RESEARCH ARTICLE

Potential constraints on evolution: sexual dimorphism and the problem of protandry in the butterfly *Bicyclus anynana*

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Abstract

The earlier mean adult emergence between males and females, protandry, has been well studied mathematically and in comparative studies. However, quantitative and evolutionary genetic research on protandry is scarce. The butterfly, *Bicyclus anynana* exhibits protandry and here we selected for each of the different combinations of male and female development time in this species, thus including direct selection on protandry (i.e., FAST, fast males and fast females; SLOW, slow males and slow females; FMSF, fast males and slow females; and SMFF, slow males and fast females). After eight generations of selection there was no significant response for increased or decreased protandry, whereas selection for increased or decreased development time in both sexes (FAST or SLOW) was successful. Continued selection (> 30 generations) for decreased or increased protandry showed a significant difference between the $FMSF_C$ and $SMFF_C$ lines (subscript c for continued selection), which was of the same magnitude as the nonsignificant difference observed between the FMSF and SMFF lines at generation eight. This indicated that the initial selection was successful, but that the difference between the lines did not increase with continued selection. Our results also indicate that the genetic covariance across sexes for development time is near unity. Interestingly, lines selected for decreased protandry (SMFF) had lower egg-to-adult survival, and broods from these lines had lower rates of egg hatching. This suggests that interactions with fertility might constrain certain directions of change in patterns of protandry. Moreover, selection yielded a change in the ratio of male to female development time for slow lines, suggesting that some amount of sex-specific genetic variance for development time is still present in this population. The $FMSF_C$ line showed the largest effect of selection on protandry, mainly through an effect on female developmental time. Lastly, our results show that temperature has an effect on the amount of protandry in the selected lines. These results are discussed in relation to the ecology of this species and the evolution of protandry.

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Introduction

One trait which frequently exhibits sexual dimorphism is developmental time; many animal species show an earlier adult emergence (or arrival) of males than of females, a phenomenon called protandry. Patterns of protandry have been well described in various animal taxa such as birds, fish and insects (reviewed in Morbey and Ydenberg 2001). Most research on protandry has tended to be descriptive, although some experimental work on factors affecting protandry has been done, mainly on arthropods (e.g., Nylin *et al*. 1993; Bradshaw *et al*. 1997).

Theoretical studies predict that protandry can be advantageous for males because it increases their probability of mating, but this can be balanced by an increased chance of death before reproduction (Wiklund and Fagerström 1977). Models suggest that it is these two selective forces that primarily shape the distribution of male and female development time, and that the distribution for males should be truncated at a point determined by pre-emergene and post-emergence mortality (Bulmer 1983; Iwasa *et al*. 1983; Iwasa and Hac-

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cou 1994; Holzapfel and Bradshaw 2002). This hypothesis is also called the 'adaptive' explanation (Wiklund and Solbreck 1982; Morbey and Ydenberg 2001), and protandry is expected to evolve when males are able to mate multiple times, when the first male to mate with a female gains reproductive benefits, and when the overlap between generations is minimal (Wiklund and Fagerström 1977; Singer 1982; Zonneveld 1992).

An alternative to the 'adaptive' explanation is that females, but not males, profit from a longer development time in the form of a higher fecundity due to larger body size, also called the 'incidental' explanation; (Wiklund and Solbreck 1982) or 'indirect selection' (Morbey and Ydenberg 2001). In this view, protandry is thought to be a by-product of asymmetric fitness benefits to the sexes (Thornhill and Alcock 1983). Other factors have also been postulated to shape protandry. It might, for example, be a female tactic to decrease the time they remain unmated and thus minimize prereproductive mortality (Fagerström and Wiklund 1982; Zonneveld and Metz 1991; Morbey and Ydenberg 2001). This advantage, in turn, could be balanced by the fact that female quality may also have a temporal component, so that females emerging later have a lower fitness and for example lay fewer eggs (Kleckner *et al*. 1995; Carvalho *et al*. 1998).

The adaptive and incidental hypotheses have been investigated somewhat independently in different taxa because they are more suited for some mating systems than for others (Morbey and Ydenberg 2001). In this brief introduction, we focus on work done on arthropods, where most of the relevant work has been mainly done on butterflies. Some work on sexual differences in development time has been done on *Drosophila* (Chippindale *et al*. 1997; Prasad *et al*. 2000) but did not specifically aim at changing the difference between the sexes. *Drosophila* is further not really suitable for studying protandry because females typically emerge before males.

In the case of butterflies, the debate between the 'adaptive' or sexual selection hypothesis and the 'incidental' natural selection hypothesis has been resolved to a large extent by comparative work (Nylin *et al*. 1993). Sexual selection seems to be the primary influence, although some argue for a combination of natural and sexual selection (Kleckner *et al*. 1995; Bradshaw *et al*. 1997). At least part of the reconstruction of the evolutionary history of protandry can be deduced from the genetic architecture of the traits involved (Roff 1997). However, the quantitative and evolutionary genetics of protandry have not been well studied. The implications of protandry for other life-history and correlated traits are unknown, and the issue of how these contrasting demands on the two sexes have been integrated into a single genome also remains largely open (Lande 1980; Reeve and Fairbairn 1996; Rhen 2000). To infer evolutionary constraints on divergent (or convergent) evolution between the sexes, genetic parameters alone are not enough; the nature of individual allelic effects is also critical (Rhen 2000).

The main question of the present study is: how constrained, in the short term, is the relationship between male and female development time within a single species? To investigate this, we have used artificial selection experiments on the African butterfly, *Bicyclus anynana*, which is consistently protandrous (Zijlstra *et al*. 2002a). Artificial selection is an especially powerful tool to explore the range of possible phenotypes and ask which phenotypes are more likely than others, and why (Brakefield 2003). Previous selection experiments for fast or slow development time in both sexes showed that there is substantial additive genetic variation for this trait, and that these selection lines retained protandry at all temperatures. However, females of the slow selected lines took relatively longer to develop than males, compared to females from unselected or fast selected lines (Zijlstra *et al*. 2002a). This sex-specific genetic variation suggests that it might be possible to change the relationship of development time between the sexes by selection.

To address the question of how integrated or coupled male and female development time are, we selected for male and female development time in four different combinations of gender and selection for either faster or slower development. For example, by taking the slowest males and mating those to the fastest females we selected for a decrease in protandry. By studying the response to selection we can begin to elucidate the evolutionary genetics of protandry. Is it possible to alter established patterns of protandry based on responses to selection of standing genetic variation? And if so, what are the consequences for the related traits, size at maturity and growth rate (see Berner and Blanckenhorn 2007)?

Material and methods

Butterflies

The stock of *B. anynana* originated from 80 gravid females caught at a single locality in Malawi. It has been kept in the Leiden laboratory for over 10 years at large census sizes in climate controlled rooms with high relative humidity. Caterpillars are reared on young maize plants and adults feed on moist banana.

Protandry selection

From the base stock we established selection lines at 25°C for each combination of male and female egg-to-adult development time. Both sexes were selected for faster development in the FAST lines, and for slower development in the SLOW lines. The FMSF lines (i.e. selected for fast male, and slow female development time) were selected for an increased magnitude of protandry, the SMFF (slow male, fast female) lines for decreased protandry. All selection lines were replicated twice. Approximately 500 eggs per replicate per generation were set up (eggs were always counted) and the mean number of emerged butterflies per replicate cage was approximately 250. We selected the 30–40 males and fe-

males with the most (appropriate) extreme development time for eight generations at 25°C. Virgin adults were kept at a lower temperature (18°C) prior to selection and mating. The FAST lines were reared independently after the first generation and had gained one generation at generation five relative to the other lines (i.e. generation six for FAST). Lines were then again reared concurrently.

The magnitude of protandry is a population measure, and thus we obtained only one data point per line per generation. To obtain multiple estimates per line, we used multiple smaller cages (four per replicate of a selection regime) with ~100 eggs per cage in generations five and eight. In addition, in generation eight we also used an internal mutant control that could be distinguished from the selected lines as adults on the basis of the ventral wing pattern elements (Zijlstra *et al*. 2002b). This latter technique entails that mutants from the same mutant stock are distributed over all cages and reared concurrently with the experimental animals. Hence, they can serve as an internal benchmark to become highly reliable estimates of male and female developmental time per cage, as described in detail by Zijlstra *et al*. (2002b).

Selection continued

After eight generations of selection no statistically significant difference for protandry between the selection lines was found (see Results). In order to investigate whether this resulted from the fact that the duration of selection was limited to eight generations, we continued the selection experiment. However, using the full set of lines was logistically (both in manpower and in space) not feasible. Therefore, after eight generations, selection was continued on the two most divergent FMSF and SMFF lines (now labelled $FMSF_C$ and $SMFF_C$).

Selection was performed as described above, and here we report on the results up until the 33rd generation of selection. In addition, after 20 generations of selection, the $FMSF_C$ and $SMFF_C$ lines were reared in five replicate smaller cages each, together with five replicate smaller cages originating from the outbred parental stock. The lines were reared at 20°C and 27°C, and female and male developmental times were measured for each of the 30 smaller cages.

Slow male – fast female incompatibility

The experiments to test for slow male – fast female incompatibility were set up because of low egg-to-adult survival of the SMFF lines (see Results). Egg-to-adult survival during protandry selection was calculated in each generation using the total number of emerged adults and dividing this by the number of eggs used to start the generation. To test for incompatibilities between slow males and fast females, we mated males and females with varying development times (but same adult age). These butterflies were derived from different selection lines of the eighth generation, but the origin never explained any differences in measured traits. We assessed fecundity (number of eggs laid per day in the first three days) and fertility (number of eggs hatched) of the first laid eggs. These measures are good indicators in our rearing conditions of lifetime fecundity, and egg-to-pupation survival, respectively (Brakefield *et al*. 2001). We also tested mating ability of the different selection lines at generation six. Only males from lines diametrically opposite to each other competed with each other, i.e., FAST versus SLOW and FMSF versus SMFF. Five virgin females from one line were put in a cylindrical hanging cage (0.3 m diameter) with five unmated males from both the same selection line and the other, opposing line. Males from the competing selection lines were differentially marked with a black permanent marker pen on the ventral side of their left hind wing to allow their identification in mating. Copulating pairs were removed and replaced with a new virgin male and female from the appropriate line, thus keeping the numbers of males and females constant during the experiment. Males and females were at least two-days-old and differed no more than one day in development time from their competitors. All individuals used were reared at 25°C and pupal weight was measured one day after pupation to the nearest 0.01 mg using a microbalance. Mating tests were also carried out at this temperature.

Statistics

Protandry was calculated as the mean development time of the males (in days) subtracted from the mean development time of the females. This provides a robust measure of the divergence in male and female emergence time for this species (Zijlstra *et al*. 2002a), and is also in line with what is customary in the literature. Alternatively, and also to calculate confidence intervals, we used bootstrapping; the difference in development time was taken 1000 times between a randomly drawn (with replacement) male and a female from a certain group. The average of these differences is calculated and, together with 999 other such obtained averages, this leads to an estimate for protandry with 95% confidence intervals. Protandry estimates were very similar between both methods, so the easier subtraction method was ordinarily used.

For all analyses of (co)variance, the factors replicate and smaller cage were always random factors, and nonsignificant terms were removed from the model (minimal adequate model, MAM). Realized heritabilities were calculated by regressing the response on the cumulative selection differential (Falconer and Mackay 1996). To obtain estimates of realized heritability for protandry, we used the FMSF and SMFF lines, for divergence in development time we used the difference between FAST and SLOW lines (Falconer and Mackay 1996). Fertility (percentage of hatching eggs) was analysed using logistic regression, and the likelihood-ratio (L-R) χ^2 has one degree of freedom, unless otherwise stated.

To estimate the genetic correlation (r_A) between male and female developmental time that would fit the observed outcome of uncoupling two-trait selection (i.e. FMSF and SMFF selection), we constructed G-matrices and P-matrices

using phenotypic variances (V_P) and covariances based on the FAST, SLOW and base populations. This was done on a population level ($N = 36$) because the covariance between male and female development time cannot be calculated at an individual level. The phenotypic variances thus obtained (V_P males = 6.03, V_P females = 7.48) agreed well with estimates based on the founder population, and also agreed well with previously published results (V_P females = 6.90; Zijlstra *et al.* (2003)). Genetic variances were obtained using the realized heritability for development time and the formula $h^2 = V_A/V_P$ (Falconer and Mackay 1996). We assumed equal heritabilities for the sexes, and a symmetric response to selection. The genetic correlation (r_A) between male and female development time can be calculated using Lande's formula $\Delta\bar{z} = GP^{-1}s$ (Lande 1979; Lande and Arnold 1983; Via and Lande 1985), where s is a vector consisting of the selection differentials and $\Delta\bar{z}$ describes the changes in male and female development time. Thus, we can calculate the minimum value of r_A that can still explain the observed response to uncoupling selection.

The data for the continued selection were analysed using analysis of variance (ANOVA), as described above. The data for the replicate smaller cages at two temperatures were analysed in two ways. Firstly, an ANOVA was performed for developmental time with fixed factors temperature, line and sex, and with smaller cage as a random factor nested within line. A significant temperature × line × sex interaction in the MAM was taken as an indication for changes in the relative developmental time of males and females. Further, a similar analysis was carried out for each temperature to estimate the significance of the line × sex interaction. Secondly, protandry was calculated for each smaller cage to allow the analysis of protandry at the population level using a one-way ANOVA with the smaller cages as the data points.

Results

Initial response to selection

Protandry did not change over the initial eight generations when comparisons are made between the selection lines (figure 1). When each generation was tested separately, the ANOVA on development time never yielded a significant sex × line interaction ($P > 0.14$). Realized heritabilities for protandry ranged from −0.017 to 0.034, and did not differ significantly from zero.

Additive genetic variation was present for development time. Differences in environmental conditions between generations made it difficult to estimate realized heritabilities directly (figure 2). However, we can use the difference between FAST and SLOW selected lines (divergence) to obtain realized heritability for development time: males: $h^2 = 0.11 \pm 0.03$, females $h^2 = 0.12 \pm 0.04$ (both significantly different from zero, $P < 0.05$). The heritability estimates for males and females did not differ from each other; the combined (sexes pooled) estimate is $h^2 = 0.12 \pm 0.04$. Only those generations were used where FAST and SLOW were reared concurrently, although including the other generations did not alter the results.

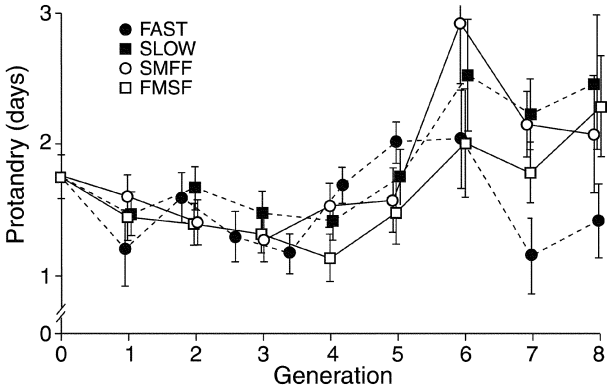

Figure 1. Protandry (difference between mean female and male development time) at 25°C for the different selection lines (replicates pooled ±s.e.). Note that the FAST lines have an extra generation between generations one and five. Thus generation five is in fact the sixth generation for FAST.

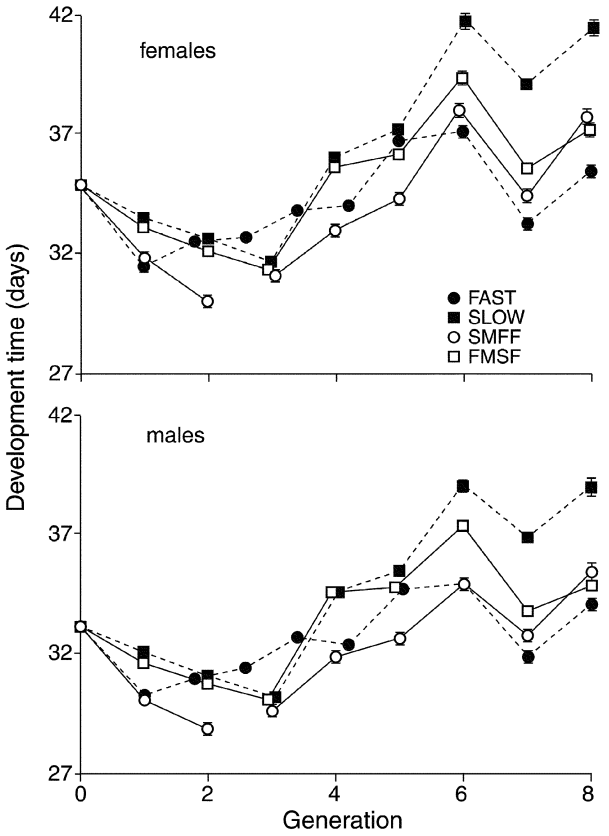

Figure 2. Development times (±s.e.) at 25°C for the different selection lines for females (top) and males. The FAST lines have an extra generation between generations one and five, and the SMFF lines were re-established from the stock, effectively making generation three the second generation 0 for these lines.

Four smaller cages were reared per replicate selection line in generation five (generation six for FAST) thus enabling a comparison of development time. Smaller cages (nested within selection line) differed significantly from each other ($F_{12,1463} = 4.28$, $P < 0.0001$). In contrast, replicates of selection lines did not differ from each other ($F_{4,24} = 1.73$, $P = 0.18$) and could thus be pooled. Males always developed faster than females ($F_{1,1463} = 320.14$, $P < 0.0001$). Selection lines also differed significantly from each other ($F_{3,12} = 7.27$, $P = 0.0049$); the SLOW selected lines were significantly slower than both FAST and SMFF lines, but did not differ significantly from the FMSF lines (figure 3).

In the eight generation of selection we used a mutant as an internal control (benchmark) and development times differed significantly between mutant-corrected selection lines ($F_{3,4} = 51.27$, $P = 0.0012$). Tukey comparisons revealed the following pattern: FAST < SMFF = FMSF < SLOW. The effect of replicate, nested within line, was also significant ($F_{4,1319} = 3.37$, $P = 0.0093$), mainly because of differences between replicates for SMFF. Further, males were consistently faster than females ($F_{1,1319} = 105.09$, $P < 0.0001$).

Continued selection

Figure 4 shows protandry in the $FMSF_C$ and $SMFF_C$ lines for generations eight until 33. With the exception of generation 23, the level of protandry was higher in the $FMSF_C$ line compared to the $SMFF_C$ line, in correspondence with the direction of selection in these lines. Averaged over these generations, protandry in the $FMSF_C$ and $SMFF_C$ line was 2.35 (s.e. 0.18) and 1.57 (s.e. 0.12) days, respectively. Strikingly, this number was nearly identical with the estimated protandry for these lines after eight generations of selection (2.36 and 1.86, respectively; table 1). This indicates that the lack of significant differences in protandry during the eight generations of initial selection was most likely due to lack of statistical power.

Although the results over more than 30 generations of selection indicate that the two selected lines differ in their level of protandry, the difference in protandry does not appear to increase over the generations (analysis not shown). Indeed, if the different generations are considered independent measurements on the same populations, the lines significantly differ in protandry ($F_{1,40} = 6.38$, $P < 0.001$). Interestingly, this differences in protandry appears to be caused by the divergence between the lines of female developmental time ($F_{1,40} = 24.40$, $P < 0.025$), but not of male developmental time ($F_{1,40} = 5.83$, $P > 0.05$; see figure 4).

Response after 20 generations

At generation 20, protandry was measured in replicate populations at two temperatures, and the data are shown in figure 5. The minimum adequate model for developmental

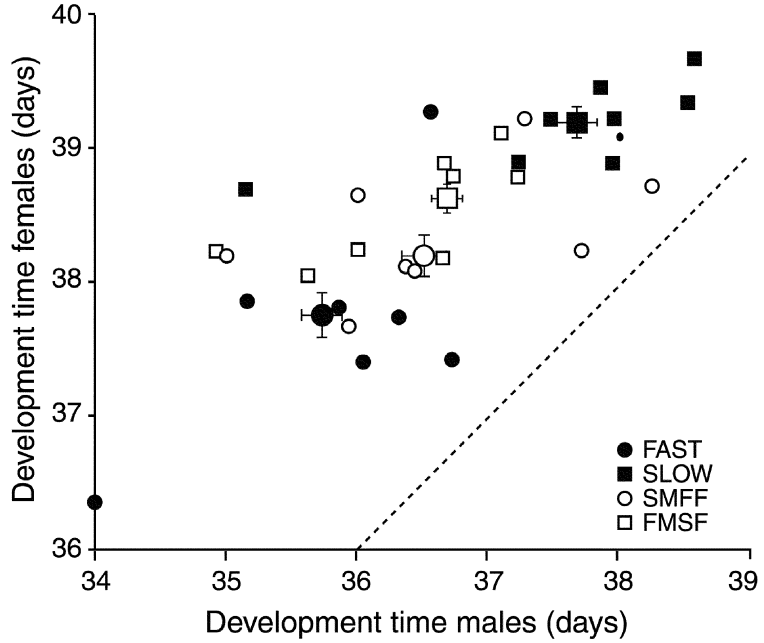

Figure 3. Female development time in days plotted against male developmental time at 25°C for generation five (generation six for FAST). Small symbols are means for single smaller cages, large symbols (± s.e.) are overall means per selection line. Dotted line depicts equal development times of the sexes.

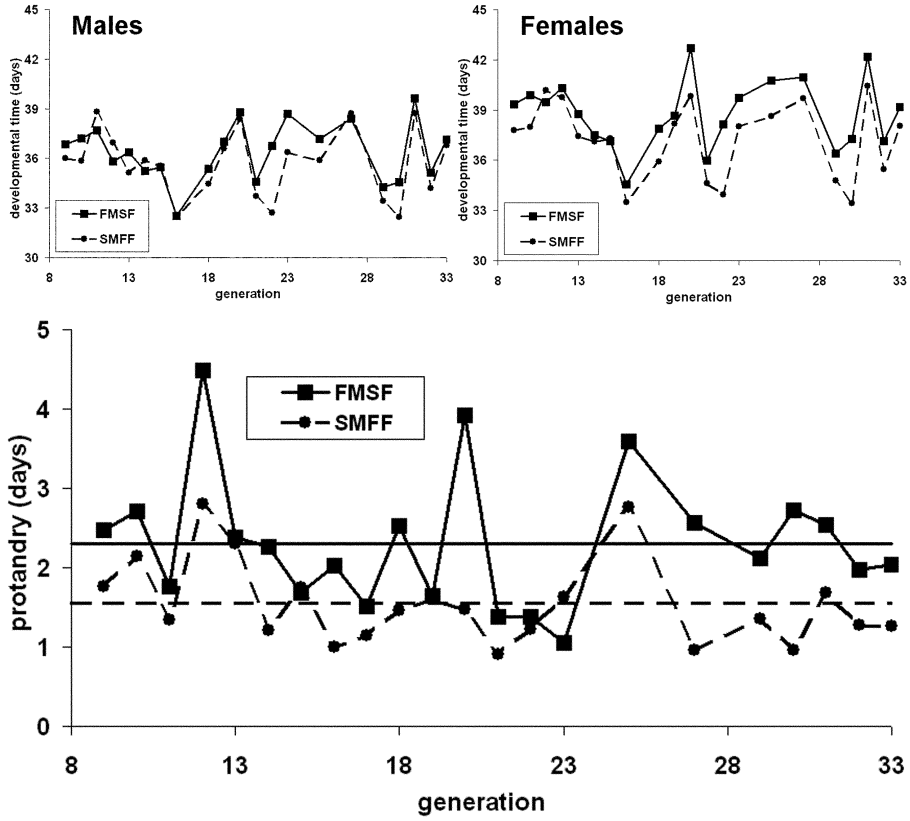

Figure 4. Mean female and male developmental time and protandry (mean difference between female and male developmental time) for the continued selection lines for increased ($FMSF_C$) and decreased ($SMFF_C$) protandry for generation nine until 33 (see Materials and methodss for details). The top graph depicts the average male and female developmental time per generation, and the bottom graph the mean protandry per generation. In the bottom graph, the horizontal lines represent the mean protandry for the lines over this range of generations. From these graphs it becomes clear that the difference in protandry for the selection lines are caused by a difference in the mean female developmental time and not by a difference in male developmental time.

Table. 1 Protandry values (in days, based on bootstrapping) and confidence intervals (CI) at generation eight for the most extreme replicates of lines selected for an increase in the difference between male and female development time (more protandry, FMSF), and lines selected for a decrease in protandry (SMFF). Bold values were used to calculate the minimal genetic correlation (r_A) that could still account for the observed (lack of) response.

	Protandry	Lower 95% CI	Upper 95% CI	Minimum r_A
FMSF (more protandry)	2.36	2.09	**2.63**	0.951
SMFF (less protandry)	1.86	**1.60**	2.13	0.979

time, including temperature, sex, line and smaller cage nested within lines, showed a three-way interaction between temperature, line and sex ($F_{2,1506} = 4.11$, $P < 0.025$). Interaction profiles showed that intercepts were different for the line × sex interaction, and the effect of selection was the same for all lines. Also, these profiles indicated that the effect of selection on developmental time for the lines was different for the two temperatures. In the case of the temperature × line interaction, there was an effect of selection on developmental time which additionally showed different effects on each of the lines. When done separately for each temperature, the analysis revealed no effect of the line × sex interaction at 20°C ($F_{2,14} = 0.72$, $P > 0.05$), but a significant line × sex interaction ($F_{2,14} = 6.07$, $P < 0.025$) at 27°C.

When the data were analysed for protandry (using the smaller cages as replicates), the lines did not differ significantly for protandry at 20°C ($F_{2,12} = 0.51$, $P > 0.05$). In line with the analysis for developmental time, lines differed

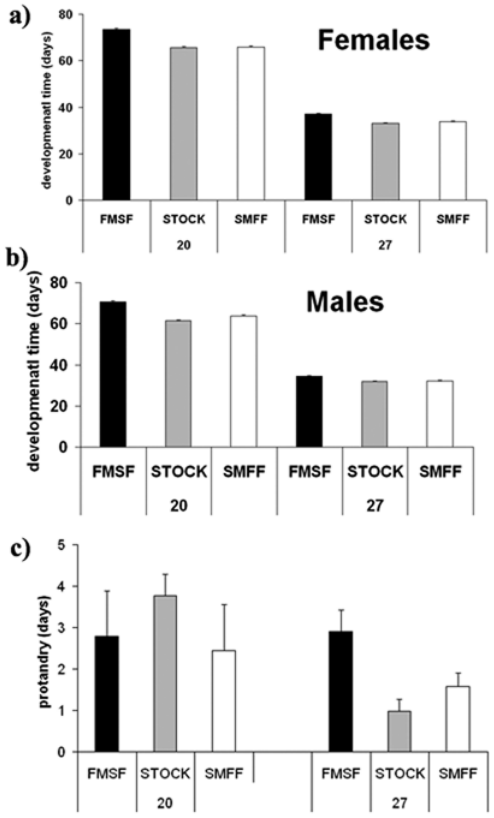

Figure 5. Developmental time (± s.e.) of (a) females and (b) males, and (c) protandry (± s.e.) of the $FMSF_C$ $SMFF_C$ lines at two developmental temperatures (20°C and 27°C).

significantly for protandry at 27°C ($F_{2,12} = 6.29$, $P < 0.025$; Tukey's HSD, FMSF > STOCK).

Genetic correlation

Using quantitative genetic theory, we can estimate the genetic correlation between male and female development time that is necessary to explain the observed response to uncoupling selection. Because only a limited response was observed in the direction of increased or decreased protandry, the value for r_A is likely to be close to unity. We can address the question of what is the lower limit to r_A that could still explain our results? This can be done on the full set of lines, hence we have performed this analysis for the eighth generation of selection.

Table 1 shows the protandry values and confidence intervals for lines selected for increased (FMSF) or decreased protandry (SMFF). Using the upper 95% value of the most extreme protandry value for FMSF (2.63 days), the genetic correlation between male and female development time has to be at least 0.95 to explain the observed response. Similarly, taking the lower 95% value of the SMFF replicate with the lowest value (1.60 days), the minimal r_A equals 0.98.

Fertility in selection lines

During the initial selection procedure, we had to restart the SMFF lines because of low numbers in generation two (figure 6). Egg-to-adult survival percentage was significantly lower for SMFF than for the other lines (logistic regression with line as the predictor variable, likelihood-ratio (L-R) $\chi^2 = 270.25, df = 3, P < 0.0001$). The odds of an egg developing to an adult were 1.64 to 2.40 times higher for the other selection lines compared to SMFF. Egg-to-adult survival also declined with generation of selection (L-R $\chi^2 = 109.41$, $P < 0.0001$) with the odds of reaching adulthood decreasing by a factor averaging 0.96 per generation (95% confidence interval of odds ratio: 0.92–0.99). In addition, the selection line by generation interaction was significant (L-R $\chi^2 = 60.23, df = 3, P < 0.0001$), due to a slightly larger decrease in egg-to-adult percentage with generation for FMSF and SLOW (odds ratios are 0.95 and 0.96, respectively).

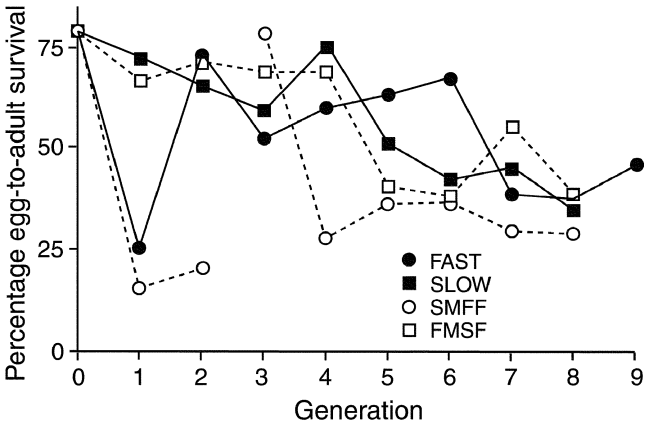

Figure 6. Egg-to-adult survival during the selection expreriment. The SMFF lines were re-established from the stock in generation three.

Fecundity (number of eggs laid per day) for the matings specifically set up to test for slow male – fast female incompatibility only depended on female traits. Although male and female pupal weight did not differ between the selected lines ($F_{3,169} = 2.2807$, $P > 0.05$), the number of eggs laid per day increased with increasing (pupal) weight of the female ($F_{1,81} = 7.95$, $P < 0.0059$) and decreased with female development time ($F_{1,81} = 19.36$, $P < 0.0001$). Neither male pupal weight nor development time were significant ($F_{1,81} = 0.19$ and 0.14, respectively, both $P > 0.05$).

Egg hatching probability was dependent on both male and female development time (figure 7). Fertility decreased with development time of the father (L-R $\chi^2 = 36.86$, $P < 0.0001$, odds ratio is 0.69 (0.61 – 0.78) per day), and increased with development time of the mother (L-R $\chi^2 = 91.95$, $P < 0.0001$, odds ratio is 1.64 (1.48 – 1.81) per day). In other words, the odds of an egg hatching decreased with 69% for every extra day the father needed to

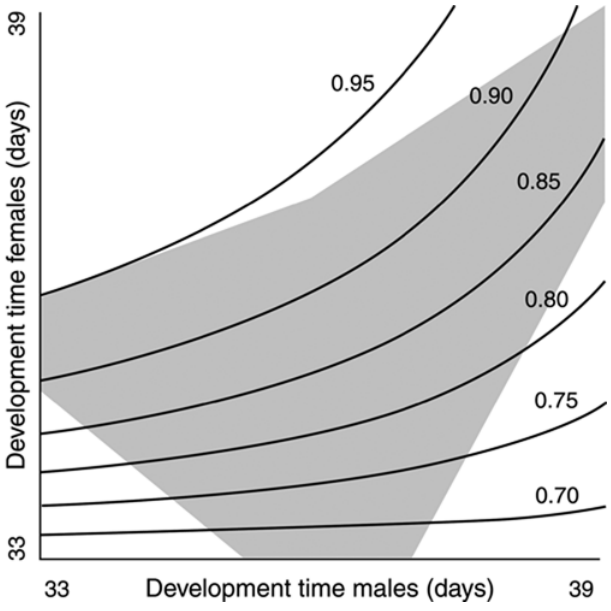

Figure 7. Logistic regression model for fertility. Lines connect combinations of parental development times with the same predicted proportion of hatching eggs. For the other effects in the model, fecundity and male weight, mean values were used (22.5 eggs per day, and 172.5 mg, respectively). Gray area outlines those combinations of male and female development time actually used to produce the data set.

develop, whilst it increased with 64% for each day increase in maternal development time. This is not the same as the probability of an egg hatching. If we use the mean fertility of 85%, then fertility will increase roughly 5% per day of increase in maternal development, and decrease 5% per day of increase in paternal development time (see figure 7). This pattern, slow males and fast females laying egg batches with the lowest chance of hatching, was exactly what was found during the protandry selection experiment (see low egg-to-adult survival for SMFF). However, the interaction complicates matters (figure 7). Interaction between male and female development time was also significant (L-R $\chi^2 = 9.54$, $P = 0.002$). This interaction implies that female development time has more profound effect on fertility than male development time, especially for development time combinations that have lower predicted fertilities. For example, fertility of a fast female with a development time of 33 days, changed very little from ~ 70% with increasing paternal development time, whilst the fertility of a brood from a slower developing female decreased with increasing development time of the male (see figure 7). Other factors (positively) influencing egg hatching were fecundity (L-R $\chi^2 = 502.41$, $P < 0.0001$), and paternal pupal weight (L-R $\chi^2 = 16.49$, $P < 0.0001$). The model explained 21.1% of the variation in fertility. Maternal pupal weight did not affect fertility (L-R $\chi^2 = 1.90$, $P = 0.1685$). A small number of sterile broods were included in our analyses (four out of 85 broods only contained unfertilized eggs), but the results did not change when they were omitted.

No differences in mating success were observed in the mate choice experiments with FAST and SLOW lines (table 2). Males from the FMSF lines, however, mated significantly more than SMFF males, irrespective of the origin of the females (FMSF or SMFF) ($\chi^2 = 4.17$, $df = 1$, $P < 0.05$; $N = 29$ matings).

Discussion

Response to selection

No significant response for more or less protandry was observed after the first eight generations of selection, although selection for increased or decreased development time in both sexes (FAST and SLOW lines) was successful. Heritability estimates for development time were similar in each sex and significantly different from zero. However, continued selection showed a significant difference between the $FMSF_C$ and $SMFF_C$ lines indicating that selection was successful. However, the difference between these lines did not increase with continued selection, suggesting that there was an early response that plateaued out, and that the lack of significant differences between the selected lines after eight generations of selection was most likely due to lack of

Table. 2 Number of matings in the mate choice experiment at 25°C. Density in mating cages was constant with five females from one selection line, and five males from each tested selection line (10 males in total) because mated individuals were removed and replaced. For the lines selected for developmental time, no differences in mating success were observed for either males or females. For the lines selected for protandry, males from the FMSF lines mated significantly more than SMFF males, irrespective of the origin of the females (FMSF or SMFF).

		Females				Females	
		FAST	SLOW			FMSF	SMFF
Males	FAST	16	17	Males	FMSF	12	8
	SLOW	13	15		SMFF	6	3

FMSF, fast males slow females; SMFF, slow males fast females.

statistical power. The results thus suggest that genetic variation for protandry was fixed in the first eight generations of selection. There was a significantly longer developmental time for females of the $FMSF_C$ compared to the $SMFF_C$ line but for males there was no difference between the lines. This leads us to the conclusion that developmental time alleles may have a stronger effect in females than in males, or that selection intensity was perhaps greater in females.

The genetic architecture across the sexes for development time might be too tightly integrated, with the genetic covariances near unity, to obtain stronger opposite responses in the sexes (slow males and fast females or vice versa). A sex-specific component of genetic variation in development time must have been present at one time in order for protandry to arise, but in our base population only a very moderate amount of additive genetic variation to for a strong sex-specific reaction to development time selection seems to be present. Indeed, it appears that in our set-up, with the selection differentials we obtained and used, the correlation between female and male developmental time was at least 0.95. In this short-term study, using selection directly targeted at protandry, the genetic correlation of development time across the sexes appears to constrain strong sex-independent evolution of developmental time.

The ratio of male to female development time did show a change for other slow lines selected for a longer period (>30 generations) from the same base population, suggesting that some form of sex-specific genetic variance for development time still exists in the base stock (Zijlstra *et al*. 2002a). Many artificial selection experiments in directions antagonistic to the genetic correlation have produced responses, albeit often erratic (Roff 1997). Some recent experiments aimed at uncoupling traits that were genetically and phenotypically correlated were successful, showing that independent evolution of correlated traits is possible (Beldade *et al*. 2002; Zijlstra *et al*. 2003); no apparent constraints on short-term responses to artificial selection were observed. In other artificial selection experiments (mainly on body size), changes in sexual dimorphisms were present, but variable (reviewed in Reeve and Fairbairn 1996; Rhen 2000).

In the 'adaptive' explanation, protandry is beneficial for males through increased mating opportunities (Wiklund and Fagerström 1977), as well as for females through decreased prereproductive death (Fagerström and Wiklund 1982; Zonneveld and Metz 1991). The 'incidental' explanation depends on selection only in the females, through increased fecundity (Wiklund and Solbreck 1982; Thornhill and Alcock 1983). Thus, in the latter hypothesis, sex-specific variation in development time of males is effectively a selectively neutral trait. It should also be noted that many genes that underlie sex differences are not sex-linked (Rhen 2000). Our results show that only a limited amount of sex-specific variation is present for development time in our population, either in females or in males. The absence of a strong response to artificial selection on patterns of protandry therefore lends further support to the hypothesis that protandry in itself is adaptive, and shows little genetic variation due to a long history of past selection.

Effects of temperature

The results of the present study show that temperature has an effect on the degree of protandry in the selected lines. The $FMSF_C$ line showed the largest effect of selection on protandry, mainly through an effect on female developmental time which may be an indication of sex-specific plasticity. However, the effect of selection in this line was only significant at 27°C. This interaction between selection response and temperature can be relevant to the ecology of this species. During the wet and warm season there is a strong selection for protandry, and it is desirable in this season to reproduce early to take advantage of the bountiful resources for the caterpillars. During the dry season, however, survival is more important than early emergence. Early emergence would decrease the chances of survival because of a lower body-weight and/or fat reserves (Zijlstra *et al*. 2002c). Further research is needed to determine the importance of these factors.

Factors limiting the response to selection: correlated traits

Our selection for protandry has resulted in significant differences between the selected lines when we consider the full 33 generations. However, the response is limited and our data also suggest that the response very quickly levels off. This could result from a limited amount of sex-specific genetic variation for protandry (see above), or could be due to the involvement of correlated traits. For instance, alterations in development time could also be strongly limited through interactions with growth rate and/or pupal weight that form a tightly interconnected triangle with development time (Chippindale *et al*. 1997; Prasad *et al*. 2000; Berner and Blanckenhorn 2007). This would buffer protandry from temporal or spatial variation in the environment. However, we have not observed significant differences in pupal weight between the selection lines (including the FAST and SLOW lines). This is consistent with observations from selected lines of *B. anynana* for egg size, developmental time, and pupal size, the three traits that largely determine growth rate. Analysis of these lines does not provide evidence for significant genetic correlations between developmental time and either egg size or pupal weight (Fischer *et al*. 2007). Therefore, the lack of response for protandry is unlikely to result from strong genetic correlations between traits related to growth rate.

Factors limiting the response to selection: fertility in selection lines

Our data do suggest another factor potentially limiting the response to selection: fertility incompatibility. Selection lines for decreased protandry (SMFF lines) had to be restarted because of low numbers due to low egg-to-adult survival (fig-

ure 6). Such a decline in egg-to adult survival could be an indication of inbreeding depression; however there are several reasons for this to be unlikely. Firstly, in *B. anynana* inbreeding depression manifests itself in complete male sterility after only a few generations of inbreeding (Saccheri *et al*. 2005). Although hatching rate of eggs probably contributed to the decline in egg-to-adult survival, results from the fertility compatibility experiment shows that the percentage of sterile broods was not increased and is about 5%, which is normal for a noninbred population in this species (Saccheri *et al*. 2005). Secondly, the selection protocol dictated that either one or both of the sexes were mated at later ages. Because of the synchronization between the lines, this is especially true for the fast developing lines and sexes. We have repeatedly observed that increasing the age at first mating decreases the fertility of both males and females. Indeed, when we controlled for age in the fertility compatibility experiment, we found that fertility depended on the developmental times of the female and male, but egg hatching was never lower than 70%. Therefore, it is implausible that inbreeding depression can explain the differences between the full set of selection lines, as well as for the continued selection lines. In addition, although the restart of the SMFF lines decreased the selection intensity, this will probably have a relatively small impact on the selection response after 33 generations.

The incompatibility found between slow males and fast females is very interesting and may act as an additional factor maintaining, or even favouring an increase in protandry. Both a decrease in fertility with increasing paternal development time, and an increase in fertility with increasing maternal development time, act to increase protandry (see figure 7). The number of hatching larvae (fertility) is a nearly perfect predictor of number of pupae (correlation = 0.98), and hence of egg-to-adult survival in our rearing conditions (Brakefield *et al*. 2001). Therefore, brood fertility can be viewed as an additional factor with the potential to shape protandry. A positive effect of paternal weight on brood fertility could be accounted for by the transfer of a larger spermatophore of potentially higher quality by larger males. However, the absence of paternal weight in explaining total number of eggs laid counters this argument, but larger spermatophores may influence remating of the inseminated female. In the field, approximately one-third of the *B. anynana* females remate (Brakefield and Reitsma 1991), and in the laboratory about one in four females remate, without strict last male precedence (Brakefield *et al*. 2001).

Previous work on differential female fecundity in the mosquito *Aedes sierrensis* and the tropical butterfly *Brassolis sophorae*, respectively, has shown that protandry is favoured because early females lay more eggs than later females (Kleckner *et al*. 1995; Carvalho *et al*. 1998). In both cases, later emerging females had a smaller size and lower fecundity in the field. This pattern is identical to that found in the laboratory here: fecundity in *B. anynana* declined with development time and increased with weight. However, in this species, the relationship between development time and weight for females shows an intermediate optimum, i.e. females with an intermediate development time from egg to pupa have the highest pupal weight (Zijlstra 2002). Therefore, variation in female quality will result in selection on protandry that lies somewhere between directional selection for increased protandry (increase in male mating success and decrease in female prereproductive death) and stabilizing selection (because intermediate females are most fecund).

We also found that males from the protandry-decreasing combination of parents mated less successfully when in competition with those from the conversely selected lines. This could be a further factor working in the wild against a decrease in protandry if males from such combinations (i.e. SMFF) have lower mating success. In our selection experiment this factor was less important because all competing males had the same selective history. Slow males may have enjoyed a slight competitive advantage because they were younger at the time of mating during the selection experiment. The relative importance in nature of these selective factors (i.e. differential fertility based on male and female development time or differential fecundity based on female development time) on protandry in comparison to other factors, as well as the underlying causal mechanisms, remains to be examined.

Conclusions

To summarize: (i) heritable genetic variation is present for development time in *B. anynana*; (ii) there is only a limited amount of genetic variation available to mediate changes in the difference between the sexes in development time. A small but significant change in protandry was achieved in response to 33+generations of selection; (iii) males from lines selected for a decrease in protandry had a lower mating success when competing against males from lines selected for increased protandry; and (iv) broods from males with a relatively long development and relatively fast females have a lower egg hatching probability, resulting in additional selection in favour of protandry.

Theoretical studies predict a longer time-scale for evolution of sexual dimorphism than for the average phenotype, but also suggest that there is potential for evolution in sexual dimorphism (Lande 1980; Rhen 2000). While substantial amounts of sex-specific genetic variation for developmental time in *B. anynana* must have been present at some point in time to establish the earlier male emergence, our results suggest that it has largely been eroded by long-term natural selection. The current genetic architecture may pose a constraint on the short-term evolution of protandry in this species. This suggests that when the current level of protandry becomes suboptimal, for instance due to changing climates, it may require long periods of time and mutational input to produce evolutionary change.

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