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**Author(s):** Nokelainen, Ossi; van Bergen, Erik; Ripley, Brad S.; Brakefield, Paul M.

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## Adaptation of a tropical butterfly to a temperate climate

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Complete List of Authors:	Nokelainen, Ossi; University of Jyväskylä, The Department of Biological and Environmental Science van Bergen, Erik; Instituto Gulbenkian de Ciência Ripley, Brad; Rhodes University, Department of Botany Brakefield, Paul; University of Cambridge, Department of Zoology
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3 1 GENERAL INFORMATION  
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7 3 **Title:** Adaptation of a tropical butterfly to a temperate climate  
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910 4  
11 5 **Authors:** Ossi Nokelainen<sup>1,2#\*</sup>, Erik van Bergen<sup>1,3#\*</sup>, Brad S. Ripley<sup>4</sup> & Paul M. Brakefield<sup>1</sup>  
12  
13 614  
15  
16 7 **Addresses:**17  
18 8 <sup>1</sup> Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ,  
19  
20  
21 9 United Kingdom22  
23 10 <sup>2</sup> Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box  
24  
25 11 35, 40014 University of Jyväskylä, Finland26  
27 12 <sup>3</sup> Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, P-2780 Oeiras, Portugal28  
29 13 <sup>4</sup> Department of Botany, Rhodes University, P.O. Box 94, 6140 Grahamstown, South Africa  
30  
31 1432  
33  
34 15 # These authors contributed equally to this work.35  
36 16 \* Authors for correspondence: Ossi Nokelainen (ossi.nokelainen@jyu.fi), Erik van Bergen  
37  
38 17 (erikvanbergen.science@gmail.com)  
39  
40 1841  
42  
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49 22 phenology, seasonal polyphenism  
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## 23 ABSTRACT

24 Developmental plasticity enables organisms to cope with environmental heterogeneity, such  
25 as seasonal variation in climatic conditions, and is thought to affect a species' capability to  
26 adapt to environments with novel seasonal and ecological dynamics. We studied  
27 developmental plasticity of the widespread tropical butterfly, *Bicyclus safitza*, which reaches  
28 the southern edge of its distribution in the temperate zone of South Africa. In wet-dry  
29 seasonal environments in tropical Africa, adults of *Bicyclus* butterflies are present all-year-  
30 around and exhibit discrete seasonal forms in alternating generations. We demonstrate that a  
31 population that colonized a more temperate climate region has adopted a different strategy to  
32 cope with local environment as no active adults were encountered during the temperate  
33 winter. The flight season coincided with a period when evaporation stress was lowest and  
34 temperatures were higher in the South African population. Butterflies collected from the field  
35 did not express seasonal polyphenism or show full expression of the tropical wet season form  
36 phenotype. Reaction norm experiments comparing stocks from South Africa and Uganda  
37 indicated that local adaptation of this tropical butterfly to a more temperate climate involved  
38 the evolution of developmental plasticity, such that a more robust development in response to  
39 thermal variation was observed for a broad suite of morphological and life history traits. Our  
40 findings have implications for understanding the mechanisms that facilitate expanding into a  
41 novel ecological niche under seasonally variable climatic conditions.

## 42 INTRODUCTION

43 Natural environments are heterogeneous and many insect species have evolved strategies to  
44 cope with spatial and temporal environmental variation. Typically these involve dispersal  
45 from unfavourable to favourable conditions, either in space by means of migration, or in time  
46 by inducing a state of arrested development or diapause (Bohonak and Jenkins 2003).  
47 Compelling examples of both phenomena include the annual migrations of monarch  
48 butterflies (*Danaus plexippus*) and the winter diapause in many other temperate butterflies,  
49 such as the speckled wood butterfly (*Pararge aegeria*), which is induced when changes in  
50 day length indicate that winter is coming (Aalberg Haugen and Gotthard 2015). An  
51 alternative strategy to cope with environmental variability is seasonal polyphenism; an  
52 extreme example of developmental plasticity in which different forms of a species are  
53 produced at different times of the year. Seasonal forms have adaptive traits specific to the  
54 environment in which they occur (Shapiro 1976). In contrast to spatially or temporally  
55 dispersing insects in the tropics, seasonally polyphenic species remain active throughout the  
56 year and, thus, demonstrate less dramatic seasonal peaks in activity and abundance.

57 *Bicyclus* butterflies have become a hallmark example of adaptive developmental  
58 plasticity and seasonal polyphenism as they have proved a tractable system in which to study  
59 the environmental regulation of development in both natural (Brakefield and Reitsma 1991,  
60 Windig et al. 1994) and laboratory populations (de Jong et al. 2010, Oostra et al. 2014, van  
61 Bergen et al. 2017). The genus consists of over 100 extant species that inhabit a wide range  
62 of tropical habitats in sub-Saharan Africa (Aduse-Poku et al. 2015). Recent work has  
63 supported that a single *Bicyclus* species began to colonize more open and seasonal habitats  
64 during the Miocene epoch, when much of the trans-continental forests began to open up  
65 (Aduse-Poku et al. 2015). Such environments, with highly distinct wet and dry seasons, are  
66 characterized by predictable patterns of variation in temperature, rainfall and humidity that

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3 67 are closely associated with changes in vegetation cover and host plant availability (e.g.  
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5 68 Brakefield and Larsen 1984). The rains of the wet season result in a luxuriant growth of  
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7 69 herbs, including species of grass, which the larvae of most *Bicyclus* species use as host plants.  
8  
9 70 Adult butterflies in the middle of the warm, wet season are highly active and reproduce  
10  
11 71 quickly (Brakefield and Reitsma 1991). Larvae of this generation develop in increasingly arid  
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13 72 and cooler conditions towards the dry season when the ground vegetation, including larval  
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15 73 host plants, dies back to become a layer of brown leaf litter. The next generation of adult  
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17 74 butterflies emerges around the transition between wet and dry season, and then survives the  
18  
19 75 unfavourable conditions as active, but reproductively dormant, adults before reproducing at  
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21 76 the beginning of the next wet season (Brakefield and Reitsma 1991, Windig et al. 1994, van  
22  
23 77 Bergen et al. 2016).

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27 78 In the laboratory, development of phenotypes similar to the seasonal forms found in  
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29 79 nature can be induced by manipulating the temperature during a sensitive phase of pre-adult  
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31 80 development (Kooi and Brakefield 1999). Larvae reared at low temperatures, which represent  
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33 81 the environmental conditions of the tropical dry season, develop into relatively large  
34  
35 82 individuals, which allocate resources toward a more durable body and demonstrate cryptic  
36  
37 83 patterning of the ventral wings that are exposed when at rest. In contrast, wet season form  
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39 84 individuals, which are induced by high developmental temperatures, have a series of  
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41 85 conspicuous marginal eyespots on their ventral wing surfaces, and demonstrate an increased  
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43 86 investment in reproduction. The distinct wing patterns serve an important fitness function in  
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45 87 terms of coping with changing predatory threats between seasonal environments (Lyytinen et  
46  
47 88 al. 2004, Prudic et al. 2015).

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50  
51 89 The common bush brown, *Bicyclus safitza* (Westwood, 1850), is one of the most  
52  
53 90 widely distributed species of *Bicyclus* butterflies (e.g. Larsen 2005). Whereas all other  
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55 91 species of *Bicyclus* are restricted to tropical climate zones, the distribution of *B. safitza*  
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3 92 extends far into more temperate climate zones in southern Africa. One of the most  
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5 93 fundamental differences between temperate and tropical ecosystems is the ambient  
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7 94 temperature during winter, which can even drop below freezing in temperate regions, and  
8  
9 95 populations of tropical butterflies which successfully colonized more temperate climate  
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11 96 regions are predicted to have evolved a suite of adaptations to cope with local environmental  
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13 97 conditions. For example, recent field studies have revealed that populations of *B. safitza* in  
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15 98 these temperate regions have a strong preference for shaded forests habitats (Nokelainen et al.  
16  
17 99 2016), which may buffer seasonal fluctuations in temperature and humidity, whereas in  
18  
19 100 tropical biomes *B. safitza* is mainly found in semi-open woodland and forest edge habitats  
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21 101 (Brakefield and Reitsma 1991, Windig et al. 1994).  
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25 102 Here, we explore whether a population of *Bicyclus safitza* that occurs in Eastern Cape  
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27 103 of South Africa has become locally adapted to temperate climatic conditions. We also aim to  
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29 104 find explanations for the strong population-specific preference for more shaded habitats  
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31 105 (Nokelainen et al. 2016). To address these questions, we study the phenology and the  
32  
33 106 expression of seasonal polyphenism of temperate population by conducting a longitudinal  
34  
35 107 survey in the Eastern Cape, South Africa, and compare these to a well-studied tropical  
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37 108 population of *B. safitza* (Brakefield and Reitsma 1991, Windig et al. 1994). Secondly, using  
38  
39 109 the ratios of stable isotopes of oxygen obtained from the exoskeleton of field-trapped  
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41 110 individuals as well as measurements of local water evaporation during the larval stage, we  
42  
43 111 explore whether evaporation stress may constrain the butterfly niche. Finally, we conducted a  
44  
45 112 comparative reaction norm study, using populations from temperate and tropical climate  
46  
47 113 regions, to investigate the extent of local adaptation in response to different thermal  
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49 114 environments. Using data from these two populations, we aim to provide insights on the  
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51 115 mechanisms that facilitate the colonization of novel ecological niches under seasonally  
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53 116 variable climatic conditions.  
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5 118 MATERIALS AND METHODS6  
7 119 *Field sites and butterfly monitoring*

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9 120 We monitored butterflies at the southern-most southern edge of the species' range in the  
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11 121 Eastern Cape province of South Africa. The Eastern Cape spans over a multitude of climatic  
12  
13 122 regions including cold and temperate interior parts as well as temperate and sub-tropical  
14  
15 123 coastal regions (Mucina and Rutherford 2006). Our study sites were predominantly in a  
16  
17 124 temperate zone that is characterised by open, semi-arid grasslands, whereas afro-montane  
18  
19 125 forests and coastal thickets provide more humid, shaded-habitats. The field sites in the  
20  
21 126 vicinity of Grahamstown and more humid coastal and riverine environments were chosen  
22  
23 127 using satellite images (Google Earth, Google Inc., Mountain View, CA, USA) to detect  
24  
25 128 suitable habitats after which the areas were visited to confirm the presence of *B. safitza*.  
26  
27 129 Three field sites were used in this study: Bathurst (33°30'S, 26°46'E), Kapriver (33°21'S,  
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29 130 26°52'E) and Kasouga (33°39'S, 26° 44'E). The Bathurst site represents a riparian bush  
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31 131 habitat with more open areas along the edges. The Kapriver site is an open, grassy hilltop,  
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33 132 which transitions into riparian forest in a lower lying river gorge. The Kasouga site is  
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35 133 characterised by coastal thickets and bordered by pastureland to the north, and the shores of  
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37 134 the Indian Ocean to the south.

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39 135 To investigate the seasonal phenology of *B. safitza* in temperate regions, we  
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41 136 monitored field populations by conducting monthly trapping sessions between November  
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43 137 2014 and October 2015. Nine traps (Megaview, DC0017, Pop-up Butterfly Bait Trap, cone  
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45 138 type) were placed at each of the three field sites and equally distributed among three habitat  
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47 139 types, open grasslands, forest fringes and under shaded canopy, within each site (for further  
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49 140 details and habitat preference comparisons, see Nokelainen et al. 2016). These traps were  
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51 141 baited with fermented banana once a month and emptied on the following day. Wild-caught  
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3 142 individuals were stored in entomological envelopes until further processing. Measurements of  
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5 143 relative humidity and temperature were obtained from each of the three habitats at all three  
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7 144 sites using data loggers (Maxim DS1922T iButton Temperature Logger, San Jose, CA, USA)  
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10 145 to enable comparisons of climatic conditions. Data on the phenology and wing pattern  
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12 146 plasticity of tropical populations have been published by Brakefield and Reitsma (1991) and  
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14 147 Windig *et al.* (1994), and here we use the same methodologies to quantify habitat use and  
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16 148 variation in butterfly occurrence and wing patterning.  
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19 14920  
21 150 *Evaporation stress measures*

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23 151 Evaporation stress from wild-caught butterflies was first studied using the ratio of stable  
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25 152 isotopes of oxygen ( $\delta^{18}\text{O}$ ) present in the exoskeleton, which have been shown to reflect the  
26  
27 153 mean atmospheric conditions surrounding the insect before moulting (Ellwood *et al.* 2011).  
28  
29 154 Briefly, the more common, lighter  $^{16}\text{O}$ -isotope evaporates more readily than the  $^{18}\text{O}$ -isotope,  
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31 155 which leads to enrichment of  $^{18}\text{O}$  in sample tissues and thus, more positive  $\delta^{18}\text{O}$ -values. To  
32  
33 156 quantify the  $\delta^{18}\text{O}$ -values of the specimens collected at the field sites, two legs were placed  
34  
35 157 into silver capsules, sealed and loaded into an auto-sampler. The tissue within the capsule  
36  
37 158 was pyrolysed at  $1200^{\circ}\text{C}$  using a Thermo Finnigan TC/EA attached to a Thermo Delta V  
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39 159 mass spectrometer via a ConFlo 3. Reference standards from IAEA in Vienna were run at  
40  
41 160 intervals throughout the sequence and these values are used to calibrate to the international  
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43 161 standards of  $^{18}\text{O}/^{16}\text{O}$  ( $\delta^{18}\text{O}$  V-SMOW). Analyses were conducted at the Godwin Laboratory  
44  
45 162 for Palaeoclimate Research, Department of Earth Sciences at University of Cambridge, UK.

46  
47 163 We measured larval rates of water loss with Li-Cor 6400 photosynthesis system (Li-  
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49 164 Cor Biosciences, Lincoln, NE, USA). Three separate runs were conducted using a total of  
50  
51 165 nine similar-sized 3<sup>rd</sup> instar larvae of *B. safitza* ( $\bar{x} = 22.5$  mg). Larvae originated from an F1-  
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53 166 laboratory stock, initially collected from the Kasouga field site, and were used only once. For  
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3 167 the measurements, three larvae were placed together (for better measurement accuracy) in a  
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5 168 small mesh cage that was inserted into the leaf chamber of the photosynthesis system in order  
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7 169 to obtain rates of water-loss. The device measures the exchange of CO<sub>2</sub> and H<sub>2</sub>O between  
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10 170 organism and atmosphere, controlling ambient CO<sub>2</sub> concentration, temperature and relative  
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12 171 humidity and hence, the vapour pressure deficit (VPD, the difference between the amount of  
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14 172 moisture in the air and how much moisture the air can hold when it is saturated). The  
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16 173 conditions under which an organism maintains its water balance during temperature changes  
17  
18 174 are more clearly shown by noting the VPD than the relative humidity (Anderson 1936).  
19  
20 175 Based on this, we measured larval evaporation rates in response to five different VPD  
21  
22 176 conditions (VPD [kPa] = 1.5, 2, 2.5, 3, 3.5) at a constant ambient temperature of 25°C.  
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24  
25 177 Evaporation measures were started at the lowest VPD and successively increased. Once a  
26  
27 178 target VPD was attained, five water loss measures (over ten seconds duration) were recorded  
28  
29 179 and averaged. Values were used to calculate average water loss as a percentage of larval body  
30  
31 180 mass per hour (% H<sub>2</sub>O g<sup>-1</sup> hr<sup>-1</sup>). Climate data at the study sites was then used to calculate  
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33 181 natural range of VPD's (Table 1) and predicted larval water loss based on the relationship  
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35 182 established in the laboratory (see supplementary information).  
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#### 41 *Temperature reaction norm experiment*

42  
43 185 To study the geographic variation in the degree of developmental plasticity we conducted a  
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45 186 reaction norm experiment using two populations of *B. safitza* and four constant thermal  
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47 187 regimes. The laboratory populations of *B. safitza* were established in 2013 from eggs  
48  
49 188 collected at a single location in the Semuliki National Park in Uganda (0°50'N, 30°9'E) and  
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51 189 the Kasouga field site in South Africa (33°39'S, 26°44'E). The eggs from at least ten females  
52  
53 190 contributed to each stock population. Thus, the Ugandan colony was derived from a  
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55 191 population in the tropics whereas the South African colony originated from a temperate  
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3 192 population at the poleward margin of the species range. After about four generations of  
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5 193 laboratory rearing, eggs were collected from both laboratory stocks and larvae were randomly  
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7 194 divided over four climate-controlled chambers (21°C, 23°C, 25°C and 27°C) within one day  
8  
9 195 after hatching. In these chambers (Sanyo/Panasonic MLR-350H, 70% RH, 12:12 L:D cycle),  
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11 196 larvae were reared in sleeve-like gauze cages on young wheat (*Triticum aestivum*) plants: a  
12  
13 197 host plant that is frequently used to rear newly established laboratory populations of *Bicyclus*  
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15 198 butterflies (Oostra 2014, van Bergen 2017). Pre-pupae were collected daily and one day after  
16  
17 199 pupation they were weighed to the nearest 0.1 mg (Fisherbrand PS-60) and individually  
18  
19 200 placed in transparent pots until they eclosed. On the first day after eclosion the adults were  
20  
21 201 sacrificed by freezing and carefully dissected. In addition to wing pattern measurements (see  
22  
23 202 below) we recorded the larval and pupal development times, calculated the growth rate, and  
24  
25 203 measured adult dry mass, relative fat content and abdomen ratio. Data from the Ugandan  
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27 204 population were included in a comparative study on developmental plasticity in mycalesine  
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29 205 butterflies, for details on methodology see van Bergen *et al.* (2017).  
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36 207 *Wing pattern measurements*

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38 208 The ventral surface of one hind- and one forewing of each individual were photographed  
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40 209 using a Leica DFC495 digital camera with a Leica M125 stereomicroscope. The images were  
41  
42 210 analysed with the image processing package Fiji (Schindelin et al. 2012). We followed the  
43  
44 211 Comstock-Needham system to refer to wing veins and cells (see Miller 1970). On the ventral  
45  
46 212 hind wing, the area of the yellow outer ring, the black inner-disc, and white focus of the  
47  
48 213 eyespot in cell Cu1 were measured. The relative distance of the proximal edge of the median  
49  
50 214 band along the second wing vein was taken as a measure of the width of the band. The  
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52 215 measurements on the ventral forewing included the yellow, black and white areas of the  
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54 216 eyespot in cell M1 as well as area of the black inner-disc of the larger eyespot in cell Cu1.  
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3 217 For all wings an area enclosed by three clear landmarks was used as a proxy of wing size  
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5 218 (Figure 1). The same protocol was used for both experimental and field-caught individuals.  
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9 220 *Statistical analyses*

10 221 Prior to analyses, all wing pattern elements were corrected for wing size and the nine ventral  
11  
12 222 wing-pattern measurements (traits 1-5; see Fig. 1) were reduced using a principal component  
13  
14 223 analysis, pooling all available data. The first principal component (PC1) explained 60 per  
15  
16 224 cent of the total variation and was strongly associated with the effect of the developmental  
17  
18 225 temperature and month of capture. PC2 explained 15 per cent of the variation and was  
19  
20 226 correlated with sex rather than seasonality. In addition, all development times from  
21  
22 227 temperature reaction norm experiment were log-transformed to improve normality. Statistical  
23  
24 228 analyses were performed with the R Statistical Package v 3.1.2 (R Development Core Team  
25  
26 229 2014) and IBM SPSS Statistics (v22). For the field-collected data, 3-way ANOVAs were  
27  
28 230 used to analyse the effect of monthly mean temperature, sex and sampling site on wing  
29  
30 231 pattern morphology (PC1) and butterfly dry mass. Full models were fitted including  
31  
32 232 temperature, sex, population and their interactions, before successive removal of non-  
33  
34 233 significant terms. To investigate seasonal phenology of the butterflies, we tested expected  
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36 234 equal monthly occurrence of butterflies (i.e. frequency of captured butterflies) across months  
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38 235 using Chi-Square tests.  
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45 236 We used 3-way ANOVAs to investigate evaporation stress. For stable oxygen isotope  
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47 237 data collected from adults, we used the  $\delta^{18}\text{O}$  values as the dependent variable and monthly  
48  
49 238 mean temperature, sex and sampling population and their interactions as explanatory  
50  
51 239 variables. In addition, 3-way ANOVAs were used to analyse the effect of monthly mean  
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53 240 temperature, population and habitat on predicted larval water loss (i.e. proportional loss of  
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55 241 body mass per hour).  
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3 242 For the reaction norm experiment, 3-way ANOVAs were used to analyse the effect of  
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5 243 developmental temperature, sex and population on each phenotypic trait of interest. Full  
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7 244 models were fitted initially before successive removal of non-significant terms. The degree of  
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9 245 plasticity was estimated by calculating the effect size (Hedges's  $g$ ), using the means and  
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11 246 standard deviation of the data from 21°C and 27°C, for each sex and population separately.  
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16 248 RESULTS17 249 *Seasonal phenology and plasticity in the wild*

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20 250 Butterfly monitoring revealed that the number of butterflies varied across months ( $\chi^2 =$   
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22 251 353.46,  $df = 8$ ,  $p < 0.001$ ), with a clear absence of adult activity between May and August  
23  
24 252 (Figure 2). Out of 490 *B. safitza* recorded, males were overrepresented with approximately  
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26 253 3:1 ratio in comparison to females.  
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29 254 Variation in ventral wing pattern morphology (PC1) of field-collected specimens of  
30  
31 255 South African *B. safitza* was best explained by the monthly average temperature ( $F_{5,161} =$   
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33 256 12.20,  $p < 0.001$ ) while adult dry mass showed a three-way-interaction: apart from one  
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35 257 sampling site, females were heavier than males and female dry mass varied significantly  
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37 258 depending on the month of capture, whereas it remained similar in males throughout the  
38  
39 259 survey. However, dry mass was also dependent of the sampling site and the month of capture  
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41 260 varied between sites (3-way-interaction among sex, month and site,  $F_{4,202} = 9.87$ ,  $p < 0.001$ ).  
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46 262 *Evaporation stress*

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48 263 Stable isotopes of oxygen ( $\delta^{18}\text{O}$ ) indicated that month ( $F_{5,227} = 26.24$ ,  $p < 0.001$ ) and site  
49  
50 264 ( $F_{2,227} = 12.20$ ,  $p < 0.001$ ) both influenced evaporation stress as measured from adult  
51  
52 265 butterflies. However, there was also an interaction between the month and site ( $F_{8,227} = 2.66$ ,  
53  
54 266  $p < 0.008$ ), and the main flight season coincided with a period when  $\delta^{18}\text{O}$ -values were lowest  
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56 267 (min = 20.03) in contrast to the end of the season with highest  $\delta^{18}\text{O}$ -values (max = 32.84).  
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3 268 This effect, however, was not as strong in the Kapriver population. The lowest  $\delta^{18}\text{O}$ -values  
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5 269 were recorded at Kasouga ( $\bar{x} = 24.84$ ,  $n = 160$ ,  $s.d. = 2.57$ ), followed by Bathurst ( $\bar{x} = 25.24$ ,  
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7 270  $n = 7$ ,  $s.d. = 2.28$ ), whereas butterflies from Kapriver mirrored a higher and more variable  
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9 271 evaporation stress ( $\bar{x} = 25.61$ ,  $n = 76$ ,  $s.d. = 2.63$ ).

10  
11 272 To explore further how evaporation stress may constrain the butterfly niche, we used  
12  
13 273 the laboratory-established physiological relationship to predict larval water loss with respect  
14  
15 274 to abiotic conditions through the monitoring period. We found two interactions that  
16  
17 275 significantly influenced larval water loss: an interaction between site and month ( $F_{8,8857} =$   
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19 276  $5.00$ ,  $p < 0.001$ ), and between site and habitat ( $F_{3,8857} = 14.02$ ,  $p < 0.001$ ). The main effects of  
20  
21 277 site ( $F_{2,8857} = 143.39$ ,  $p < 0.001$ ), month ( $F_{5,8857} = 10.98$ ,  $p < 0.001$ ) and habitat also  
22  
23 278 influenced predicted larval water loss ( $F_{2,8857} = 103.71$ ,  $p < 0.001$ ). Both temperature and  
24  
25 279 humidity fluctuations through the survey period were less dramatic under the shaded-forest  
26  
27 280 canopy than in forest fringes or grasslands (Table 1).  
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### 33 282 *Plastic responses to developmental temperature*

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35 283 In total, 728 individuals were reared in the temperature reaction norm experiment. We found  
36  
37 284 significant interactions between population and temperature for most life history traits and  
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39 285 wing pattern elements, indicating that populations respond differently to developmental  
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41 286 temperature (see supplementary material for all minimum adequate models). For all traits,  
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43 287 except for the pupal development time in females, the degree of plasticity was larger in the  
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45 288 Ugandan population (Figure 3) and the phenotypic differences between populations were  
46  
47 289 wider at higher temperatures. Not all traits were equally plastic in their response to  
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49 290 developmental temperature. Relative to other wing pattern elements, the plastic response of  
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51 291 the large eyespot on the forewing (2), as well as the width of the ventral bands (4-5) was less  
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53 292 pronounced in both populations (Figure 3).  
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3 293 The relationships between developmental temperature and phenotypic variation in the  
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5 294 South African population were linear or continuous in most traits. In contrast, the Ugandan  
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7 295 population showed more discontinuous responses to temperature (Figure 4). For example, the  
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9 296 conspicuousness of the wing pattern elements (PC1) increased dramatically between 23°C  
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11 297 and 25°C in Ugandan population and, as a consequence, all individuals reared at the extremes  
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13 298 of the temperature gradient showed a close phenotypic resemblance. The difference in the  
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15 299 shape of the reaction norm was even more pronounced when using developmental time as a  
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17 300 proxy for environmental variation (Figure 4).  
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## 302 DISCUSSION

303 We show that a natural population of *Bicyclus safitza* in temperate South Africa displays a  
304 different seasonal phenology and reduced developmental plasticity in comparison to a  
305 population from a tropical region in Uganda. In the tropics, populations of *B. safitza* are  
306 typically found in wet-dry seasonal habitats and active butterflies are present here throughout  
307 the year. They exhibit discrete seasonal forms, one with conspicuous, and one with cryptic  
308 wing patterns (Windig 1991, Brakefield and Reitsma 1991, van Bergen et al. 2016). In  
309 contrast, our data show that field-collected specimens in South Africa displayed continuous  
310 morphological variation throughout the year with no individuals showing the large eyespots  
311 typical of the wet season form in the tropical regions. Moreover, a flight season, in which  
312 adult butterflies were active, was followed by a period when no active adults were  
313 encountered.

314 Populations of *B. safitza* that inhabit tropical environments typically survive the  
315 unfavourable conditions of the dry season as semi-active adults that will feed  
316 opportunistically on fruit, and freely and continuously entered fruit-baited traps (Brakefield  
317 and Reitsma 1991, Windig et al. 1994). Our results reveal that a population that colonized a



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3 318 more temperate climate region in southern Africa has adopted a different strategy to cope  
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5 319 with local environmental conditions. In South Africa two large activity peaks in November  
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7 320 and February, and a smaller increase in butterfly numbers in April, were followed by a  
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9 321 quiescent period in which no butterflies were caught until late September. These data suggest  
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11 322 that this tropical butterfly species takes on a different ‘overwintering’ strategy in temperate  
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13 323 climates, possibly with butterflies aestivating as fully dormant individuals in shelters or  
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15 324 surviving in an arrested stage of pre-adult development (Stålhandske et al. 2017).

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18 325 The adjusted phenology as well as the preference for more shaded habitats at the  
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20 326 range margin of the species’ distribution may be associated with different evaporation  
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22 327 constraints. Stable isotopes of oxygen, which in butterflies reflect evaporation rates during  
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24 328 the late larval development (van Bergen et al. 2016), indicated that individuals caught at the  
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26 329 beginning of the flight season experienced high evaporation rates during development. In  
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28 330 contrast, we observed less evaporation stress in the middle of summer months while larval  
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30 331 evaporation rates rose again closer towards the end of the flight season. The quiescent period  
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32 332 of adults could thus reflect coping with evaporation stress and as well as targeting the more  
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34 333 favourable environmental conditions for reproduction (Brakefield and Larsen 1984,  
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36 334 Brakefield and Reitsma 1991). Moreover, larval evaporation rates were predicted to be  
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38 335 significantly higher in forest fringes and open grassland compared to more shaded habitats. In  
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40 336 the shaded forests the temperature was mild, humidity high and vapour pressure deficit low,  
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42 337 which provides a buffer against the weather extremes (Addo-Bediako et al. 2001, Chown et  
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44 338 al. 2011). Thus, it is possible that the maintenance of the body water balance, together with  
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46 339 plant–insect co-evolution (Braschler and Hill 2007, Nokelainen et al. 2016), constrains  
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48 340 populations of *B. safitza* to microclimates provided by shade-habitats in temperate zone.

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50 341 Our results show that local adaptation of this tropical butterfly species to more  
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52 342 temperate climatic conditions in southern Africa involved the evolution of developmental  
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3 343 plasticity. Field surveys confirmed the absence of polyphenism in the wild and, when  
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5 344 compared in the laboratory to a population from the tropics, the South African population  
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7 345 showed more robust development in response to thermal variation for a broad suite of  
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9 346 morphological and life history traits. In addition to geographical variation in the degree of  
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11 347 plasticity (i.e. the steepness of the reaction norm), we also observed clear differences in the  
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13 348 shapes of reaction norms between the two populations. The expression of ventral wing  
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15 349 pattern elements responded in a discontinuous manner to the temperature gradient, which is  
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17 350 typical of polyphenism, while the relationship between developmental temperature and  
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19 351 phenotypic variation in the South African population was more or less linear for these traits.  
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23 352 The evolution of developmental plasticity in this group of butterflies has been studied  
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25 353 by conducting artificial selection experiments using *B. anynana*, a species closely related to  
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27 354 *B. safitza* and a model system in the field of eco-evo-devo (Brakefield et al. 2009). Rapid  
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29 355 responses to selection were observed with respect to the height of reaction norms (intercept),  
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31 356 while developmental plasticity for eyespot size was retained in the selection lines (Brakefield  
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33 357 et al. 1996). In contrast, attempts to change the slope (steeper or shallower) or the shape of  
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35 358 the reaction norms were largely unsuccessful (Wijngaarden et al. 2002), which suggested that  
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37 359 the slope of reaction norms is unlikely to evolve as readily as the intercept. Subsequent  
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39 360 studies using two different tropical populations of *B. anynana* from different latitudes  
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41 361 revealed parallel reaction norms for a suite of traits and no obvious genotype-by-environment  
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43 362 interactions (de Jong et al. 2010), confirming the results obtained in the laboratory.  
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45 363 Moreover, recent work confirms that intra-population genetic variation for plasticity is highly  
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47 364 depleted in *B. anynana* (Oostra et al. 2017), which may hinder expansions of this species into  
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49 365 environments with different seasonal and ecological dynamics. The striking differences in  
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51 366 plasticity among populations described in the present study indicate that natural populations  
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53 367 of *B. safitza* contain sufficient genetic variation in the response to thermal variation for  
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3 368 developmental plasticity to evolve. In insects, including *Bicyclus anynana* (Koch et al. 1996,  
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5 369 Mateus et al. 2014, Monteiro et al. 2015), developmental plasticity is often mediated by  
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7 370 endocrine signalling (Nijhout 1999, Zera et al. 2007). The evolution of environmentally  
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9 371 sensitive traits, as shown here for *B. safitza*, likely involved evolutionary changes in the  
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11 372 levels and timing of systemic hormone titres in response to external cues or in the degree and  
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13 373 timing of the sensitivity of hormonal receptors in the developing target tissues.

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16 374 Finally, phenotypic plasticity not only enables organisms to cope with environmental  
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18 375 heterogeneity, such as seasonal variation in climatic conditions, but it may also enable  
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20 376 dispersal into regions with climates to which organisms are not adapted to at source (West-  
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22 377 Eberhard 2003, Wund et al. 2008, Gibert 2017). Upon exposure to novel environmental  
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24 378 variation, plasticity provides an immediate shift in phenotypic variation, leading to increased  
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26 379 population persistence and providing time for adaptive evolution to take place. Based on the  
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28 380 data from the two populations studied here, we postulate that developmental plasticity of the  
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30 381 ancestral population of *B. safitza* may have facilitated the process of local adaptation to  
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32 382 temperate climates. Interestingly, individuals from the extant populations mate and produce  
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34 383 viable offspring in the laboratory (personal observations). However, hybrid females  
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36 384 demonstrated signs of reduced fertility, which is in line with Haldane's rule: the preferential  
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38 385 hybrid sterility of the heterogametic sex. This may imply that the South African population of  
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40 386 *B. safitza* is becoming genetically isolated and may be on its way to evolving full  
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42 387 reproductive isolation.

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52  
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55 392 experiments. ON and EvB performed the experiments, analysed the data and wrote the  
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## 399 FIGURE LEGENDS

400 Figure 1: Wing pattern elements measured in all specimens of *Bicyclus safitza*. The images  
401 represent the typical phenotype of individuals reared at 27°C (row 1 and 3) and 21°C (row 2  
402 and 4). The first two columns represent females (left) and males (right) of the population  
403 from the Kasouga field site in South Africa. The last two columns represent individuals of the  
404 population from Semuliki National Park in Uganda. For each individual, we obtained 11  
405 wing measurements corresponding to three categories of traits: ventral eyespots (1-3), ventral  
406 bands (4-5) and wing areas (6-7). Different letter codes were used to refer to the  
407 corresponding yellow rings (y), black discs (b) and white pupils (w). References to wing  
408 veins and cells follow the Comstock-Needham system (Miller 1970). The icons were  
409 provided by Manuel Marques-Pita and adjusted from Mateus et al. (2014).

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411 Figure 2: Seasonal phenology of *Bicyclus safitza*. A) Monthly numbers of butterflies captured  
412 during the longitudinal survey in the Eastern Cape, South Africa (2014-2015) are given in  
413 log<sub>2</sub>-scale while the red dots represent monthly mean temperatures for a period of 30 years  
414 (1980-2009). In the temperate climate zone a flight period (Oct-Apr) was followed by a  
415 period in which no active adults were encountered (May-Aug). B) In tropical climate zones,  
416 here represented by data from Zomba in Malawi (1988-1989), adults of *B. safitza* are actively  
417 present throughout the year. Data presented in panel B were derived from Windig *et al*  
418 (1994).

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420 Figure 3: The degree of plasticity for a suite of phenotypic traits in populations of *Bicyclus*  
421 *safitza* from temperate South Africa (blue) and tropical Uganda (red). Open symbols  
422 represent the degree of plasticity, calculated as the effects size (Hedges' g) between 21°C and  
423 27°C, in females while males are represented by filled symbols. The black circles in the

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3 424 legend at the bottom of the figure represent the effect size in Hedge's  $g$ . For details of the  
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5 425 icons and codes for the wing pattern elements see Figure 1.

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9 427 Figure 4: Effects of developmental temperature (A, B) and development time (C, D) on the  
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11 428 first principal component (PC1) of nine ventral wing pattern elements (see Fig. 1) of *Bicyclus*  
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13 429 *Safitza* butterfly. Panels A and C represent the data from the South African population  
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15 430 whereas panels B and D represent the Ugandan population. Coloured dots represent the  
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17 431 values for individuals reared at 21°C (purple), 23°C (blue), 25°C (pink) and 27°C (red). In  
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19 432 graphs C and D, non-linear sigmoidal curves were fitted and explained over 49% and 80% of  
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21 433 the variation in the population from South Africa and Uganda, respectively.  
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## 434 TABLES

435 Table 1: Variation in climatic conditions during a field survey of *Bicyclus safitza* in Eastern  
 436 Cape, South Africa. Table shows air temperature (C °), relative humidity (RH %) and vapour  
 437 pressure deficit (VPD kPa) range values and their standard deviations in three habitat types  
 438 along the coastal range. Vapour-pressure deficit is the difference between the amount of  
 439 moisture in the air and how much moisture the air can hold when it is saturated, and thus  
 440 important in understanding the conditions under which an organism maintains its water  
 441 balance during temperature changes.

Abiotic factor	min	mean	max	s.d.
Temperature				
Open	11.05	21.50	43.54	4.80
Edge	8.05	21.40	52.08	5.21
Closed	9.10	20.49	39.60	3.90
Relative humidity				
Open	12.59	77.80	100	16.75
Edge	12.57	79.54	100	16.08
Closed	17.25	84.16	100	13.45
Vapour pressure deficit				
Open	0	0.71	7.41	0.82
Edge	0	0.67	12.01	0.86
Closed	0	0.45	6.00	0.51

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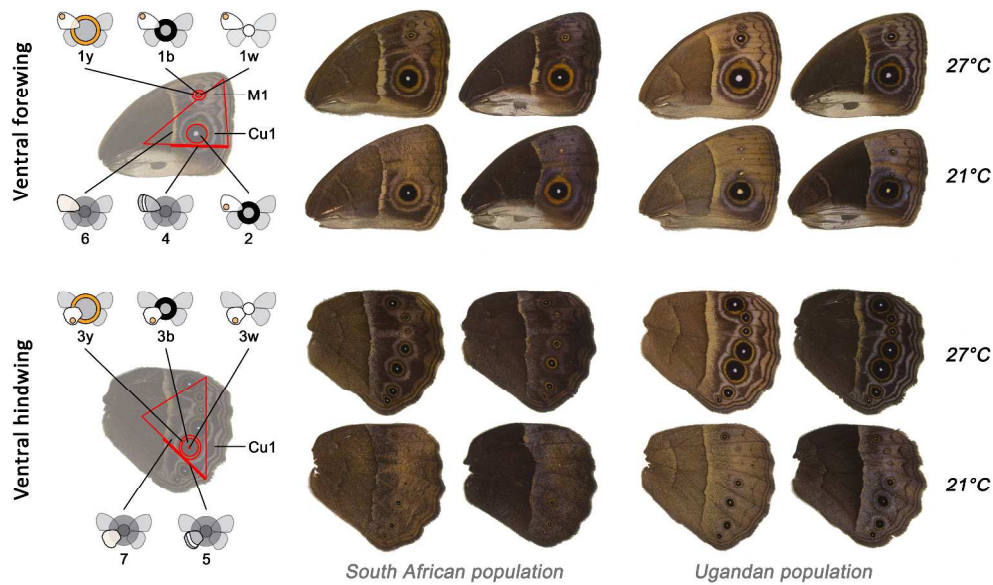


Figure 1: Wing pattern elements measured in all specimens of *Bicyclus safitza*. The images represent the typical phenotype of individuals reared at 27°C (row 1 and 3) and 21°C (row 2 and 4). The first two columns represent females (left) and males (right) of the population from the Kasouga field site in South Africa. The last two columns represent individuals of the population from Semuliki National Park in Uganda. For each individual, we obtained 11 wing measurements corresponding to three categories of traits: ventral eyespots (1-3), ventral bands (4-5) and wing areas (6-7). Different letter codes were used to refer to the corresponding yellow rings (y), black discs (b) and white pupils (w). References to wing veins and cells follow the Comstock-Needham system (Miller 1970). The icons were provided by Manuel Marques-Pita and adjusted from Mateus et al. (2014).

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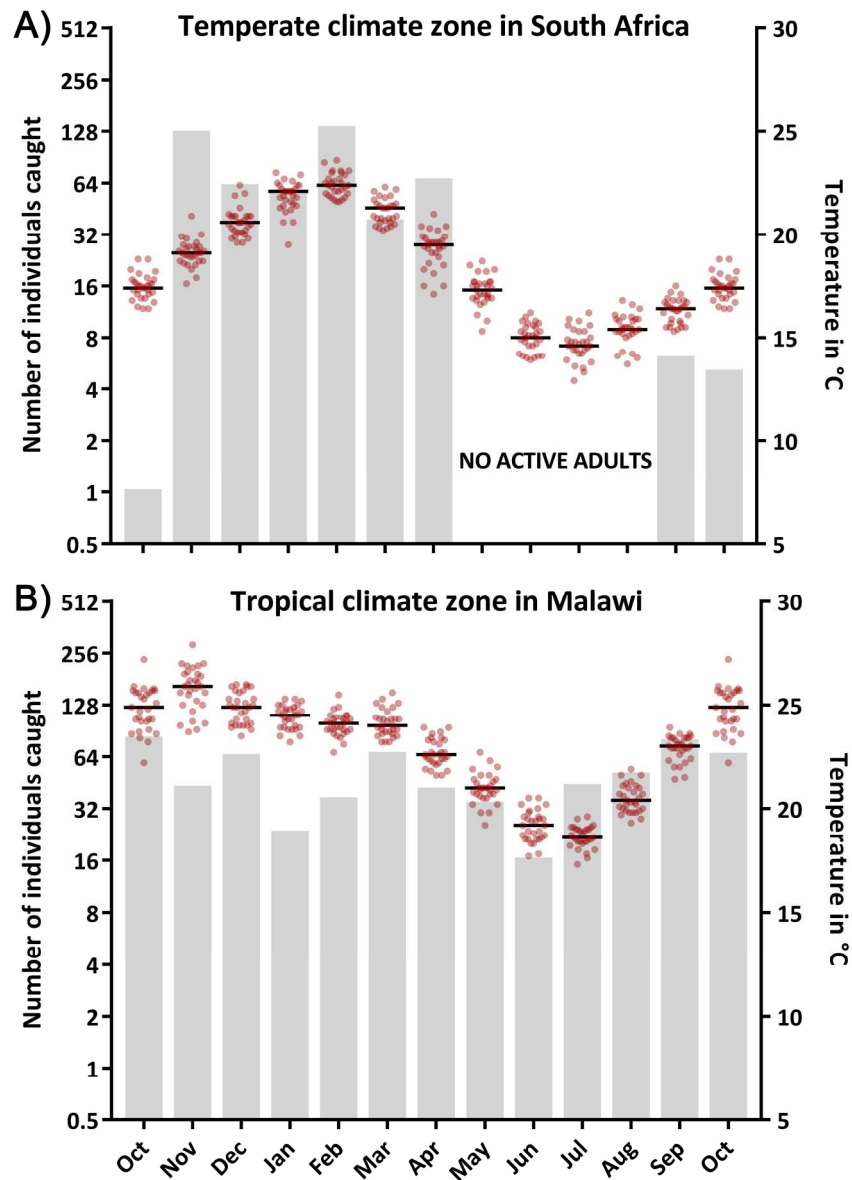


Figure 2: Seasonal phenology of *Bicyclus safitza*. A) Monthly numbers of butterflies captured during the longitudinal survey in the Eastern Cape, South Africa (2014-2015) are given in log<sub>2</sub>-scale while the red dots represent monthly mean temperatures for a period of 30 years (1980-2009). In the temperate climate zone a flight period (Oct-Apr) was followed by a period in which no active adults were encountered (May-Aug). B) In tropical climate zones, here represented by data from Zomba in Malawi (1988-1989), adults of *B. safitza* are actively present throughout the year. Data presented in panel B were derived from Windig et al (1994).

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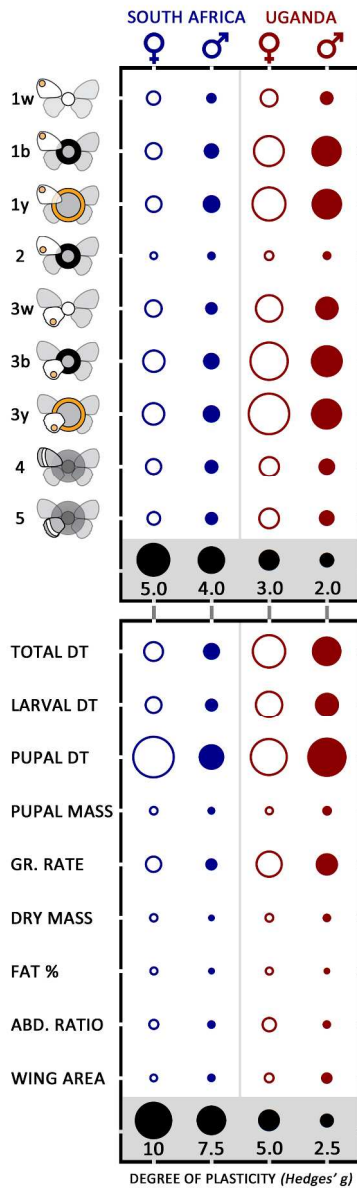


Figure 3: The degree of plasticity for a suite of phenotypic traits in populations of *Bicyclus safitza* from temperate South Africa (blue) and tropical Uganda (red). Open symbols represent the degree of plasticity, calculated as the effects size (Hedges' g) between 21°C and 27°C, in females while males are represented by filled symbols. The black circles in the legend at the bottom of the figure represent the effect size in Hedges' g. For details of the icons and codes for the wing pattern elements see Figure 1.

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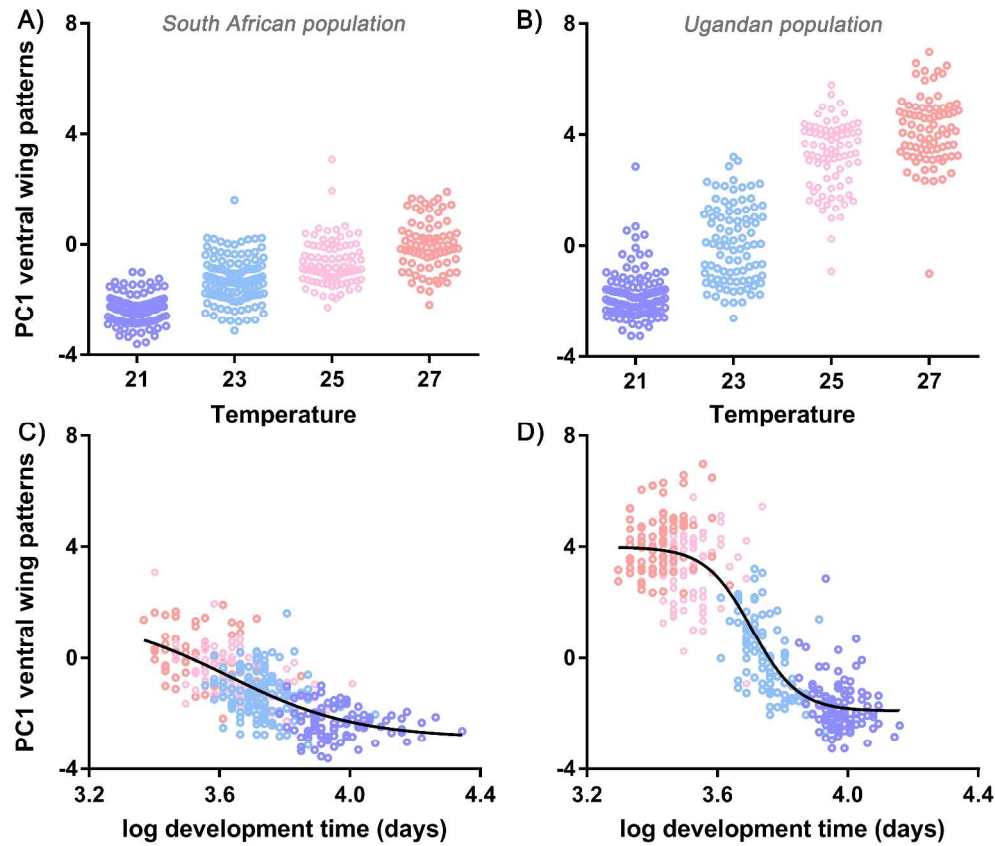


Figure 4: Effects of developmental temperature (A, B) and development time (C, D) on the first principal component (PC1) of nine ventral wing pattern elements (see Fig. 1) of *Bicyclus Safitza* butterfly. Panels A and C represent the data from the South African population whereas panels B and D represent the Ugandan population. Coloured dots represent the values for individuals reared at 21°C (purple), 23°C (blue), 25°C (pink) and 27°C (red). In graphs C and D, non-linear sigmoidal curves were fitted and explained over 49% and 80% of the variation in the population from South Africa and Uganda, respectively.

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Table 1 Minimum adequate models of the effect of population, developmental temperature and sex on a suite of phenotypic traits in populations of *Bicyclus safitza* from Uganda and South Africa, related to figure 3 in the main text and figure 1 in the supplementary material. Significant interactions between population and temperature are denoted in bold, indicating both populations respond significantly different to developmental temperature. The codes for the wing pattern element refer to figure 1 in the main text. Statistical significance is indicated as: \* P <0.05, \*\* P <0.01, \*\*\* P <0.001. Data of the population from Uganda have previously been used in another study (van Bergen et al. 2017).

Trait	(transformation)	fixed effects	F	df	df	P
Total development time (d)	(log)	Population	51.3	1	715	***
		Temperature	1057.2	3	715	***
		Sex	27.3	1	715	***
		<b>Population x Temperature</b>	43.8	3	715	***
Larval development time (d)	(log)	Population	86.8	1	719	***
		Temperature	572.4	3	719	***
		Sex	49.7	1	719	***
		<b>Population x Temperature</b>	28.9	3	719	***
Pupal development time (d)	(log)	Population	68.7	1	715	***
		Temperature	2785.3	3	715	***
		Sex	100.3	1	715	***
		<b>Population x Temperature</b>	66.3	3	715	***
Pupal mass (mg)		Population	492.5	1	719	***
		Temperature	21.0	3	719	***
		Sex	518.5	1	719	***
		Temperature x Sex	4.9	3	719	**
Growth Rate (ln mg/d)	(log)	Population	190.5	1	719	***
		Temperature	459.8	3	719	***
		Sex	4.4	1	719	*
		<b>Population x Temperature</b>	37.4	3	719	***



Table 1 *Continued*

Trait	(transformation)	fixed effects	F	df	df	P
Adult Drymass (mg)		Population	232.3	1	714	***
		Temperature	11.0	3	714	***
		Sex	1836.8	1	714	***
		Population x Sex	7.8	1	714	**
Adult Fat content (%)	(arcine)	Sex	127.8	1	713	***
Abdomen Ratio (%)	(arcine)	Population	9.5	1	712	**
		Temperature	35.2	3	712	***
		Sex	3082.7	1	712	***
		<b>Population x Temperature</b>	3.4	3	712	*
Total Wing Area (mm <sup>2</sup> )		Population	1299.6	1	710	***
		Temperature	25.4	3	710	***
		Sex	1384.5	1	710	***
		<b>Population x Temperature</b>	4.6	3	710	**
		Population x Sex	4.1	1	710	**
Wing pattern element 1y	(size corrected)	Population	135.1	1	715	***
		Temperature	363.2	3	715	***
		<b>Population x Temperature</b>	88.0	3	715	***
Wing pattern element 1b	(size corrected)	Population	490.1	1	714	***
		Temperature	325.2	3	714	***
		Sex	4.5	1	714	***
		<b>Population x Temperature</b>	113.2	3	714	***
Wing pattern element 1w	(size corrected)	Population	605.3	1	713	***
		Temperature	119.4	3	713	***
		Sex	7.5	1	713	**
		<b>Population x Temperature</b>	26.2	3	713	***
		Population x Sex	7.4	1	713	**
Wing pattern element 2	(size corrected)	Population	188.5	1	716	***
		Temperature	29.8	3	716	***
		Sex	100.1	1	716	***
		Population x Sex	16.1	3	716	***

Table 1 *Continued*

Trait	(transformation)	fixed effects	F	df	df	P
Wing pattern element 3y	(size corrected)	Population	776.2	1	717	***
		Temperature	490.2	3	717	***
		Sex	4.0	1	717	*
		<b>Population x Temperature</b>	113.6	3	717	***
Wing pattern element 3b	(size corrected)	Population	1197.9	1	718	***
		Temperature	447.7	3	718	***
		<b>Population x Temperature</b>	145.9	3	718	***
Wing pattern element 3w	(size corrected)	Population	1456.8	1	716	***
		Temperature	335.9	3	716	***
		Sex	16.0	1	716	***
		<b>Population x Temperature</b>	168.6	3	716	***
		Population x Sex	6.0	1	716	*
Wing pattern element 4	(size corrected)	Population	214.3	1	716	***
		Temperature	175.3	3	716	***
		Sex	467.6	1	716	***
		Population x Sex	4.2	1	716	*
Wing pattern element 5	(size corrected)	Population	18.7	1	719	***
		Temperature	146.4	3	719	***
		Sex	192.5	1	719	***
		Population x Sex	9.8	1	719	**

Figure 1 Reaction norm representations of the effect of population, developmental temperature and sex on a suite of phenotypic traits in populations of *Bicyclus safitza* from Uganda and South Africa, related to figure 3 in the main text and table 1 in the supplementary material. Data from males is given in the left hand panel, females in the panel on the right. The South African population is represented by the filled symbols and dashed lines. Ugandan population is represented open symbols and solid lines. Codes for the wing pattern element refer to figure 1 in the main text and error bars represent 95% confidence intervals.

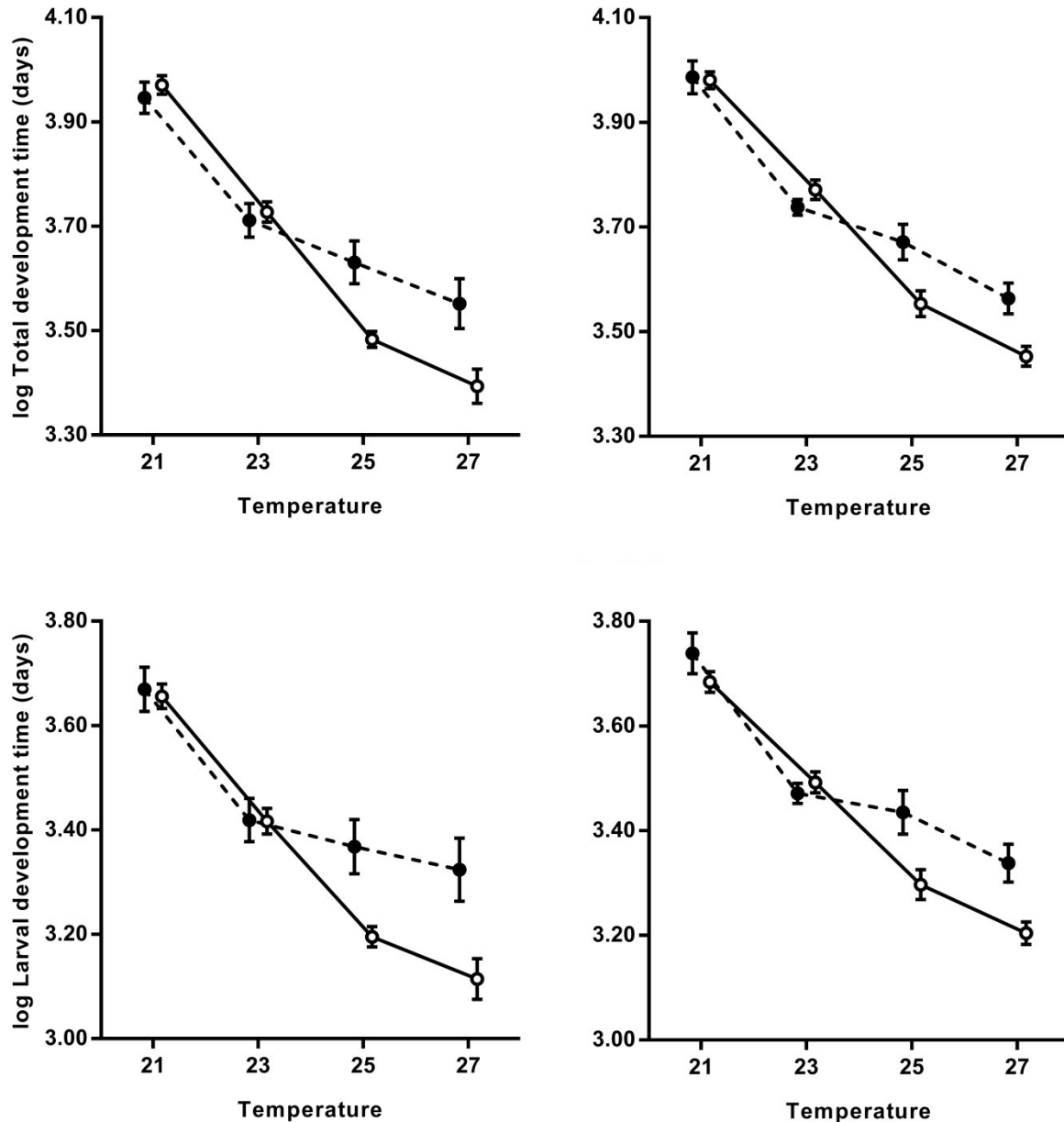


Figure 1 *Continued*

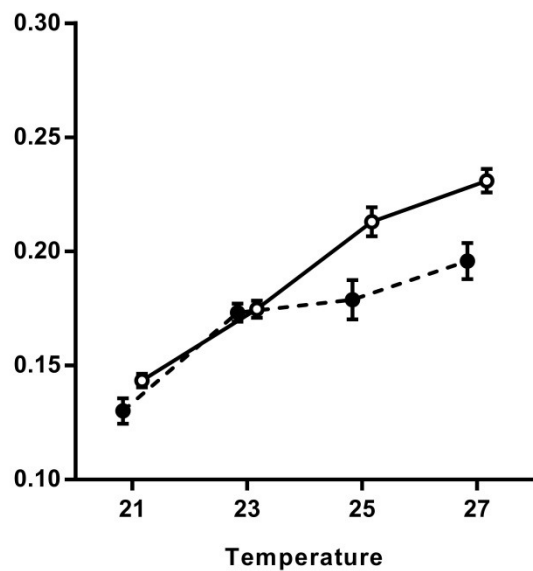
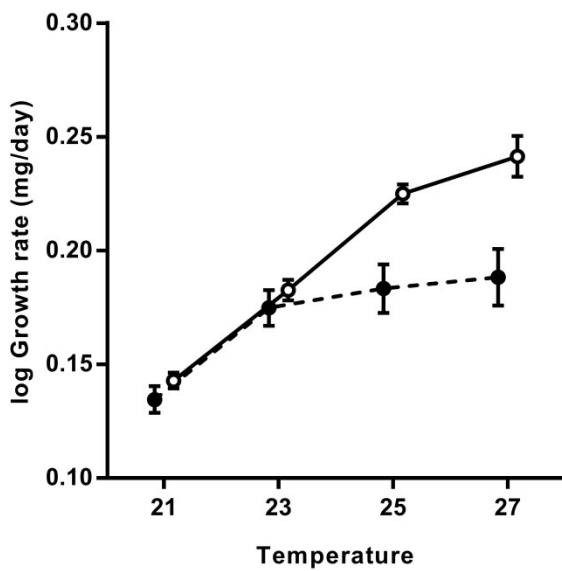
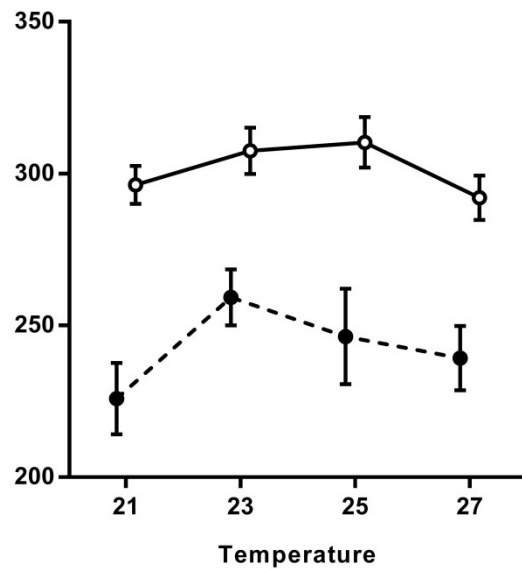
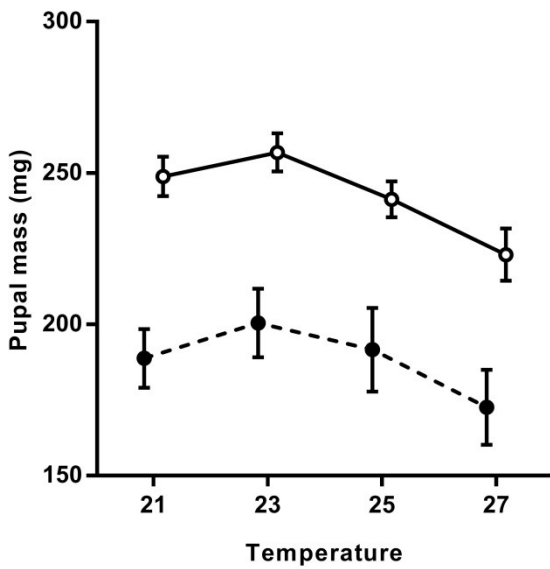
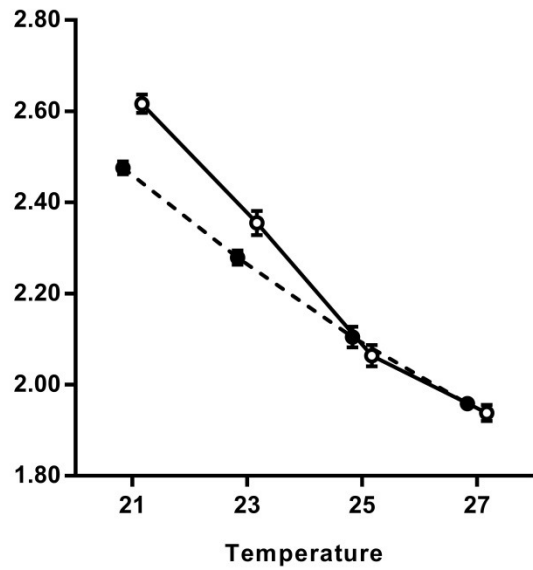
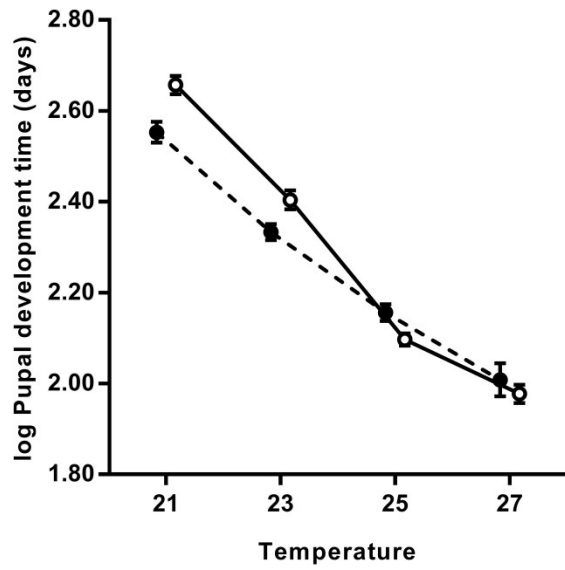


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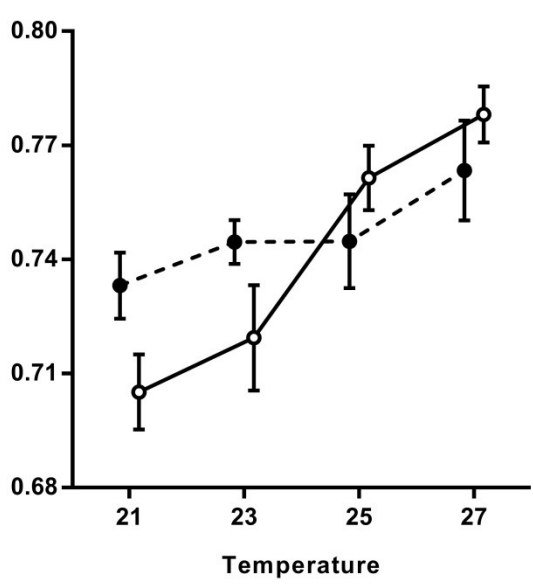
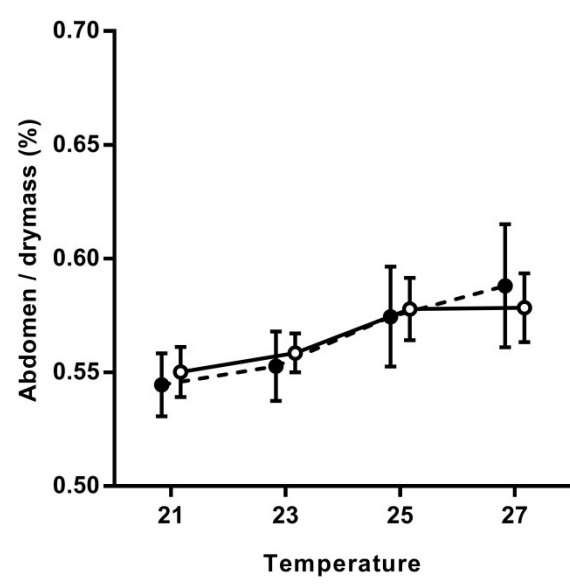
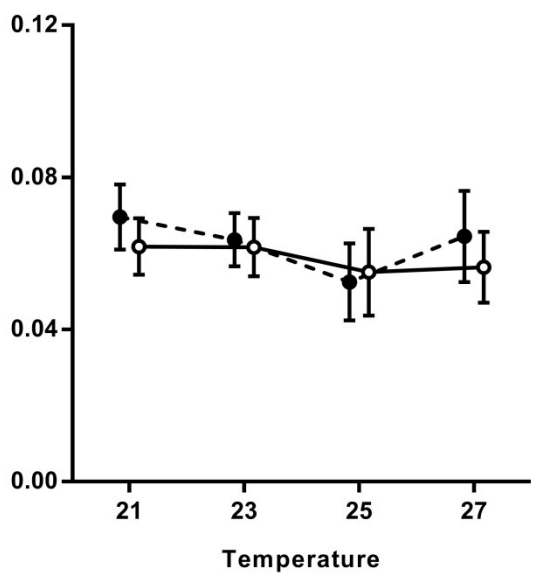
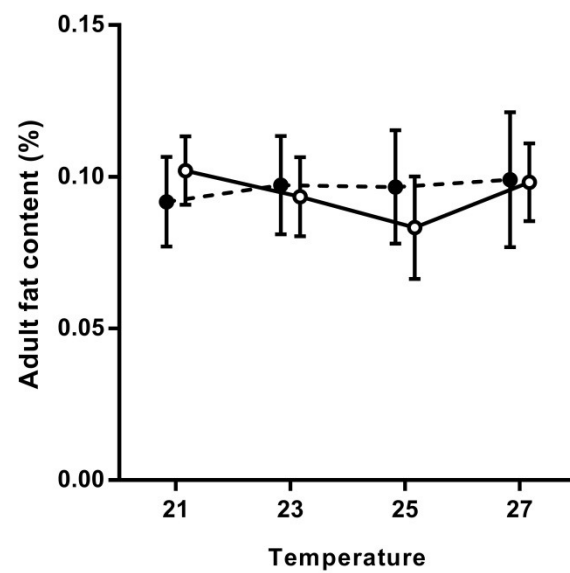
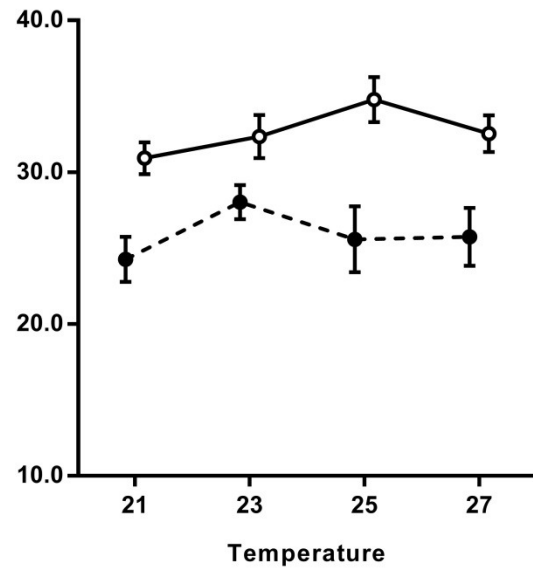
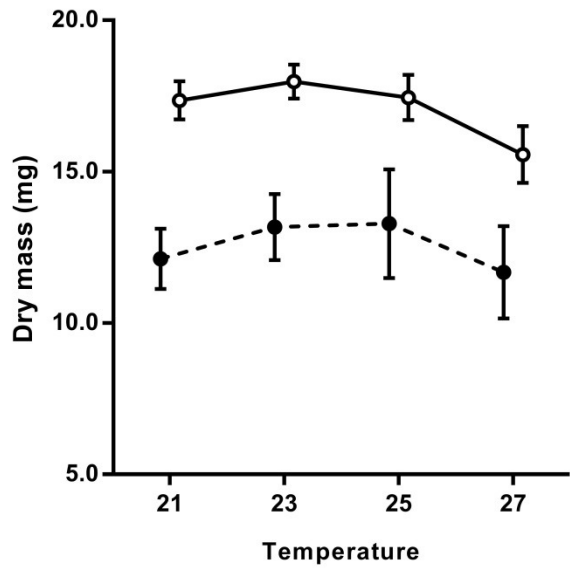


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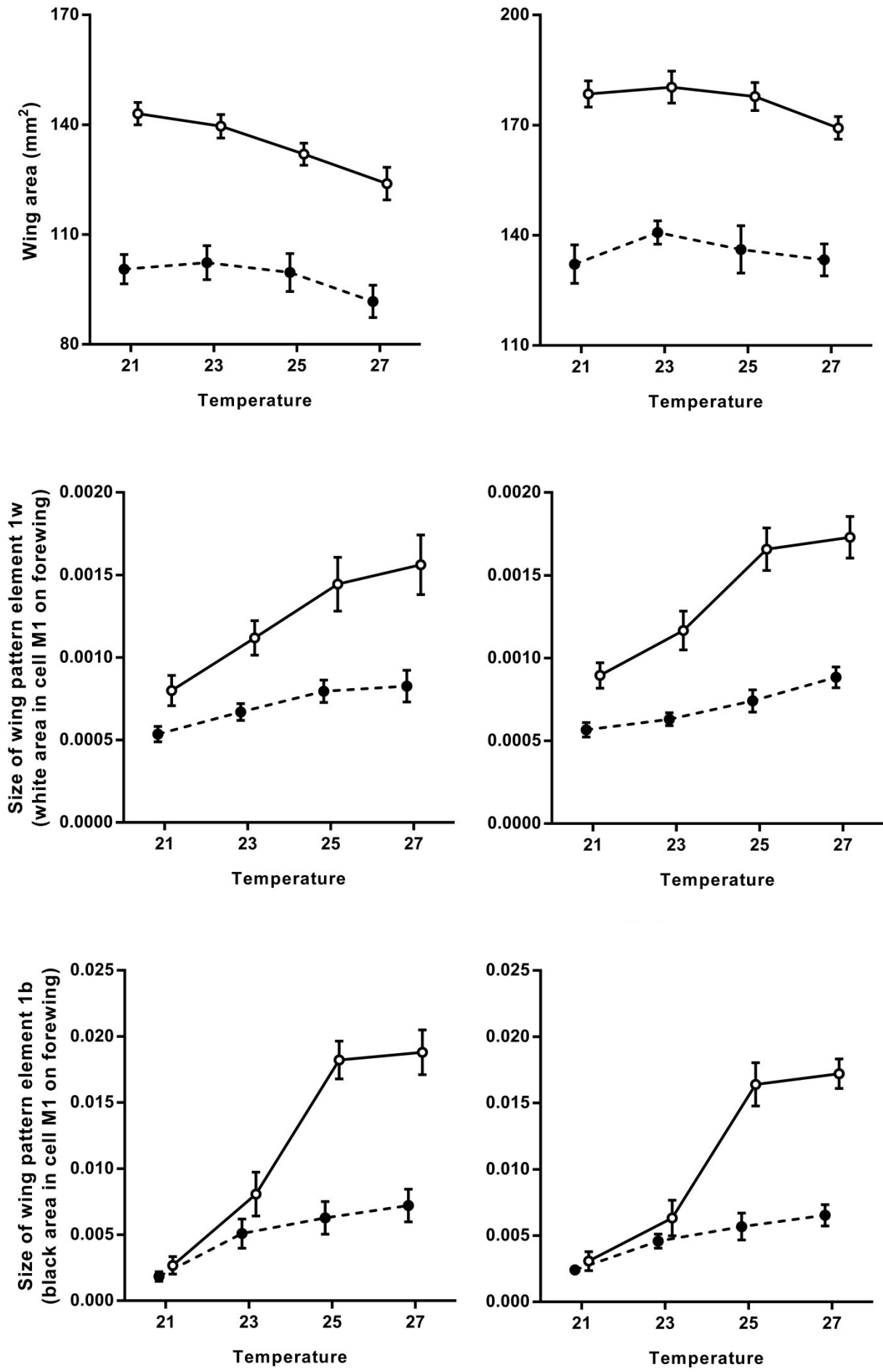


Figure 1 *Continued*

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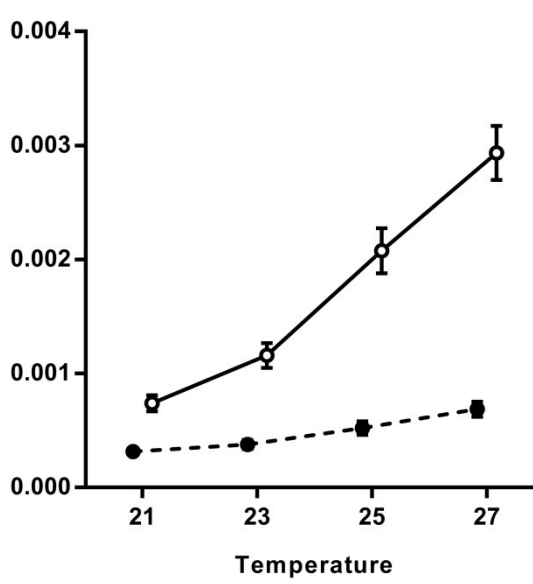
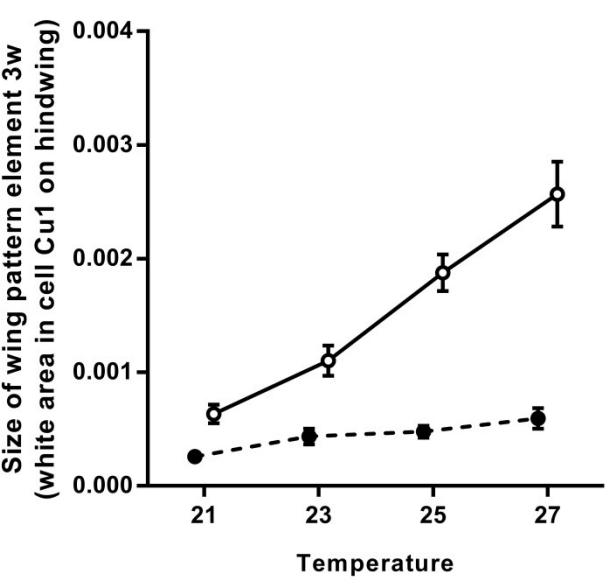
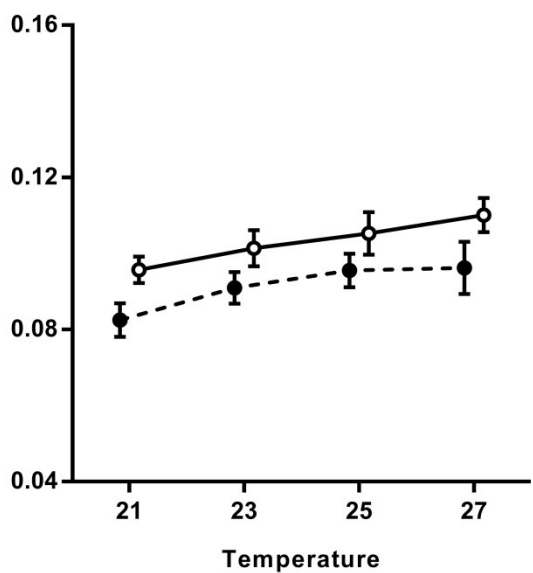
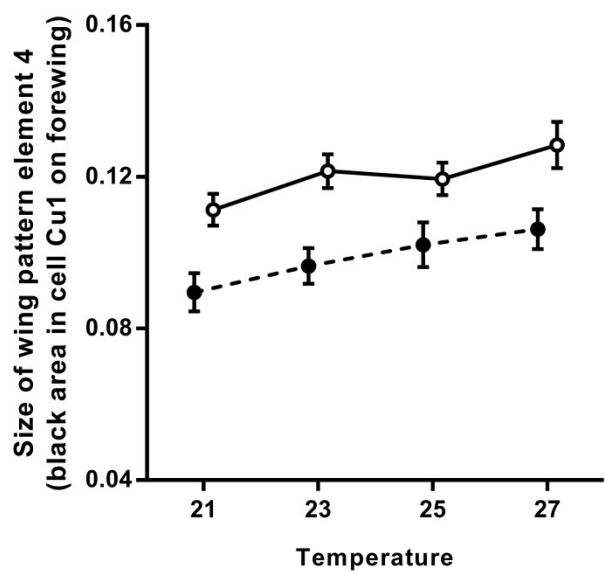
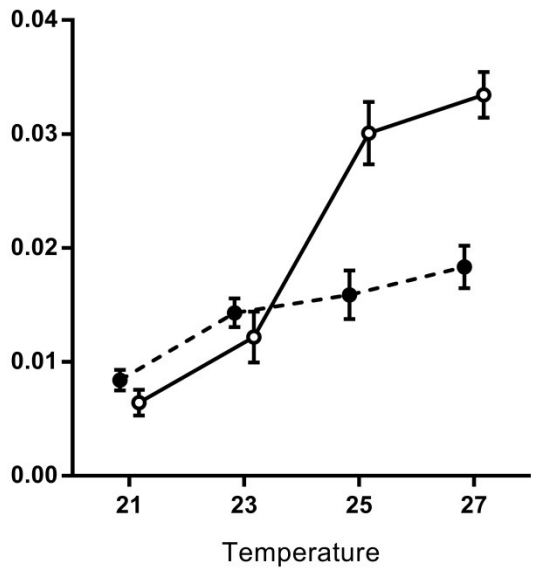
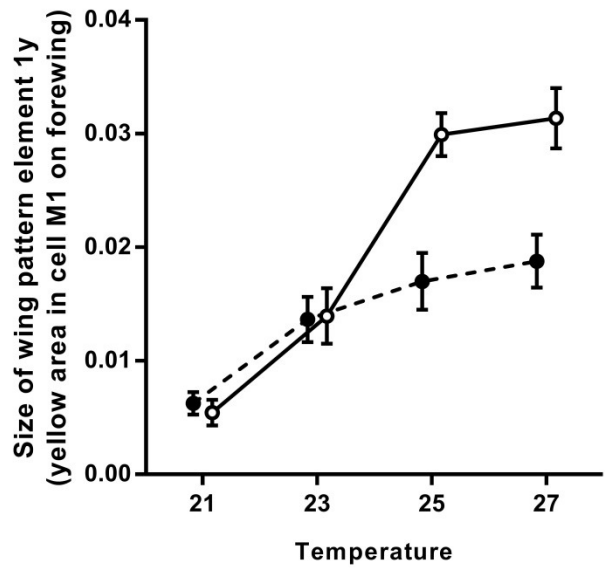


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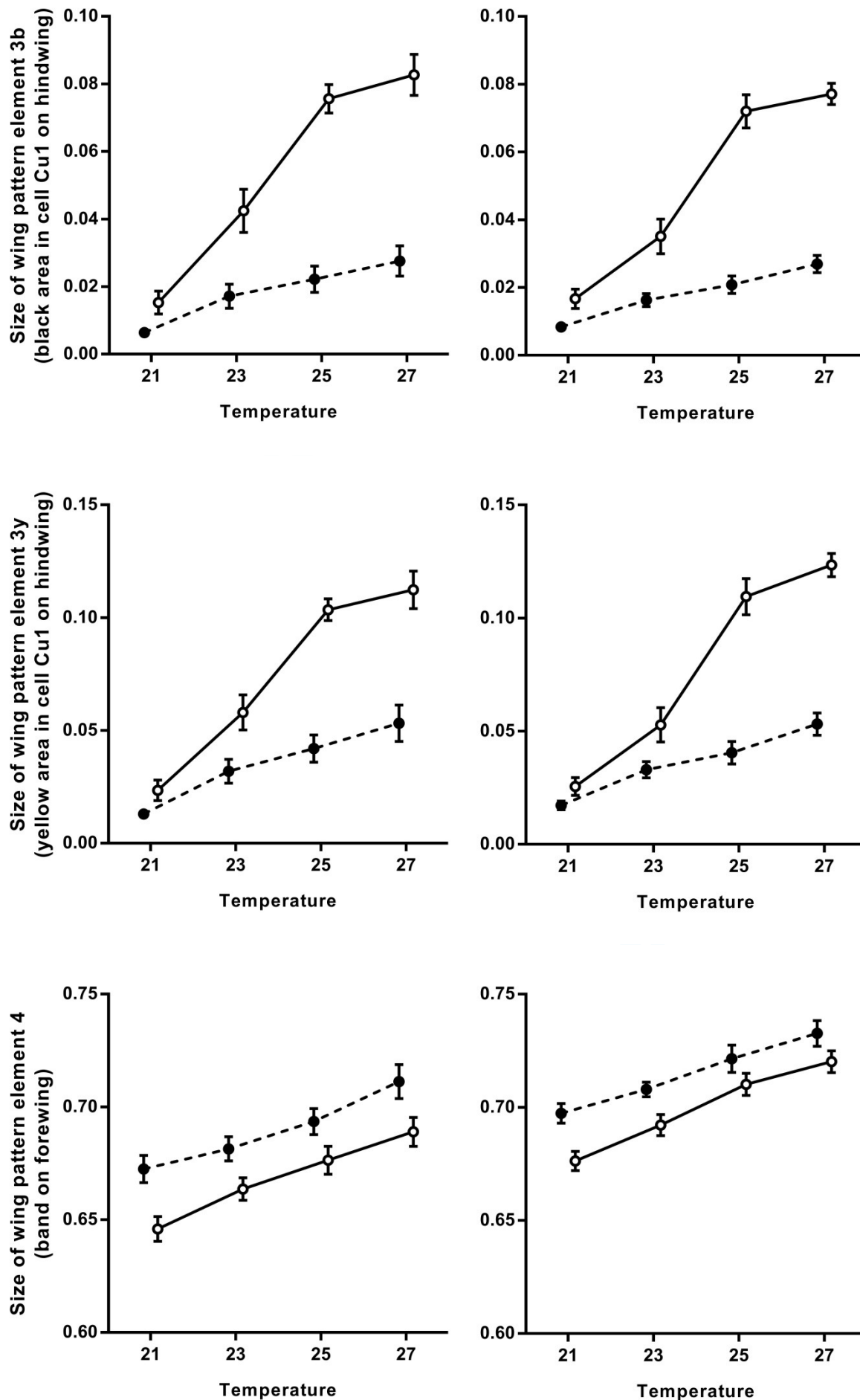




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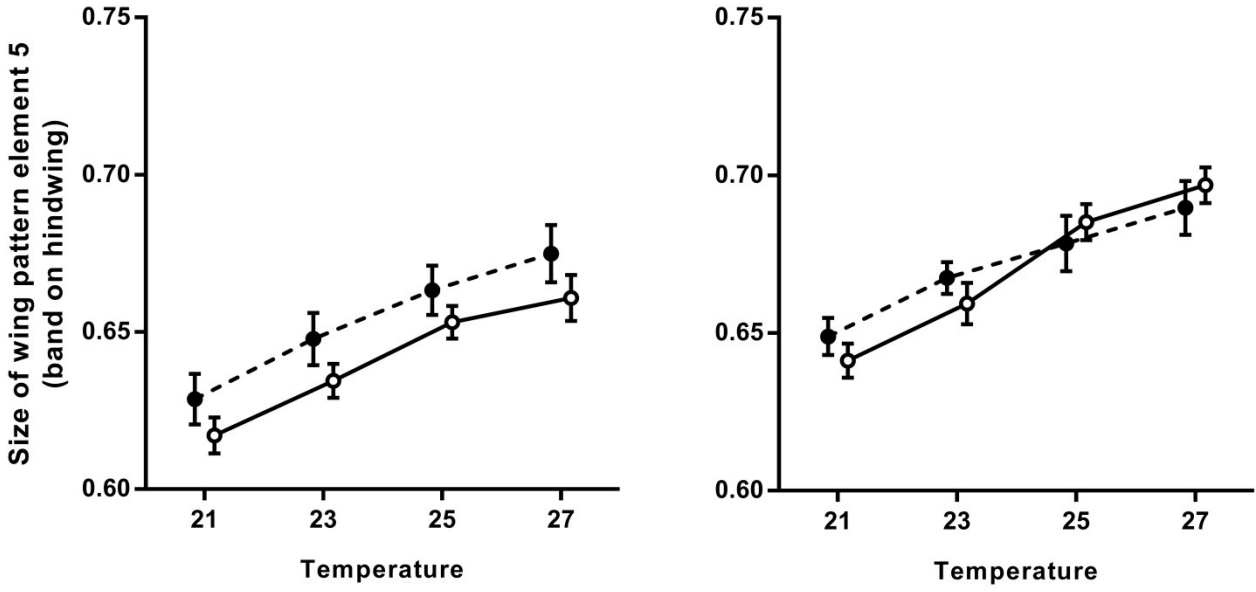


Table 2: Extrapolated larval water loss of *Bicyclus safitza* in Eastern Cape, South Africa. Water loss was calculated as a percentage of larval body mass per hour (% H<sub>2</sub>O g<sup>-1</sup> hr<sup>-1</sup>). Negative values indicate water uptake (i.e. weight gain), while positive values indicate water (or weight) loss in relation to body weight (i.e. 100 would indicate the loss of entire body weight within an hour).

Larval water loss	min	mean	max	s.d.
Habitat				
Open	-10.46	-5.11	38.52	5.36
Edge	-11.11	-5.38	68.47	5.66
Closed	-10.44	-6.80	29.35	3.36
Site				
Kapriver	-10.46	-4.62	68.47	6.66
Kasouga	-11.11	-6.69	16.53	2.89
Bathurst	-10.44	-6.52	24.84	3.99
Month				
November	-9.79	-5.79	5.06	3.13
December	-10.11	-6.04	19.14	4.20
January	-10.45	-5.28	30.90	5.27
February	-11.11	-6.24	35.09	4.45
March	-10.51	-5.75	68.47	5.90
April	-10.46	-6.08	27.47	4.12

\*Negative values imply water uptake and positive values water loss.