20
4	27 °C M × 27 °C F	27
5	20 °C M × 27 °C F	20
6	20 °C M × 27 °C F	27
7	27 °C M × 20 °C F	20
8	27 °C M × 20 °C F	27

Eggs were collected for one (27 °C) or two (20 °C) days and subsequently measured (see below). These eggs were the first ones laid within the females' adult lifespan, thus effectively controlling for any confounding effects of female age (Karlsson & Wiklund 1984; Brakefield *et al.* 1994; Braby & Jones 1995). The females were kept at the oviposition temperature they were assigned to for 10 days, and then once again set up individually for egg laying. Eggs were again collected and measured. Mean daily fecundity varies between 20 and 40 singly laid eggs during the first 10 days of the oviposition period. Throughout, females had access to maize plants for ovipositing and to moist banana for adult feeding.

(c) *Data collection and analysis*

Apart from egg size we measured the development time for all females and pupal weight for sub-samples of males and females. For the latter, individuals from the pupation peaks were used. Pupae were weighed 1 day after pupation. As the eggs of *B. anynana* are nearly perfectly spherical, egg size was measured as cross-sectional area (mm^2) using a digital camera (Leica DC200) connected to a binocular microscope. The resulting images were analysed using Scion Image public software (Scion Corporation 2000). Tight correlations between egg area (applying image analysis) and egg mass as well as hatchling size confirm that this method provides a highly reliable measurement of egg size in *B. anynana* (Fischer *et al.* 2002). To calculate egg size for individual females, the mean of about 10 eggs was used. Previous experiments showed that the means did not change substantially above a critical minimum number of seven to eight eggs (data not shown). All data were analysed using nested analyses of variance (ANOVAs), with female developmental temperature, male developmental temperature and oviposition temperature as fixed factors and replicates nested within female developmental temperature. Throughout, all means are given ± 1 s.d.

3. RESULTS

(a) *Egg size*

In the first round of egg measurements (i.e. directly after the 2-day mating period), only female developmental

Table 2. Results of nested three-way analyses of variance (ANOVAs) for the effects of female developmental, male developmental and oviposition temperature on egg size in *Bicyclus anynana*.
(Replicate populations were nested within female developmental temperature. The two parts of the table refer to the first (directly after mating, *a*) and second (10 days later, *b*) round of egg measurements. Significant *p*-values are printed in bold.)

source	sum of squares	d.f.	*F*-ratio	*p*
(*a*)				
female temperature	1.1807	1	644.01	< **0.0001**
replicate [female temperature]	0.0013	2	0.26	0.6974
male temperature	0.0008	1	0.46	0.4991
oviposition temperature	0.0017	1	0.93	0.3351
male temperature × female temperature	0.0003	1	0.16	0.6917
oviposition temperature × female temperature	0.0001	1	0.07	0.7922
oviposition temperature × male temperature	0.0002	1	0.12	0.7249
oviposition temperature × male temperature × female temperature	< 0.0001	1	< 0.01	0.9901
(*b*)				
female temperature	0.0483	1	20.51	< **0.0001**
replicate [female temperature]	0.0018	2	0.39	0.6786
male temperature	< 0.0001	1	0.04	0.8489
oviposition temperature	1.1231	1	476.60	< **0.0001**
male temperature × female temperature	0.0053	1	2.27	0.1327
oviposition temperature × female temperature	0.0026	1	1.10	0.2938
oviposition temperature × male temperature	0.0023	1	0.96	0.3277
oviposition temperature × male temperature × female temperature	0.0002	1	0.11	0.7455

temperature significantly affected egg size (tables 2*a* and 3). Regardless of male developmental and female oviposition temperature, females reared at 20 °C laid significantly larger eggs than those reared at 27 °C (*ca.* 0.74 mm^2 compared with 0.66 mm^2, equivalent to a difference of *ca.* 19% in volume). After an acclimation period of 10 days, however, the main effect on egg size is due to the prevailing temperature during oviposition, although female developmental temperature remains a significant factor affecting egg size (tables 2*b* and 3). At this stage, the females that were reared at 20 °C, but afterwards kept at 27 °C for egg laying, laid considerably smaller eggs than those reared at 27 °C, but ovipositing at 20 °C. However, even after 10 days the transfer groups did not quite reach the values of those individuals constantly kept at either high or low temperature (figure 1). Females that oviposited at the same temperature as used in rearing produced, on average, smaller eggs in the second round of measurements. This indicates the effects of the relatively advanced female age. Again, male rearing temperature did not affect egg size. The results were always highly consistent across replicates.

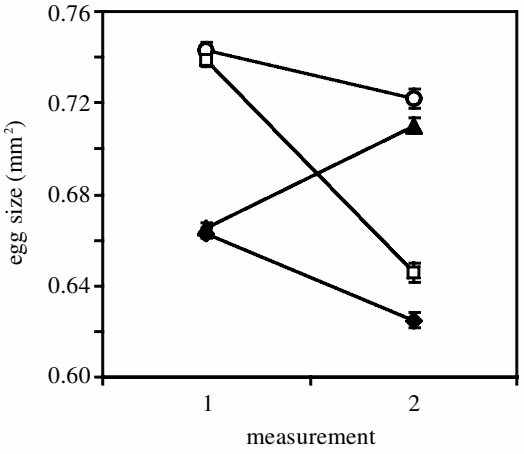

Figure 1. Effects of female developmental and ovipositing temperature on egg size (± 1 s.e.) in *Bicyclus anynana* (circles, 20–20: reared at 20 °C, oviposited at 20 °C; squares, 20–27: reared at 20 °C, oviposited at 27 °C; triangles, 27–20: reared at 27 °C, oviposited at 20 °C; diamonds, 27–27: reared at 27 °C, oviposited at 27 °C). Measurement 1 was made on the day after mating; measurement 2 was made 10 days later. The graph shows a strong effect of female developmental temperature in measurement 1, whereas the effect of oviposition temperatures is dominant in measurement 2. Note that in measurement 2 the effects of developmental temperature remain. Data are pooled for replicates and for male developmental temperature.

(b) *Pupal mass and development time*

At the lower rearing temperature, both sexes achieved slightly higher pupal weights than at the higher rearing temperature ($F_{1,401} = 8.6$, $p = 0.0035$; table 4). Throughout, females were considerably heavier than males ($F_{1,401} = 461.3$, $p < 0.0001$). The results were consistent across replicates ($p = 0.40$), and the interaction between rearing temperature and sex was non-significant ($p = 0.98$). As expected, female development time (in days) was much reduced at the higher temperature ($F_{1,1015} = 12\,552.9$, $p < 0.0001$; 31.3 ± 2.1 ($n = 248$) and 31.3 ± 2.0 ($n = 256$) at 27 °C, 62.9 ± 5.8 ($n = 255$) and 62.2 ± 6.1 ($n = 260$) at 20 °C), with replicates again behaving consistently ($p = 0.23$).

4. DISCUSSION

Our results show that differences in both female developmental and ovipositing temperature can induce very

Table 3. Egg size (s.d. in parentheses) in replicate *Bicyclus anynana* populations.
(Butterflies were reared for two generations at either 20 °C or 27 °C, afterwards mated in the four possible sex-by-parental-temperature crosses, and then exposed to either 20 °C or 27 °C for egg laying. Egg size was measured the day after mating (egg size I), and again after an acclimation period of 10 days (egg size II).)

female temperature (°C)	male temperature (°C)	oviposition temperature (°C)	replicate	egg size I		egg size II	
				(mm^2)	*n*	(mm^2)	*n*
20	20	20	1	0.743 (0.042)	39	0.728 (0.057)	40
20	20	27	1	0.732 (0.041)	51	0.659 (0.045)	37
20	27	20	1	0.746 (0.045)	49	0.723 (0.046)	47
20	27	27	1	0.735 (0.050)	48	0.637 (0.050)	39
20	20	20	2	0.742 (0.056)	47	0.719 (0.054)	38
20	20	27	2	0.748 (0.044)	51	0.645 (0.050)	42
20	27	20	2	0.739 (0.053)	47	0.722 (0.051)	39
20	27	27	2	0.741 (0.053)	47	0.645 (0.058)	31
27	20	20	1	0.668 (0.037)	52	0.717 (0.044)	48
27	20	27	1	0.663 (0.033)	58	0.622 (0.042)	48
27	27	20	1	0.666 (0.049)	49	0.712 (0.046)	46
27	27	27	1	0.660 (0.034)	57	0.615 (0.046)	48
27	20	20	2	0.665 (0.039)	57	0.697 (0.050)	52
27	20	27	2	0.667 (0.039)	56	0.626 (0.047)	42
27	27	20	2	0.663 (0.040)	51	0.715 (0.048)	52
27	27	27	2	0.662 (0.032)	56	0.638 (0.043)	48

Table 4. Pupal weight (s.d. in parentheses) for males and females of *Bicyclus anynana* raised at different developmental temperatures.
(At each rearing temperature, two replicate populations were measured. M, male; F, female.)

temperature (°C)	replicate	sex	weight (mg)	*n*
20	1	M	136.0 (15.2)	52
20	1	F	180.8 (24.1)	48
20	2	M	135.4 (17.1)	38
20	2	F	175.7 (29.6)	51
27	1	M	130.0 (18.8)	50
27	1	F	174.4 (19.3)	58
27	2	M	129.5 (13.7)	54
27	2	F	170.2 (17.6)	58

similar plastic responses in egg size. Both types of phenotypic plasticity result in larger eggs at lower temperatures, as has already been found in some other arthropods (Azevedo *et al.* 1996; Crill *et al.* 1996; Sheader 1996; Yampolski & Scheiner 1996). The interpretation in this study that the effects of rearing temperature on egg size are due to developmental plasticity is fully substantiated by the comparable results from an earlier experiment in which temperatures were switched within the pupal stage and not in the young adult (Fischer *et al.* 2003*b*). Although females were transferred between temperatures before adult eclosion and 8–14 days prior to egg measurements in that case, the effects of adult temperature on first eggs were hardly detectable.

We know of no other study that has explored the relative importance of developmental plasticity versus acclimation as well as the potential interactions between the two. As expected, only female rearing temperature affected egg size in the first round of measurements. Egg maturation is a gradual process, which in some species of butterfly begins in the pupal, or possibly even in the late larval, phase (Boggs 1997*a*,*b*). In some other species egg maturation may only begin after adult eclosion, although the ovarioles start developing earlier (Ramaswamy *et al.* 1997). For the former group of species at least, it is highly unlikely that the first eggs laid could respond plastically to prevailing temperature conditions or male contributions, because their size has presumably been determined prior to temperature changes and mating. Note that the substantial differences in initial egg size found here cannot be explained as a correlated response to the minor temperature-mediated differences in body size. Generally, female body size is a poor predictor for egg size in *B. anynana*, explaining no more than 1% of the overall variation in egg size (Fischer *et al.* 2002).

The eggs measured after an acclimation period of 10 days, however, presumably matured after females had been assigned to different oviposition temperatures and after they had mated, leaving scope for further plastic responses. At this point, egg size is principally determined by the prevailing temperature during oviposition. The results demonstrate that the effects of developmental environment, commonly assumed to be irreversible (Willmer *et al.* 2000; Wilson & Franklin 2002), can in this case be largely reversed by acclimation in the adult stage. Within 10 days, the transferred females that experienced different developmental and adult temperatures produced eggs of almost the same size as for those females kept continuously at either high or low temperature (figure 1). Whether the remaining difference is due to partial irreversibility or is just a transient stage depending on the time elapsed since temperature change, remains to be tested in future experiments.

In contrast to the previous factors, we found that females mated to males reared at either high or low temperature laid eggs of the same size. Several reasons could account for the absence of any effect of male developmental temperature. First, the temperature-mediated plasticity

in egg size might be merely a non-adaptive physiological response (and consequently there is no advantage to the male to make any contribution). Second, the amount of nuptial gifts or any other substances transferred by the male during mating could be independent of temperature. Two recent studies on dipterans showed that sperm length tended to increase with temperature, whereas testis length decreased (Blanckenhorn & Hellriegel 2002; Hellriegel & Blanckenhorn 2002). However, temperature effects specifically on nuptial gifts or accessory gland products have apparently not yet been studied.

As expected, egg size decreased with female age (figure 1), confirming the results of Brakefield *et al.* (1994) for *B. anynana* and those for many other butterflies (Karlsson & Wiklund 1984; Braby & Jones 1995). The rate of decrease was apparently very similar at both oviposition temperatures, as was found in a previous study using *B. anynana* (Fischer *et al.* 2003*a*). Consequently, it is highly unlikely that differences in physiological age between oviposition temperatures confounded any of the results presented here.

In summary, our results demonstrate that *B. anynana* is able to adjust egg size to the temperature conditions experienced during development and oviposition. The acclimation response in the adult stage is largely independent of the developmental history, i.e. the effect of developmental temperature is almost fully reversible (as is the effect of adult temperature; Fischer *et al.* 2003*a*). These findings may have important implications for designing experiments in the context of examining the beneficial acclimation hypothesis. While we agree with most criticism of previous approaches (e.g. exposure to stressful or even harmful environments subsequently degrading performance, focusing on net performance instead of on individual traits; Wilson & Franklin 2002; Woods & Harrison 2002), one should be careful when stating that the effects of developmental plasticity are very different from those of acclimation in the adult stage (Wilson & Franklin 2002). Similarity of effects does, of course, not necessarily mean that these are caused by the same mechanisms, which may well be different during development and in the adult stage.

Although some plastic responses are clearly irreversible and intimately connected to the conditions during ontogeny (e.g. different wing patterns of seasonal morphs, or body size in holometabolous insects), our results suggest that the effects of developmental plasticity and acclimation may at least in some cases be very similar. Consequently, we argue that the most important question is not at which stage a given plasticity is induced (although a clear distinction between different types will always give additional information and may in some cases even be necessary), but rather whether plastic responses to environmental change are adaptive (see also Woods & Harrison 2002). Focusing too narrowly on traditional definitions of acclimation may obscure this fascinating general problem.

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