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Ph. D. thesis Leiden University. Met een samenvatting in het Nederlands.

Printed by Haveka, Alblasserdam

EVOLUTIONARY CONSTRAINTS ON THE LIFE HISTORY OF THE BUTTERFLY *BICYCLUS ANYNANA*

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D.D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 10 oktober 2002
klokke 14:15 uur

door

Wilte Gerrit Zijlstra

geboren te Groningen
in 1973

Promotiecommissie

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Chapter 1

General introduction

Evolutionary constraints...

Definitions

A constraint, according to the Oxford English Dictionary (second edition, 1989), is a "confinement, bound or fettered condition; restriction of liberty or of free action." Merriam-Webster's Collegiate Dictionary (tenth edition) defines constraint as "the state of being checked, restricted, or compelled to avoid or perform some action."

In evolutionary biology, constraints on phenotypic evolution are "limits and preferential routes that are superimposed on the action of selection" (Schlichting & Pigliucci, 1998, p. 155). Gould even gives a positive (active) definition of constraint as "compelling or channeling phenotypic change" (Gould, 1989, p. 518). Constraint terminology is surrounded by semantic confusion, because constraints are too often invoked as *ad hoc* explanations (Perrin & Travis, 1992). The wealth of adjectives that have been adhered to constraint do not help either (Antonovics & Van Tienderen, 1991). Popular choices include mechanical, phylogenetic, genetic, and developmental constraints (Arnold, 1992; Schlichting & Pigliucci, 1998). The first, mechanical constraints, is one of the most unambiguous: organisms have to abide by the laws of physics and chemistry. The last, developmental constraint, was defined by Maynard-Smith *et al.* (1985) as "... a bias on the production of variant phenotypes or limitations on phenotypic variability caused by the structure, character, composition, or dynamics of the developmental system". Schlichting and Pigliucci (1998) argue that by substituting "genetic architecture" for "developmental system", we obtain a good definition of genetic constraint. Some authors (e.g., Arnold, 1992) also term selection a 'selective constraint', others oppose this, because the term then loses all meaning (e.g., Gould, 1989).

Constraint versus selection

Interest in the concept of constraint was triggered by Gould and Lewontin, who criticized the "adaptationist programme" and argued against adaptation through natural selection being "...the primary cause of nearly all organic form, function, and behaviour" (Gould & Lewontin, 1979, p. 585). They stress "...the importance of developmental blocks and pervasive constraints of history and architecture" (p. 597).

The key question with regard to constraints is: Why is not all seemingly possible variation seen? Although a wide range of diversity is seen in nature, many optimal and/or feasible designs are not observed. The classic example is Raup's 'cube of life' for shell

forms (see e.g., Gould, 1980; Schlichting & Pigliucci, 1998). Three parameters describing shell morphology form a cubical space, that is only partly occupied by actual shells that have occurred at one time or another. Possibly, constraints have obstructed organisms to fill the empty part of the morphological space, or, alternatively, selection has never favored individuals to enter it and they represent ill-adapted forms (Gould, 1980). The truth probably lies somewhere in the middle, and Gould's main aim is to move that middle a bit more towards the structural integration explanation that constraints prevent colonization of the unoccupied space. In his challenge to the *Allmacht* of selection he notes that "...strict selectionists maintain that (...) correlations are weak relative to the power of selection to break them down" (Gould, 1980, p. 42). The struggle between constraint and selection is the main topic of this thesis. Most pertinent to this issue are genetic constraints, i.e. the (short term) impossibility (or not) of the existence of a certain combination of traits; for example, because both traits are underpinned by the same physiological/endocrine system ('physiological constraint'). The main aim in this thesis was to attempt to break this kind of apparent constraint for combinations of life-history traits in a particular system by means of strong artificial selection; is the standing genetic variation sufficient to enable responses to selection in all directions for coupled life-history traits?

Genetic constraints can be caused by either the genetic architecture (e.g., pleiotropy) or by absence of genetic variance. The genetic (co)variance matrix (**G-**matrix) can be employed to describe genetic constraints (Schlichting & Pigliucci, 1998). In work on sticklebacks and other vertebrates, for example, Schluter showed that adaptive differentiation occurs principally along 'genetic lines of least resistance', that is, in a direction close to the direction of the greatest genetic variance (Schluter, 1996). He also noted that phenotypic lines of least resistance performed nearly equally well. This agrees with Cheverud, who observed that patterns of phenotypic correlation (**P-**matrix) were strikingly similar to genetic correlations and are likely to be good estimates (Cheverud, 1988). Roff is more cautious and calls for supporting evidence that **G**- and **P**-matrices are similar (Roff, 2002, pp. 60-61).

...on the life history...

Survival and reproduction

Again, first some definitions; life history is "an individual's pattern of allocation, throughout life, of time and energy to various fundamental activities, such as growth, repair of cell and tissue damage, and reproduction" (Freeman & Herron, 2001). And, life-history evolution is "the evolution of major features of a life cycle, principally the age distribution of birth and death rates, growth rates, and the size of offspring" (Stearns, 1992). In short, the life history of an organism is how the energy budget is distributed over reproduction and survival. Implicit is another fundamental aspect of life-history evolution, the trade-off: any resources invested in one trait (e.g., reproduction) cannot be allocated to another (e.g., survival). Or, a more concrete example for insects, body weight increases with development time, thus a faster development trades-off to a lower adult body weight. But in this context, the importance of variation in growth rate also needs to

be stressed. Growth rate is not always simply the resultant of development time and pupal weight, but can itself vary adaptively (Nylin, 1994; Nylin & Gotthard, 1998). Furthermore, there is a temporal aspect, variation over time in selection intensity will give rise to stage-specific schedules of mortality and reproduction. Key life-history traits include development time, age at maturity, body size and fecundity. In a sense, life-history traits are the prime determinants of fitness, and the age dependent investments in survival and reproduction determine the strength of selection.

Most life-history traits are continuous, and their genetic and environmental determination are studied using quantitative genetics. How are quantitative traits inherited and what are their responses to selection? A frequently used measure is heritability (h^2), the ratio of (additive) genetic variation to total variation (environmental and genetic), although **G**- and **P**-matrices (see above) also belong to the field of quantitative genetics. The fields of life history and quantitative genetics are much too broad for a complete treatment here, see for more information on quantitative genetics Falconer & Mackay (1996), Roff (1997) and Lynch & Walsh (1998), and on life history Stearns (1992) and Roff (2002). I will focus on a few aspects of both that are especially relevant to this thesis.

Two-trait selection

A few decades ago, artificial selection experiments were set up to test quantitative genetic theory of heritability, genetic variation and co-variation. Single trait selection will usually elicit correlated responses in certain other, unselected traits. To better understand the underlying genetic architecture, two correlated traits were selected simultaneously (e.g., Cockrem, 1959; Bell & Burris, 1973; Rutledge *et al.*, 1973; Sheridan & Barker, 1974, review in Roff, 1997). With agonistic, reinforcing selection, both traits are selected in the same direction as the correlation (cf. along the lines of least resistance), whilst antagonistic lines are selected against the correlation. Roff cautions against too much optimism in the prediction of the evolutionary trajectory of multi-trait selection because of erosion of genetic variation, drift, asymmetry of response, and the difficulty of estimating genetic parameters (Roff, 2002, p. 61). Some studies reported constraints on the response to antagonistic selection (Rutledge *et al.*, 1973), whereas others did not (Cockrem, 1959; Sheridan & Barker, 1974). The main conclusion of these studies taken together is that response to two-trait selection is erratic, especially in the antagonistic direction. Most antagonistic selection experiments performed to date involved morphological traits without substantial impact on fitness, such as bristle number in flies or tail length in mice. In this thesis, I studied the response to two-trait selection on life-history traits. Such traits are under (strong) selection and, therefore, may yield fundamentally different results due to of the effects of selection on genetic and phenotypic variance patterns. Is a suite of life-history traits that seems tightly integrated and adapted to specific circumstances flexible enough to adapt to new (euphemistically called) environmental challenges, such as global warming? More encouragement comes from Roff, who notes as a topic for further study that in the study of life-history evolution "...there are virtually no experiments in nondomesticated species in which two or more traits were simultaneously selected" (Roff, 2002, p. 460).

Protandry

Darwin already observed the phenomenon called protandry: "Throughout the great class of insects the males almost always emerge from the pupal stage before the other sex", and: "those males (...) first ready to breed (...) would leave the largest number of offspring" (Darwin, 1871, p. 260). Interest in this subject has been rekindled by Wiklund and Fagerström (Wiklund & Fagerström, 1977; Fagerström & Wiklund, 1982) who hypothesized that males emerge before females to increase their probability of mating, and females emerge later to minimize prereproductive death. For males, selection to increase mating probability (i.e. to emerge earlier) is counterbalanced by the increased chance of dying before mating. More theoretical work followed (e.g., Bulmer, 1983; Iwasa *et al.*, 1983; Zonneveld, 1996), and Iwasa and co-workers, for example, argued on theoretical grounds for a truncated emergence pattern of males, given a smooth, one peak emergence pattern for females.

The mating system must comply to several conditions for the sexual selection theory to explain the evolution of protandry. Selection for protandry can only occur with discrete generations, because males cannot be selected to emerge before females when receptive females are continuously present (Singer, 1982). Furthermore, males should be able to mate multiple times, and there should be an advantage for males to be the first to mate with a female, *in extremis* because of monandry (Zonneveld, 1992). Another factor that shapes protandry is temporal variation in female quality (Kleckner *et al.*, 1995; Carvalho *et al.*, 1998). If later emerging females are of lower quality, for example because they have a lower fecundity, this will strengthen the selection on males to emerge earlier, thus increasing protandry. This could explain why although early emerging males of the butterfly *Euphydryas editha* did not achieve more matings in the field, nevertheless protandry is favored in this species (Baughman, 1991).

An alternative to the sexual selection hypothesis was suggested by Thornhill and Alcock (1983): because female, but not male (or less so), fecundity increases with body weight, females are selected for a longer development (assuming a positive correlation between body weight and development time), hence the difference in emergence. Protandry is not adaptive itself, but more a by-product of asymmetric fitness benefits to the sexes. Protandry as an inbreeding-avoidance scheme (Petersen, 1892) seems less likely. Protogyny (earlier emergence of females) would then be expected in half of the cases, but it is seldom seen. It could be favored if males are better dispersers, but this has not been well studied.

Comparative studies to evaluate the sexual selection and the natural selection (protandry as by-product) hypotheses generally support the former (e.g., Nylin *et al.*, 1993). However, larger size does seem more important for females than for males (e.g., Fischer & Fiedler, 2001), so there is scope for the sex-specific influence of natural selection (Kleckner *et al.*, 1995; Bradshaw *et al.*, 1997).

Protandry is an integral part of the life history of many insects, but the quantitative and evolutionary genetics are, with some exceptions (e.g., Bradshaw *et al.*, 1997), not well studied. The last part of this thesis attempts to change that, by asking questions such as "Does protandry respond to artificial selection, that is, can male and female development time evolve independently?" This can be seen as a special case of

two-trait selection, with male development time and female development time being the two selected traits.

...of the butterfly *Bicyclus anynana*:...

Seasonal polyphenism

The tropical butterfly *Bicyclus anynana* (Butler, 1879) occurs in highly seasonal environments in sub-Saharan Africa. The wet season in Malawi, from November to April, is characterized by substantial rainfall, high temperatures (>22ºC) and abundant food plants for the caterpillars (Brakefield & Reitsma, 1991; Brakefield & Mazzotta, 1995). In the dry season, temperatures are lower and hardly any foodplants are available.

Wing patterns differ markedly between the wet and dry season as a result of seasonal polyphenism. In the wet season, butterflies have large, conspicuous circular eyespots on the margins of the wings. The (ventral) eyespots are considered to function in deflecting predatory attacks of birds or lizards away from the vulnerable body (Brakefield & Larsen, 1984). Butterflies show active, reproductive behavior in the lush green wet season to fully exploit it. In the dry season, butterflies mainly rest on the dead, brown leaf litter that covers most of the ground, and are effectively in an adult reproductive diapause. Eyespots are very much reduced in dry season butterflies, they rely on camouflage to survive the disadvantageous dry season. This seasonal polyphenism is externally cued by temperature in the final fifth instar and during the early pupal stage (Kooi & Brakefield, 1999); high temperatures (>22ºC) lead to large eyespots, low temperatures (<20ºC) to a more cryptic, uniformly-colored wing pattern, with nearly absent eyespots. When reared in the laboratory, a continuous, non-linear reaction norm across temperature is obtained (figure 1.1), with intermediate phenotypes that are not frequently observed in nature (Brakefield *et al.*, 1996).

Artificial selection experiments on eyespot size showed that additive genetic variation is present for this trait (Holloway *et al.*, 1993; Monteiro *et al.*, 1994; Brakefield *et al.*, 1996; Beldade *et al.*, 2002b). Heritability estimates for size of the dorsal fifth eyespot range from 0.47 to 0.67, with other eyespots showing a correlated response (Monteiro *et al.*, 1994). Realized heritabilities for the second eyespot on the ventral forewing are larger than 0.4 (Holloway *et al.*, 1993). Lines selected for ventral eyespot size lost the ability to produce both seasonal forms (figure 1.1). Phenotypic plasticity remains, but small eyespot size selected lines show a camouflaged wing pattern at high, wet seasonal, temperatures, and the converse is true for large eyespot selected lines (Brakefield *et al.*, 1996, figure 1.1). However, selection specifically applied to change the shape of the reaction norm was not successful (Wijngaarden & Brakefield, 2001). This is contrary to what was expected based on the non-significant genetic correlations for seasonal form across temperatures obtained in single generation studies (Windig, 1994), but in line with predictions from Holloway *et al.* (1993). Other selection lines that have been successfully established include lines for faster and slower development time, and for pupal weight (P. M. Brakefield and F. Kesbeke, unpubl. Results; B. J. Zwaan, unpubl. results).

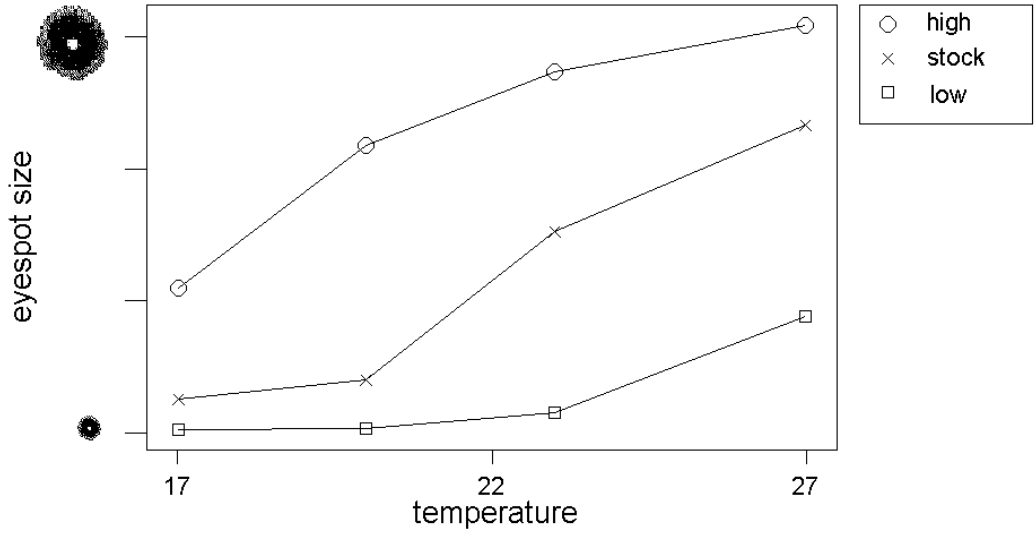


Figure 1.1 Reaction norms for eyespot size. The high line was selected for large ventral eyespot size, the low line for small eyespot size. Data from Brakefield *et al.* (1996).

Endocrinology

Many, if not all, polyphenisms in insects are hormonally regulated (Nijhout, 1994, 1999). Like other butterflies, such as *Araschnia levana* (Koch & Bückmann, 1987) and *Precis coenia* (Rountree & Nijhout, 1995; see Koch, 1992 for review), the seasonal polyphenism of *B. anynana* is (partly) under endocrine control, specifically via the ecdysteroids.

Lines selected for larger (HIGH) or smaller (LOW) ventral eyespot size showed different hormonal dynamics; the HIGH lines showed an earlier increase in ecdysteroid titers after pupation than the stock, pupae from the LOW line a later increase (Brakefield *et al.*, 1998). The differences between HIGH and LOW lines are already significant in the first hours immediately after pupation. Peak values in hormone levels were similar for selection lines and stock. Similar patterns were found for development time selected lines: fast selected lines had an earlier increase in ecdysteroid levels than controls (Koch *et al.*, 1996). These results match 20-hydroxyecdysone injection experiments. Eyespot size increased by hormone injection at the sensitive period, and pupal time was shortened (Koch *et al.*, 1996; Brakefield *et al.*, 1998). For instance, injected animals from the LOW line had larger eyespots, although not nearly as large as those for the HIGH lines, suggesting that other mechanisms also play a role in determining eyespot size. Continuous administration of 20-hydroxyecdysone showed qualitatively similar results (Brakefield *et al.*, 1998).

Both development time and wing pattern are (partly) regulated by a common endocrinological system, perhaps explaining the (phenotypic) correlation between these two traits (see below; figure 1.2). Hormones may function as a manifestation of pleiotropy, and thus constrain evolution (Ketterson & Nolan, 2000).

Methods

The stock population of *B. anynana* was founded in 1988 by approximately 80 gravid females, caught at a single locality in Nkhata Bay, Malawi. It has been kept in the Leiden laboratory at high census size (>500 individuals) and has retained substantial genetic variation on neutral molecular markers, indicating no fixations by genetic drift (Saccheri & Bruford, 1993). In the early generations, *Oplismenus* grasses were used for the feeding of caterpillars and oviposition, but for practical purposes this has gradually shifted to young maize plants. Adults of this fruit-feeding butterfly feed on mashed banana with moist cotton wool. Rearing occurs in climate controlled rooms with a 12h:12h light:dark regime. Several different types of cages are used for housing: cylindrical hanging cages (0.3m diameter) for adults, sleeve cages for families or small populations (0.1m × 0.2m, containing two maize plants, up to 100 eggs), and larger cages for large populations (0.5m × 0.5 m, ± 500 eggs, maximum of 16 maize plants). Pupae can emerge individually in small pots (125ml), and adults can be individually monitored using markings on the wings.

an outline.

This thesis consists of two major blocks, relating to two selection experiments. Chapters 2 and 3 deal with simultaneous selection on a morphological trait with a clear adaptive value (eyespot size) and on overall development time, a key life-history trait. In the stock population, there is a phenotypic correlation between these two traits: faster developing individuals tend to have larger eyespots than butterflies that take longer for their development (figure 1.2; Brakefield & Reitsma, 1991; Brakefield & Kesbeke, 1997). This correlation is observed over a wide range of temperatures (17ºC to 27ºC), representative for both dry and wet seasons. **Chapter 2** describes the response to selection in the same direction as this correlation ('phenotypic line of least resistance') and against this correlation (antagonistic selection). The aim was to obtain more insight into the genetic basis of the coupling between wing pattern and development time. In **chapter 3**, I compared the endocrinology of the selected lines. A possible mechanistic basis for the coupling of eyespot size and development time could be a common hormonal system. Single trait selection on both wing pattern and development time showed that the dynamics of ecdysteroid titers after pupation changed. Because some of my selection regimes posed contrasting selection pressures on ecdysteroid dynamics, it is cardinal to examine the result of antagonistic selection at the endocrine level and what it tells us about the nature of constraints.

Crucial to chapters 2 and 3 was to accurately establish development times, however, environmental factors such as temperature and food quality substantially influence development time. Because most interest is in the genetic component of development time, we wish to correct for environmental differences. In **chapter 4**, I describe a novel approach to tackle this problem. By using phenotypic mutants as internal

controls, we have a benchmark for all experimental cages. Thus we can compare genetic differences between cages. A prerequisite to this method is that the mutants should be comparable to the wild type in development time and competitive ability.

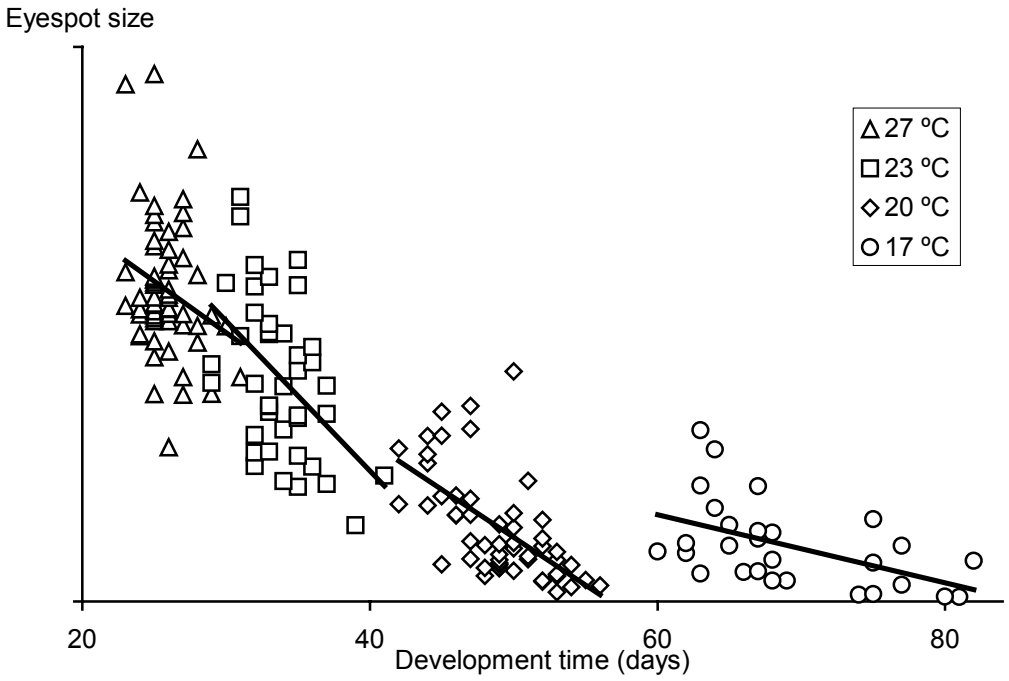


Figure 1.2 The relationship between eyespot size and development time for the stock population at different temperatures, with trend lines.

The final three chapters (chapter 5-7) all pertain to protandry, the earlier mean emergence of males before females, which is a common feature of insect mating systems. Its relation to fitness and the roles of natural and sexual selection for protandry has been the subject of much debate. We can view male and female development time as two traits that can be agonistically and antagonistically selected. **Chapter 5** contains a description of protandry in the stock at various temperatures (i.e. seasons), and also a comparison of protandry in selection lines for development time and pupal weight. This forms the framework for **chapter 6**, where I describe a selection experiment on the trait protandry. By selecting all combinations of male and female development time, we obtain more insight into the genetic basis of development time across the sexes. Because development time can respond to selection and because there is a difference in development time between males and females, protandry seems likely to respond to selection. Finally, **chapter 7** describes correlated responses to protandry and development time selection on such traits as pupal weight and growth rate. Trade-off theory suggests that faster development carries a cost in the form of a lower pupal weight, which is in turn predicted to have repercussions for other traits such as fecundity.

Chapter 2

Simultaneous selection on two life-history traits in the butterfly *Bicyclus anynana*[†]

Abstract

Theory about the role of constraints in evolution is abundant, but few empirical studies describe the consequences that a bias in generation of phenotypic variation has for (micro-)evolution. Responses to natural selection can be severely hampered by a (genetic) correlation among a suite of traits. Constraints can be studied using antagonistic selection experiments, that is, two trait selection in opposition to this correlation. The two traits studied here were development time and wing pattern (eyespot size) in the butterfly *Bicyclus anynana*, both of which have a clear adaptive significance. Realized heritabilities were higher for eyespot size than for development time, but were independent of the concurrent selection (either in the same direction as the correlation or perpendicular to it). Lines differed in both traits in all directions after 11 generations of selection. The patterns for eyespot size (reaction norms) were consistent across different rearing temperatures. Differences in lines selected for fast and slow development time were more pronounced at lower temperatures (irrespective of the direction of joint wing pattern selection). Furthermore, correlated responses in pupal weight and growth rate were observed; lines selected for a slower development had higher pupal weights, especially at lower temperatures. We detected no limiting effects of genetic covariances on the response to artificial selection in different directions. This suggests that the structure of the genetic architecture does not constrain the short term, independent evolution of both wing pattern and development time.

[†] Zijlstra, W. G., Steigenga, M. J., Brakefield P. M. & Zwaan, B. J., *Evolution*, submitted.

Introduction

The concept of constraint in evolutionary biology has received considerable attention, especially on a semantic level (Maynard Smith *et al.*, 1985; Antonovics & Van Tienderen, 1991; Schlichting & Pigliucci, 1998). Various potential explanations exist for a bias in the patterns of phenotypic variation, for example a lack of genetic variation or a particular pattern of genetic architecture. It seems clear that not all evolutionary trajectories are possible, but empirical data on the mechanisms of constraints are scarce. One interesting way to study constraints is to perform (ant)agonistic selection experiments, that is to apply two-trait selection in the same direction as a correlation or opposite a correlation. This approach can investigate the balance between selection and constraint on a micro-evolutionary scale. The main question is then: is it possible to uncouple traits that are initially phenotypically and genetically linked?

The tropical butterfly *Bicyclus anynana* is an interesting organism to study potential constraints in. It occurs in a highly seasonal environment which poses contrasting demands in the different seasons. Development in the warm (>23ºC), wet season is rapid and the ventral wing pattern, exposed when at rest, is conspicuous and likely to function in deflecting predatory attacks (Brakefield & Larsen, 1984). The cooler (<20ºC) dry season is associated with a camouflaged wing pattern, in which the ventral wing pattern is absent. In the laboratory, the nature of the reaction norms has been established by rearing butterflies at a range of temperatures. Butterflies have large eyespots at high temperatures, and small eyespots at low temperatures. At intermediate temperatures, intermediate wing patterns are obtained, although these are rarely observed in nature (Brakefield *et al.*, 1996). The wing pattern is regulated by temperature cues during the final larval stage and the beginning of the pupal stage (Kooi & Brakefield, 1999). The negative correlation between development time and wing pattern is not only seen across temperatures, but also within one temperature, i.e. faster developing animals tend to have larger eyespots (Brakefield & Reitsma, 1991). Since this relationship has persisted in a laboratory population kept for >100 generations, linkage disequilibrium can be ruled out.

Both development time and wing pattern are important for the life history of the butterfly and clearly have major ecological effects. Brakefield and Reitsma (1991) suggested that development time may be the key trait underlying phenotypic plasticity, and that temperature influences on wing pattern are mediated via this trait. There is substantial genetic variability available for both traits: laboratory selection experiments on one trait yielded realized heritabilities of 0.47 - 0.67 for (dorsal) eyespot size (Monteiro *et al.*, 1994) and 0.11 - 0.12 for divergence of development time (chapter 6).

Previously, antagonistic selection experiments have been performed using domesticated animals to increase yield but decrease costs (e.g., Nordskog *et al.*, 1974), or to estimate genetic correlations, substantiate quantitative genetic theory and compare methods of selection (e.g., Cockrem, 1959; Bell & Burris, 1973; Rutledge *et al.*, 1973; Sheridan & Barker, 1974; review in Roff, 1997). In most cases, response to selection of the antagonistic lines was erratic, and results were mixed. Some authors did find impediments on responses to simultaneous selection in opposite directions (Rutledge *et al.*, 1973), whereas others did not (Cockrem, 1959; Sheridan & Barker, 1974). Most antagonistic experiments have involved morphological traits without substantial impact

on fitness, such as tail length in mice or bristle number in flies. Here we aim to study a life-history trait and a morphological trait that are important for fitness. Both traits are under (strong) selection and, therefore, this experiment may yield fundamentally different results due to of the effects of selection on genetic and phenotypic variance patterns

Our aim in this study is to establish (ant)agonistic selection lines for the coupled traits of egg-to-adult development time and ventral eyespot size. Is selection on one trait hampered by the simultaneous selection on the other trait in the direction opposite to the correlation? Furthermore, to evaluate the key role of developmental temperature in the life history of this butterfly, we compared the reaction norms of the different lines following selection by rearing them at three different temperatures.

Materials and Methods

Butterflies

The laboratory stock of *Bicyclus anynana* was established in 1988 and has been kept at generation sizes of >500 since; it has retained substantial genetic variation over the years (Saccheri & Bruford, 1993). Caterpillars feed on young maize plants, adults feed on moist banana. During our selection experiments, animals were kept at 22.5ºC ± 0.5ºC, 70% RH (± 10%), and 12:12 light:dark regime. We chose this intermediate temperature to minimize any bias in the response. That is, the characters under selection had intermediate trait values in the reaction norm. Population census size was 200-400 adults.

Selection

Emerging males and females were separated each day and transferred to a lower temperature to ensure sufficient offspring (Zijlstra *et al.*, 1999), and selection was applied to females only. Individually marked females were selected for a combination of egg-to-adult development time (FAST or SLOW) and eyespot size (WET [large eyespot size] or DRY [small eyespot size]), giving rise to four selection lines: FAST WET, FAST DRY, SLOW WET, and SLOW DRY. Thus, FAST WET [+ +] and SLOW DRY [− −] were selected in the same direction as the correlation (agonistic selection), FAST DRY [+ −] and SLOW WET [− +] were selected perpendicular to this relation (antagonistic selection). Each treatment was replicated twice, and for practical purposes, the replicates were started one generation later. In addition, three unselected control lines were used, reared in every generation together with the selected lines to be able to correct for environmental effects across cages.

The diameter of the black inner disc of the fifth ventral hindwing eyespot was measured using a digitizing tablet, and corrected for wing size by dividing by the interfocal distance (distance between first and fifth hindwing eyespot focus, which correlates highly with overall wing size). Depending on the selection regime, only females that emerged in the first (FAST) or last (SLOW) half were measured and after selection, fifty randomly chosen females from the whole line population were measured to obtain the selection line mean. Development time (egg-to-adult) and corrected eyespot

size were ranked and, depending on the selection regime, the two ranks were added or subtracted. The 30-40 females with the highest (lowest) score were mated at random using all the (non-selected) males of the same line. Females were allowed to oviposit for 1-2 days to establish the next generation. Selection was continued for 10 generations, although generation 10 was only selected for development time and reared at a lower temperature (20ºC). In the final (eleventh) generation, *Spotty* mutants were reared together with experimental animals to be able to correct development time more accurately for environmental fluctuations between cages (see methods described in chapter 4). Egg hatching did not decline with number of generations selected suggesting that there was no inbreeding depression due to the selection regime, (Saccheri *et al.*, 1996).

Reaction norms

In generation 8, 3-4 replicate cages (~100 eggs per cage) per selection line were reared at each of the following temperatures: 18ºC, 22.5ºC and 27ºC (low, intermediate, and high temperature, respectively), to compare the reaction norms for egg-to-adult development time, pupal weight and eyespot size of the different selection lines. Pupae were weighed one day after pupation, to the nearest 0.01mg. To synchronize selection lines in generation 8, parents of lines with a FAST component were reared at 18ºC, and lines with a SLOW component at 27ºC. At each temperature and for each selection regime, we used at least 59 animals (males and females, mean total = 143) for development time and one-day pupal weight, and 45 butterflies (mean = 97) for eyespot size measurements.

Statistical analysis

Realized heritabilities were calculated by taking twice the slope of regression of trait value on cumulative selection differential (CSD). We used the interaction term line × CSD in an analysis of covariance (ANCOVA), to test for the equality of slopes. Comparisons between slopes were made using contrasts. To compare response to selection between eyespot size and development time, we standardized CSD, and development times were log transformed for comparisons across temperatures. Replicate and cage (for the reaction norm comparison) were treated as random factors, and nested in line, or line and replicate, respectively. Individual growth rate was calculated using larval development time (D; egg hatching to pupation in days), pupal weight (W_p) and average egg weight (W_e; 0.408mg) with the formula: log (growth rate) = (log (W_p) − log (W_e))/D (Brakefield & Kesbeke, 1997). Multiple comparisons were corrected using the sequential Bonferroni technique (Rice, 1989).

Results

Response to selection

The changes over the generations for the different selection lines means, corrected for by the unselected control lines, are shown in figures 2.1 and 2.2b. Significantly more phenotypic variation was available to select on for the lines selected in the direction of the correlation (FAST WET [+ +] and SLOW DRY[− −]) than for the antagonistic selection lines (FAST DRY [+ −] and SLOW WET [− +]); contrasts, eyespot size, $t = 7.04$, $p < 0.001$, development time $t = 2.14$, $p = 0.036$ (figure 2.2a). Selection intensity was higher in the DRY direction of selection compared to the WET direction of selection ($t = 2.23$, $p = 0.029$) and higher for SLOW compared to FAST ($t = 2.54$, $p = 0.014$, figure 2.2a). A decrease in selection intensity on eyespot size with generation, suggesting a depletion of selectable variation was only observed in one line, FAST DRY 2 [+ −] (regression, $F_{1,7} = 20.4$, $p < 0.05$, sequential Bonferroni corrected).

		realized heritability					
selection line	replicate	Development time			eyespot size		
FAST DRY [+ −]	1	0.076	± 0.139	ns	0.801	± 0.211	**
	2	−0.042	± 0.155	ns	1.074	± 0.228	**
FAST WET [+ +]	1	0.053	± 0.096	ns	0.407	± 0.065	***
	2	−0.046	± 0.129	ns	0.253	± 0.131	ns
SLOW DRY [− −]	1	0.245	± 0.081	(*)	0.268	± 0.068	**
	2	0.173	± 0.091	ns	0.378	± 0.065	(*)
SLOW WET [− +]	1	0.189	± 0.060	(**)	0.283	± 0.059	(*)
	2	0.081	± 0.105	ns	0.928	± 0.013	***

Table 2.1 Realized heritabilities (± standard error) for development time and eyespot size at 22.5ºC. Significance of the slope of the regression is denoted as: ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Between parentheses: not significant after sequential Bonferroni correction.

Estimates of realized heritability (h^2) for the eyespot size direction of selection were nearly all significantly larger than zero, whilst only two estimates were significant for selection on development time (table 2.1). After sequential Bonferroni correction,

none of the development time heritabilities, and five out of eight h^2 estimates for eyespot size were significantly larger than zero. Comparing the estimates of realized heritability (i.e. slopes) for eyespot size, we encountered a significant replicate (nested in line) effect (ANCOVA, $F_{4,72} = 4.14$, $p = 0.005$). This was mainly due to a wide difference between the replicates of the SLOW WET treatment (see table 2.1, figure 2.1). The interaction between line and cumulative selection differential (CSD) was significant for eyespot size (ANCOVA including replicate as a random factor, $F_{3,72} = 2.81$, $p = 0.046$). Comparisons between the parameter estimates showed that the interaction estimate (i.e. realized h^2) for FAST DRY was significantly larger than that for SLOW DRY ($t = 2.80$, $p < 0.05$, sequential Bonferroni corrected). When the replicates of the SLOW WET treatment were treated separately, and the replicates of other lines were pooled, the significance of the line × CSD interaction increased ($F_{4,74} = 4.37$, $p = 0.0032$, cf. $p = 0.046$, above), pointing to differences in realized heritabilities. A contrast test ($t = 3.55$, $p < 0.0001$) indicated that realized heritabilities for eyespot size of FAST DRY [+ −] and SLOW WET 2 [− +] were higher than realized heritabilities for the other selection lines (table 2.1, figure 2.1).

For the development time selection component, there was no significant replicate effect and the interaction term line × CSD was not significant (ANCOVA, $F_{3,84} = 2.05$, $p = 0.11$), indicating no significant differences in realized heritabilities between the lines. Overall, the response to selection on eyespot size was significantly larger than the response to development time selection ($F_{1,172} = 10.62$, $p = 0.0014$, see also figure 2.3). In addition, none of the selection lines showed a significant change in the (phenotypic) correlation (mean = −0.288) between eyespot size and development time with the number of generations selected (ANCOVA on correlations, $F_{4,104} = 1.02$, $p = 0.40$).

Final generation of selection

The final, eleventh, generation of selection was reared with an internal mutant control (*Spotty*). Since the development time of *Spotty* females differed significantly amongst cages ($F_{10,172} = 7.74$, $p < 0.001$), indicating differences in environments, development times were corrected by subtracting the mean development time of the *Spotty* females of the cage (figure 2.3).

Selection regimes differed significantly in eyespot size (ANOVA, $F_{4,6} = 37.4$, $p < 0.001$) and corrected development time ($F_{4,6} = 4.89$, $p = 0.043$), and in both cases replicates differed significantly ($F_{6,671} = 5.0$ and 13.6, respectively, $p < 0.001$). Tukey tests ($p < 0.05$) revealed the following pattern for eyespot size (see figure 2.3): SLOW DRY [− −] had the smallest eyespots, both FAST DRY [+ −] and FAST WET [+ +] did not differ significantly from the unselected lines, although they did differ from each other. SLOW WET [− +] did not differ from FAST WET [+ +] but did have larger eyespots than the unselected line. None of the Tukey comparisons for development time were significant, but contrasts between unselected lines and lines with either a FAST or SLOW component of selection, showed that SLOW selected lines were significantly slower than FAST ($t = 4.06$, $p = 0.0067$) and unselected lines ($t = 3.26$, $p = 0.017$), and remained significant after sequential Bonferroni correction. Figure 2.3 clearly shows that SLOW selected lines have diverged more than FAST selected lines.

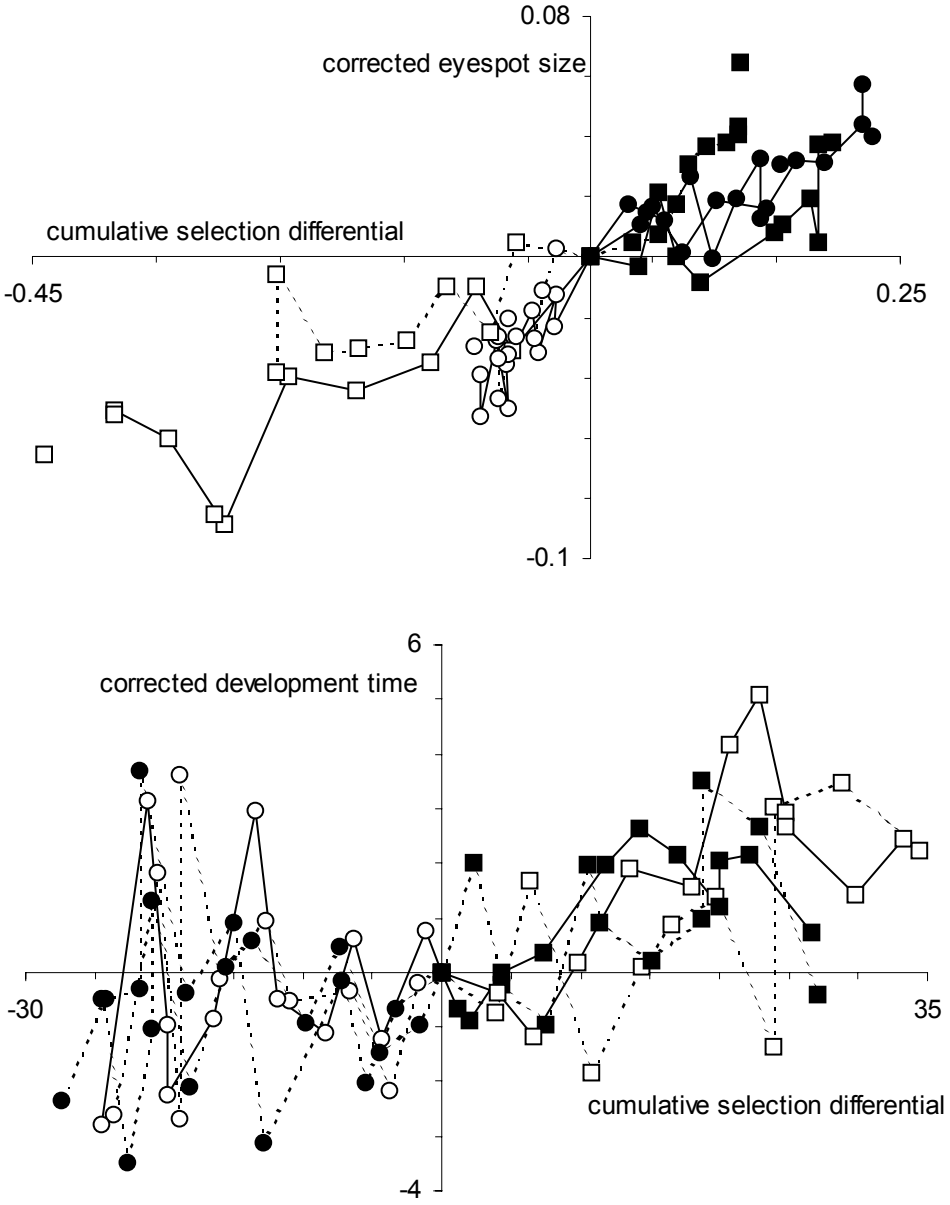


Figure 2.1 Response (corrected for by controls) to selection of females at 22.5ºC on eyespot size (top) and development time (bottom) for the joint selected lines FAST WET [+ +] (●), FAST DRY [+ −] (○), SLOW WET [− +] (■), and SLOW DRY [− −] (□). Dotted lines connect values for the second replicate. Some points in the eyespot size graph are unattached because no selection on eyespot size took place in the penultimate generation.

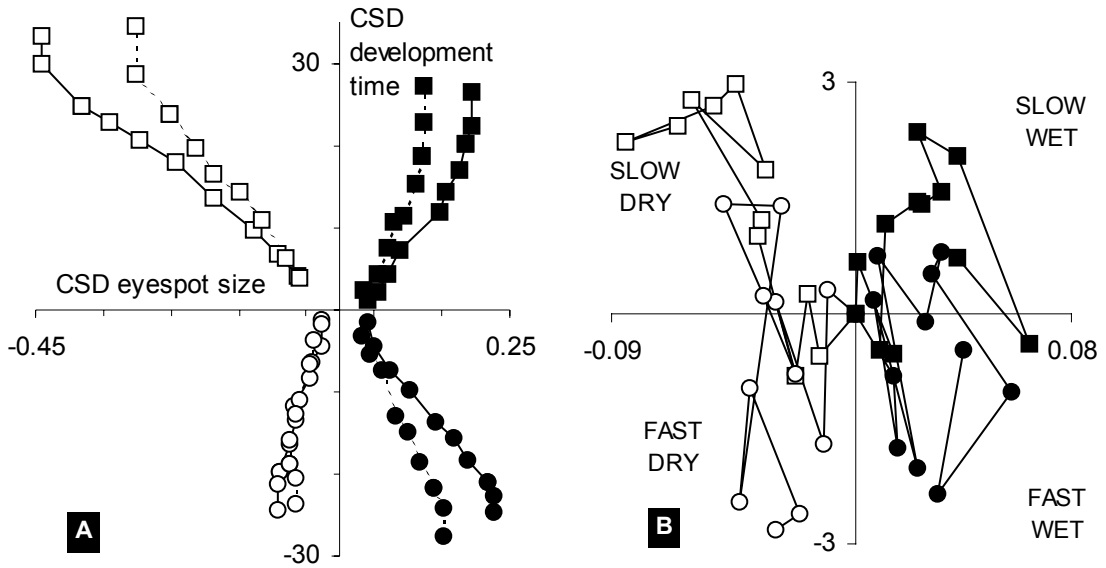


Figure 2.2 A (left): Cumulative selection differentials (CSD) for joint selection on eyespot size and development time (days) on females at 22.5ºC. Selection lines are: FAST WET [+ +] (●), FAST DRY [+ −] (○), SLOW WET [− +] (■), and SLOW DRY [− −] (□). Dotted lines connect values for the second replicate. **B** (right): Female development time (days) and relative eyespot size (both corrected for by controls) for four two-trait selection lines, selected for eleven generations at 22.5ºC.

To examine what part in the selection response could be attributed to selection on the correlated trait, we treated both development time (either FAST or SLOW) and eyespot size (either WET or DRY) as independent factors. The trait directly selected on was always significant. Development time selection (either FAST or SLOW) did not explain variance in eyespot size, but the interaction component was significant ($F_{1,4}$ = 15.6, p = 0.017); selection for a smaller eyespot phenotype (DRY) was facilitated by concurrent selection for a longer development time. This facilitation was not seen when selecting for large eyespot size (WET phenotype; see also figure 2.3). In other words, SLOW DRY has much smaller eyespots than FAST DRY, but is not much slower than SLOW WET. Graphically (figure 2.3), we would expect a more rectangular shape without facilitation, but we see a rhomboidal shape, with SLOW DRY clearly standing out in the eyespot size direction (dotted line figure in figure 2.3). The dotted lined figure of the response is less rectangular than the dashed lined figure of the cumulative selection differential in figure 2.3. Comparing these two shapes also clearly shows the larger response to eyespot size selection than to development time selection, and the larger response to SLOW selection compared to selection for FAST development time. Neither eyespot size selection, nor its interaction with development time selection were significant factors in explaining variance in development time.

The absolute response to selection was largest in the direction of SLOW DRY [− −] (figure 2.3), but realized heritabilities were higher for other directions of selection (e.g., FAST DRY [+ −]) in which selection pressures were lower (see table 2.1, figure 2.1). Indications for constraints were observed only incidentally: the decrease in response to development time selection for SLOW WET 1 [− +], and the decrease in the selection differential with generations for FAST DRY 2 [+ −]. Generally, the short term response to selection was not obstructed by the observed correlation of development time and eyespot size

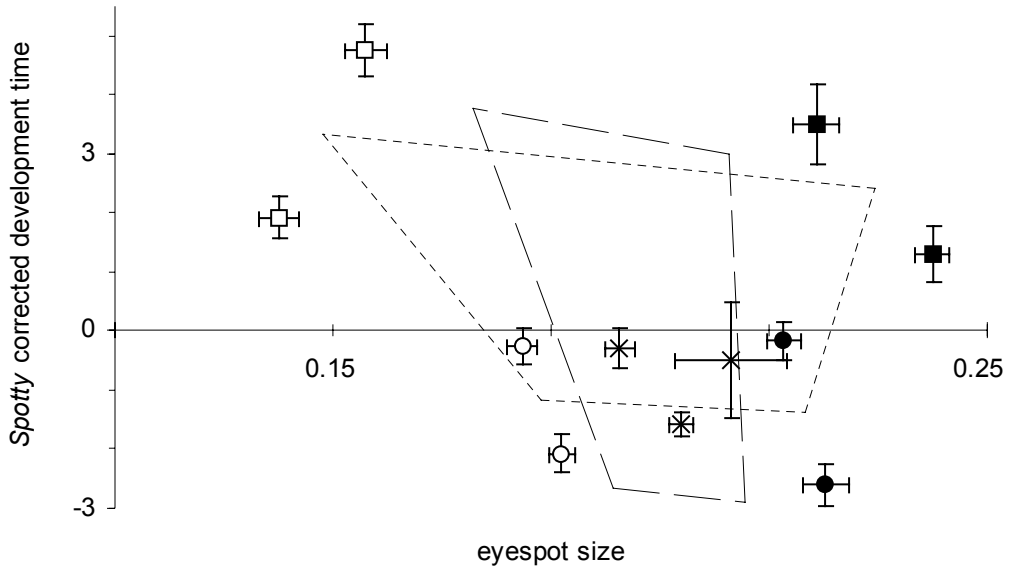


Figure 2.3 Female development time and eyespot size data (± standard errors) of the selection lines at 22.5ºC for the final generation of selection (both replicates shown). Development time has been corrected by subtracting the development time of the accompanying *Spotty* females. Selection lines are FAST WET [+ +] (●), FAST DRY [+ −] (○), SLOW WET [− +] (■), SLOW DRY [− −] (□), and UNSELECTED (×). Dotted lines connect replicate means per selection line, dashed lines depict the cumulative selection differentials (from figure 2.2a, divided by 9 to allow better comparison), see text for more explanation.

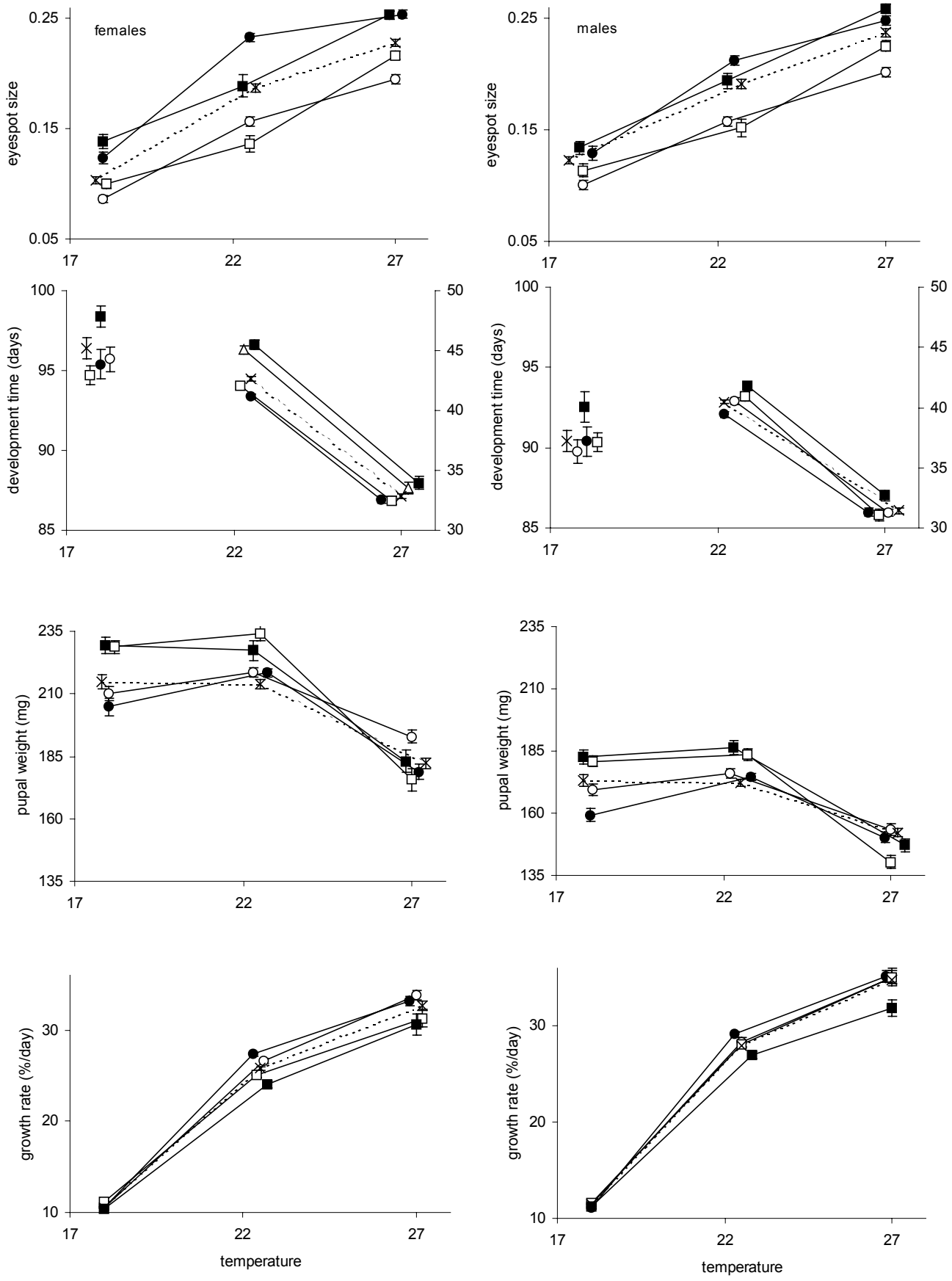
Reaction norms

Reaction norms at generation 8 for corrected eyespot size, development time, pupal weight and growth rate are shown in figure 2.4 for each selection line and sex. In the full model across temperatures, development time (log transformed) decreased with temperature ($F_{1,2086} = 9991.49$, $p < 0.0001$) and males were always faster than females because of protandry ($F_{4,2086} = 7.10$, $p < 0.0001$, see figure 2.4). The random factors, replicate (nested in line) and cage (nested in replicate) were also significant ($F_{6,33} = 2.73$, $p = 0.029$, and $F_{33,2086} = 1.69$, $p = 0.009$, respectively). The significant temperature × line

interaction ($F_{1,2086} = 34.85$, $p < 0.0001$) was due to the fact that development time of selection lines with a SLOW component increased much more with decreasing temperature than development time of partly FAST selected lines (contrast FAST versus SLOW, $t = 4.40$, $p < 0.001$). There was no significant interaction between sex and temperature ($F_{1,2086} = 0.07$, $p = 0.797$). At 22.5ºC, the difference between male and female development time was smaller for FAST selected lines than for SLOW selected lines (contrast, $t = 5.06$, $p < 0.0001$, table 2.2). At the two extreme temperatures, the factor selection line was highly significant (table 2.2), and the pattern as expected: FAST < unselected < SLOW. Correlations between development time and eyespot size did not differ within or across temperatures.

Pupal weight decreased significantly with temperature ($F_{1,2136} = 533.13$, $p < 0.0001$) and differed significantly between selection lines ($F_{4,2136} = 7.46$, $p < 0.0001$, contrast FAST versus SLOW, $t = 4.25$, $p < 0.0001$). Females were consistently (across and within temperatures) heavier than males (across temperatures: $F_{1,2136} = 1545.42$, $p < 0.0001$, figure 2.4). Interactions between temperature and selection line ($F_{4,2136} = 10.32$, $p < 0.0001$) and between temperature and sex ($F_{4,2136} = 21.35$, $p < 0.0001$) were significant. Differences in pupal weight between the sexes were more pronounced at lower temperatures (contrast, $t = 4.62$, $p < 0.0001$), and pupal weights of SLOW selected lines decreased more with temperature than FAST selected lines (contrast, $t = 5.58$, $p < 0.0001$). Neither across ($F_{4,2136} = 1.71$, $p = 0.15$), nor within temperatures were there line × sex interactions. At 18ºC and 22.5ºC, the FAST lines had the lowest pupal weights and unselected lines were of intermediate weight, but at 27ºC there was no clear pattern (see figure 2.4).

Figure 2.4 (next page) Reaction norms for eyespot size (top), development time, one-day pupal weight, and growth rate (bottom), for females (left) and males (right). Selection lines are: FAST WET [+ +] (●), FAST DRY [+ −] (○), SLOW WET [− +] (■), SLOW DRY [− −] (□), and unselected (×, dotted reaction norm). Note that for both the development time graphs (second row), the left-hand y-axis pertains to data at 18ºC, and the right-hand one to 22.5ºC and 27ºC. Some points are slightly offset for clarification.


females
males
eyespot size
development time (days)
pupal weight (mg)
growth rate (%/day)
temperature
0.25
0.15
0.05
100
95
90
85
50
45
40
35
30
235
210
185
160
135
30
20
10
17
22
27

	factor	18ºC $N = 400$	22.5ºC $N = 1038$ ‡	27ºC $N = 703$
development time	line	19.87 ***	2.62 ns	18.85 **
	sex	99.55 ***	210.03 ***	56.82 ***
	line × sex	0.18 ns	6.78 ***	0.09 ns
	factor	18ºC $N = 308$	22.5ºC $N = 412$ †	27ºC $N = 394$
eyespot size	line	24.01 ***	14.10 ***	61.62 ***
	sex	10.59 **	0.09 ns	3.82 ns
	line × sex	1.89 ns	2.97 *	1.03 ns

Table 2.2 F-statistics for ANOVA's on development time and eyespot size per temperature at generation 8. For factors line and line × sex: degrees of freedom (df) = 4, for sex: df = 1. Degrees of freedom for denominators equals *N* (number of individuals) minus 9, except for †: cage, nested in line (line denominator df = 15) was also significant, ‡: replicate (df = 6, denominator for line) and cage (df = 33), both nested in line were significant random factors. ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Growth rates significantly increased with temperature ($F_{1,2122} = 6258.70$, $p < 0.0001$), and differed between the selection lines ($F_{4,2122} = 15.23$, $p < 0.0001$). FAST WET and FAST DRY lines did not differ from each other in growth rate, and the SLOW selected lines similarly did not differ (Tukey test). Contrasts between SLOW and either FAST or unselected lines were highly significant ($p < 0.0001$), and the difference between the FAST and unselected line in growth rate was also significant (contrast, $t = 2.37$, $p = 0.017$). Males had higher growth rates than females ($F_{1,2122} = 70.20$, $p < 0.0001$), and there was a significant temperature by sex interaction ($F_{1,2122} = 6.03$, $p = 0.014$), indicating a larger increase in growth rate with temperature for males. When analyzing the sexes separately, temperature and selection line were significant factors, although differences between selection lines were less pronounced for males than for females.

Eyespot size increased with temperature ($F_{1,1094} = 2386.13$, $p < 0.0001$, figure 2.4), but in a consistent fashion for all selection lines (non-significant temperature × line interaction, $F_{1,1094} = 2.03$, $p = 0.088$). The analysis further showed that selection lines differed in relative eyespot size ($F_{4,6} = 15.15$, $p = 0.003$), with the expected pattern DRY < unselected < WET. Females had smaller relative eyespot sizes than males ($F_{1,1094} = 8.80$, $p = 0.003$), and the significant interaction between sex and selection line ($F_{4,1094} = 3.39$, $p = 0.009$) showed that the differences between lines were larger for females (the target sex for selection) than for males (figure 2.4). Replicates differed ($F_{6,1094} = 6.73$, $p < 0.0001$), but cages did not (omitted from the model). In the analyses split by temperature, there are

significant line effects at all temperatures, but only a significant sex effect at 18ºC (table 2.2). However, at 22.5ºC, line × sex is significant and there is a trend for the differences in eyespot size between WET and DRY selected lines to be larger for females than for males (contrast WET versus DRY, $t = 1.90$, $p = 0.058$).

To summarize, selection lines showed similar patterns across temperatures where eyespot size is concerned, but differed across temperatures with respect to development time and pupal weight; differences between FAST and SLOW selected lines increased with decreasing temperature. Furthermore, relationships between selection lines differed within and across temperatures for females (target of selection) and males (not selected directly).

Discussion

Response to selection

The correlation between eyespot size and development time observed for *B. anynana* within and across temperatures did not constrain the response to selection on a combination of these two traits in any direction. Realized heritabilities were comparable between agonistic (selected in the same direction as the correlation) and antagonistic (selected against the correlation) selection lines. In fact, we found some indication that antagonistic selection lines (especially FAST DRY [+ −]) had higher realized heritabilities for eyespot size than agonistic lines. The divergence in eyespot size was similar for all selection lines, but the antagonistic lines attained this with less selection, because less phenotypic variation was available in those directions (see figure 2.1). This also explains why the SLOW DRY lines, which had diverged the most (figure 2.3), did not show the highest realized heritabilities. The decrease in selection intensity on eyespot size for the FAST DRY 2 [+ −] line might point to exhaustion of genetic variation in that direction, and thus constrain additional response in the longer term (no available phenotypic variation present for selection to act on). Relatively speaking, the largest response was found in the antagonistic direction, that is, opposite the correlation. This would suggest that variation in the direction of the correlation has a larger component of environmental variation than the opposite direction. In other words, that phenotypic variation is predominantly environmental variation in the agonistic direction, and predominantly genetic variation in the antagonistic direction. How this relates to the function of genes and genetic pathways is unknown.

The larger response to selection for eyespot size versus development time is in accordance with previous realized heritability estimates: dorsal eyespot size 0.47 - 0.67 versus development time 0.11 - 0.12 (Monteiro *et al.*, 1994; chapter 6). Extrapolation of heritabilities for the dorsal eyespot size to the ventral eyespots may not be entirely appropriate because the former is not under strong natural selection, whilst the latter is. The larger divergence from the controls of SLOW compared to FAST in the final generation (figure 2.3) and the tendency for higher realized heritabilities for selection on SLOW development compared to selection for FAST development (see table 2.1, figure 2.3) is also as expected; heritability estimates for development time are commonly asymmetric

(Falconer & Mackay, 1996, p. 211). It was probably not due to the (intermediate) selection temperature, which was chosen to minimize any bias in the response.

Furthermore, the differences in eyespot size between selection lines in the final generation were wider for females, the sex that was selected on, than for males. This suggests some sex-specific factors affect wing pattern, just as development time has been shaped by sex-specific selection to lead to protandry (the adult emergence of males before females, figure 2.4). Similarly, association between alleles at the *Dll* locus and dorsal eyespot size showed sex-specific differences (Beldade *et al.*, 2002a). Why there should be sex-specific differences in wing-pattern determination is unclear, perhaps these differences are in part a by-product of protandry.

The phenotypic correlation between development time and eyespot size was not altered in the course of selection. Although we examined phenotypic correlations and not genetic correlations, there are grounds to believe that they tend to be (qualitatively) similar to each other (e.g., Cheverud, 1988). In previous studies using antagonistic selection lines, no changes (Bell & Burris, 1973), or variable and unpredictable changes (Sheridan & Barker, 1974), in genetic correlations were found. Epigenetic factors and modifier genes might explain why the pleiotropic relations persist even when selection is specifically aimed to break it. Downstream modifiers (more locally acting) of physiologically processes, as suggested by Sheridan and Barker (1974), may well be important in the *B. anynana* system as well (see below).

Reaction norms

The reaction norms for eyespot size were not altered in their shape or slope by the different selection regimes, only in elevation. All selection lines had a similar increase in eyespot size with increasing temperature; selection on eyespot size at a single, intermediate temperature (22.5ºC) changed eyespot size in the same manner at other, more extreme temperatures (18ºC and 27ºC). Wijngaarden and Brakefield (2001) did not obtain a response to selection on reaction norm shape of eyespot size (Wijngaarden & Brakefield, 2001). For development time and pupal weight, we found a line by temperature interaction (G × E): SLOW selected lines were even more slow (heavy) at lower temperatures than FAST selected lines. Development time and pupal weight scale differently with temperature for the different selection lines. Although the largest responses were observed for selection on eyespot size, there were no differences in plasticity between lines. However, we observed G × E interaction for development time, where elevation of the plasticity curve had not changed as dramatically as for wing pattern. Although there does not seem to be standing genetic variation present in our stock population to select for novel shapes of the eyespot size reaction norm (Wijngaarden & Brakefield, 2001), there is enough variation present to change the reaction norm of development time (and consequently of pupal weight). This pattern is the near opposite of patterns seen in the elevation of reaction norms, where wing patterns shows much higher realized heritabilities than development time (table 2.1).

Ecological implications

From an ecological viewpoint, we can conclude that the relation between development time and wing pattern has not become so rigidly integrated into developmental physiology that adaptation to a new combination of these traits is impossible or unlikely to evolve when favored by natural selection. These butterflies originate from Malawi, where the optimal combination is likely to be either fast development time and large eyespot size (in the wet season), or slow development and small eyespots (in the dry season). The main cue for pattern induction is temperature in the final larval and early pupal stage (Kooi & Brakefield, 1999). However, in other parts of Africa (northern hemisphere), the most advantageous combinations are different and may indeed be the reverse, because of different climatological circumstances (e.g., temperature, rainfall). For example, the more favorable season might be associated with lower temperatures and warrant conspicuous wing patterns, that is with the opposite relationship between development time and eyespot size. Furthermore, other abiotic factors such as rainfall may be better predictors of seasonality, and different cues, for example photoperiod, might yield better adapted seasonal polyphenisms (Roskam & Brakefield, 1999). The consequences of the genetic architecture of *B. anynana* we observed in the present study do not suggest strong short-term constraints would exist on adaptation to such a new combination of environmental circumstances. However, development time is influenced dramatically by temperature in ectotherms, and this universal factor is a much stronger determinant of development time than the genotype. Therefore, it is not assured that the temperature-wing pattern association can be readily changed to produce, for example, a reverse of the observed negative phenotypic correlation. However, this study shows that intermediate steps can be taken, that is a change in association within one temperature.

Further research: endocrinology

The relatively unconstrained response to antagonistic selection makes it very interesting to examine the putative hormonal system underlying the coupling between developmental time and wing pattern. One-trait selection lines for either ventral eyespot size or development time showed a shift in ecdysteroid-titer peak: fast developing, or large eyespot size selected lines had an earlier ecdysteroid peak three days after pupation at 20ºC than unselected lines or lines selected for small eyespot size (Koch *et al.*, 1996; Brakefield *et al.*, 1998). Furthermore, lines selected for large eyespot size also had higher ecdysteroid levels than small eyespot size selected lines in the first 12 hours after pupation when wing pattern determination is underway (Brakefield *et al.*, 1998). Our antagonistic selection is predicted to have exercised opposing selection pressures on the ecdysteroid dynamics: in the FAST DRY [+ −] lines, for example, the FAST component of selection would lead to an earlier peak in ecdysteroids after pupation, whilst the DRY component of selection would give rise to a later timing of the hormone peak. Because we did not observe any constraints on the short term response to selection, it is of interest to examine how these conflicting selection pressures have been accommodated at the endocrine level (see chapter 3).

To summarize our results, we did not find that the phenotypic correlation between development time and eyespot size hampered in any way, the short term response to selection on both traits simultaneously and in directions opposite to the correlation. There was no evidence for any limiting effects of genetic covariances on the response to artificial selection over ten generations in different directions.

Chapter 3

Endocrine differences between selection lines for development time and eyespot size in *Bicyclus anynana*[†]

Abstract

Hormonal mechanisms underlie many life-history traits and their interactions. We studied the role of ecdysteroids with regard to wing pattern and development time of the polyphenic butterfly *Bicyclus anynana*. Time series of ecdysteroid concentrations and sensitivity to ecdysone injection were assayed for two-trait selected lines (eyespot size and development time concurrently). Although selected lines had diverged most in eyespot size, the widest differences in ecdysteroid dynamics were observed between the development time selection regimes; fast selected lines had an earlier hormonal peak after pupation than slow selected lines. This endocrine peak was also earlier for females than for males. Furthermore, sensitivity to ecdysone injection as measured by a subsequent decrease in pupal time was significantly lower for slow selected lines than for fast or unselected lines. The observed response in eyespot size to artificial selection was achieved via other developmental mechanisms, such as changes in morphogen production or receptivity, because the dynamics of the alternative, hormonal, pathway were dictated by development time selection.

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Introduction

The study of the hormonal basis of (life-history) traits is an emerging topic in evolutionary biology (Ketterson & Nolan, 2000; Zera & Bottsford, 2001). Hormones are important in the regulation of life-history decisions, because their effects are manifold and integrate many traits. Many, if not all, polyphenisms are regulated by the endocrine system (Nijhout, 1994, 1999). For example, the winged/flightless polyphenism in *Gryllus* crickets, with its associated effects on fecundity and dispersal, is largely controlled by ecdysteroids and juvenile hormones (Cisper *et al.*, 2000; Zera & Bottsford, 2001). Not only hormone concentrations, but also changes in compounds that degrade hormones or protect them from degradation, or changes in receptivity (e.g., receptor numbers) could exert the endocrine control of such processes. Because hormones affect whole suites of traits, they can also act as constraints on evolution. Two traits controlled by the same hormonal mechanism are tightly linked and probably cannot evolve independently in the short term, or can only evolve separately until one of the two becomes coupled to another hormonal regulation pathway (Ketterson & Nolan, 2000).

To examine the evolutionary and ecological consequences of endocrinological variation on (life-history) traits and polyphenisms, we need an integrative approach. The seasonal polyphenic butterfly, *Bicyclus anynana*, has been studied at many levels, from development to ecology, and makes a good model system to study the interaction between hormones and life history (Brakefield *et al.*, 1996). This tropical butterfly occurs in nature in two seasonal forms (wet and dry), which are externally cued by (mainly) temperature in the final larval and early pupal stages (Brakefield & Reitsma, 1991; Brakefield *et al.*, 1996; Kooi & Brakefield, 1999). Wet season butterflies occur during the warm (>24ºC), wet season when larval food plants are widely available. Development is rapid, to maximize reproductive output in this favorable season. The ventral wing pattern of the adult butterflies is conspicuous (large circular marginal eyespots), and is thought to function in deflecting predatory attacks from birds and lizards away from the vulnerable body (Brakefield & Larsen, 1984). During the unfavorable, cool (<20ºC), dry season, the emphasis is on survival and butterflies have a cryptic wing pattern, relying on camouflage on a resting background of dead, brown leaves. In the laboratory at intermediate temperatures, intermediate wing pattern forms are observed that are rare in the wild (Windig *et al.*, 1994; Brakefield *et al.*, 1996). Heritable, genetic variation is available for (dorsal) eyespot size (realized $h^2 = 0.47 - 0.67$, Monteiro *et al.*, 1994). The same holds for development time (realized $h^2 = 0.12$, chapter 6). There is a strong correlation between development time and wing pattern, not only across temperatures or seasons, but also within one temperature or season; faster developing individuals have relatively larger eyespots than more slowly developing butterflies (Brakefield & Reitsma, 1991; Brakefield & Kesbeke, 1997).

As in other butterflies (e.g., *Araschnia levana*, Koch & Bückmann, 1987; *Precis coenia*, Rountree & Nijhout, 1995; see Koch, 1992 for review) the seasonal polyphenism of *B. anynana* is regulated by hormones, in particular ecdysteroids. Previous studies have shown that one-trait selection on either ventral wing pattern or development time altered the ecdysteroid titer dynamics. Butterflies selected for large ventral eyespots, or for faster development time showed an earlier ecdysone peak release after pupation than controls (Koch *et al.*, 1996; Brakefield *et al.*, 1998). Furthermore, pupae from lines selected for

large eyespots showed higher ecdysteroid titers in the first 12 hours after pupation than pupae from lines selected for small eyespots (Brakefield *et al.*, 1998). Microinjection or continuous administration of 20-hydroxyecdysone in early pupae increased ventral eyespot size and decreased pupal time (Koch *et al.*, 1996; Brakefield *et al.*, 1998).

A common hormonal system underpinning both development time and wing pattern could be the underlying mechanistic basis of the coupling of these two traits, and constrain the response to antagonistic selection. However, such (short term) constraints were not observed in a two-trait selection experiment (chapter 2). Most response to joint artificial selection was observed on eyespot size selection, irrespective of selection applied concurrently on development time . However, the selection lines did differ in development time after 11 generations of selection. These results raise the intriguing question of what happened at the hormonal level, especially in the antagonistic selected lines.

To examine this issue, we measured ecdysteroid titer dynamics after pupation for the two-trait selected lines. Changes in timing of hormone release have been documented for lines in which selection was applied to either development time or wing pattern. We also performed hormone injection analyses to test if the sensitivity for ecdysone had changed due to (ant)agonistic selection. Koch *et al.* (1996) showed that ecdysone injections affected wing pattern and pupal time in *B. anynana*, but they did not examine differences between selection lines in this reaction to ecdysteroid administration.

Materials and Methods

Butterflies

The *Bicyclus anynana* butterflies in this experiment were derived from the stock population that has been kept in the laboratory in Leiden for over 10 years. Details of the selection lines used are described in chapter 2, here we recapitulate only the most relevant aspects. Lines (each replicated twice) were created at an intermediate rearing temperature of 22.5ºC, and were selected simultaneously for both egg-to-adult development time (FAST and SLOW) and eyespot size (WET, i.e. relatively large fifth ventral hindwing eyespot, or DRY, small eyespot). FAST WET [+ +] and SLOW DRY [− −] were agonistically selected lines because selection was in the same direction as the relationship between development time and eyespot size, FAST DRY [+ −] and SLOW WET [− +] were antagonistically selected (against the observed relationship). Three unselected controls were maintained during selection. The selection lines had diverged in both traits after eight generations, when the experiments reported here were performed, and no apparent constraints on response to selection were observed (chapter 2).

Eggs laid by the selection line females over a 12h period were distributed over three temperatures, 18ºC, 22.5ºC and 27ºC (all with high relative humidity and 12:12 L:D regime), using four cages per replicate line per temperature. Caterpillars were reared on young maize plants. Cages were checked daily for pupations and one-day old pupae were weighed to the nearest 0.01mg, after which they were kept individually until emergence.

Hormone titers

To establish a time series for ecdysteroid titers at 22.5ºC, we took hemolymph samples from pupae at different times after pupation (pupations were checked every half hour and pupae were sexed). The time series consisted of 0h, 24h, 36h, 48h, 60h, 72h, 96h, and 144h after pupation and we attempted to sample four males and four females per time point per replicate selection line (summed over all four cages). To compare hormonal trends at different temperatures, we sampled at about one-third of pupal time, i.e. 96h after pupation at 18ºC, 60h at 22.5ºC, and 36h at 27ºC. At that time, the largest differences between groups are expected because of differences in the timing of ecdysteroid release across temperatures (Brakefield *et al.*, 1998). Hemolymph samples (~50µl) were mixed with phenylthiocarbamide (Sigma) and centrifuged at 0ºC, the supernatant was stored at −80ºC. Ecdysteroid titers in pupal hemolymph samples were determined using radioimmunoassay (RIA), similarly to Koch *et al.* (1996). Titers were measured for each pupa individually, except for samples taken 0h and 144h after pupation, where we pooled 2-3 pupa of the same sex. At these two extreme time points we needed more hemolymph for the assay because of low ecdysteroid concentrations.

The absolute hormone levels measured after about one-third of pupal time differed significantly among temperatures, but there was no trend in amount of ecdysteroids with temperature. Therefore, we will use within-temperature standardized values to compare selection lines across temperatures. Standardizing using the unselected ecdysteroid titers yielded near identical values to use of the overall mean and standard deviation of a temperature (correlation = 0.996). We have used data standardized by the unselected control lines.

Hormone injection

Microinjections were made in the region of the fifth abdominal segment of the pupa using a 10µl syringe with a 0.3mm needle (Hamilton). We injected 0.25µg 20-hydroxyecdysone (Sigma) dissolved in 3µl physiological salt solution (ecdysone injection), or for controls only 3µl physiological salt solution. Pupae (of known sex) were injected at physiological similar times after pupation: 20h at 18ºC, 15h at 22.5ºC, and 9h after pupation at 27ºC. Dose and administration time were thus chosen to obtain an optimal balance between hormonal effects and survival (Koch *et al.*, 1996). All pupae emerged individually and butterflies were subsequently frozen for wing pattern analysis. We measured the black portion of the fifth ventral hindwing eyespot, and the distance between the first and fifth hindwing foci (measure of wing length, to correct for overall size) for all injected animals and for a random subsample of untreated individuals.

Statistical analysis

In the analyses of variance on ecdysteroid titer, replicates of selection lines (treated as random effect) never differed significantly, and were, therefore, pooled. Unless otherwise stated, interaction terms were not significant and were omitted from the model. Temperature was always used as a discrete factor. Pairwise comparisons were made using Tukey tests or contrasts.

Results

Time series

Descriptive statistics for ecdysteroid titers in the selection lines at 22.5ºC are given in table 3.1. Comparing across lines for each time point, most analyses of variance (ANOVA) had one or two significant factors (table 3.2). Females showed an earlier peak in hormone titer after pupation than males (figure 3.1). From 24-48h after pupation, females had significantly higher ecdysteroid levels than males, but this pattern reversed at around 60h after pupation. At 96h after pupation, males had significantly higher concentrations of ecdysteroids in their hemolymph than females (table 3.2, figure 3.1). Males and females attained similar maximum levels of ecdysteroids. Accounting for the slightly longer pupal time of males compared to females, or for differences in pupal time between selection lines did not alter the time series results (data not shown). FAST WET [+ +] selected lines had an earlier increase in ecdysteroids than other selection lines, i.e. significantly higher titers 24h after pupation. SLOW DRY [− −] animals tended to have a later increase in hormones than the other lines and five days after pupation, ecdysteroid levels were significantly higher than those of FAST DRY [+ −] and unselected lines (table 3.2, figure 3.2).

For analytical purposes, we split the factor of selection line into two separate components (i.e. two new factors), one for development time selection (either FAST or SLOW) and one for eyespot size selection (DRY or WET). The unselected lines are omitted for this analysis. The development time component of selection showed significant differences in ecdysteroid concentrations 24h and 36h after pupation (FAST > SLOW, 24h: $F_{1,30} = 13.06$, $p = 0.0011$, 36h: $F_{1,24} = 4.89$, $p = 0.037$). In addition, WET selected pupa (larger eyespots) had higher levels of hormone at 24h after pupation ($F_{1,30} = 5.08$, $p = 0.032$). Seventy-two hours or more after pupation, there were significant interactions between the two components of selection; the combinations FAST WET and SLOW DRY had higher hormone concentrations than the two antagonistically selected lines FAST DRY and SLOW WET (interaction: 72h: $F_{1,24} = 5.71$, $p = 0.024$, 96h: $F_{1,25} = 7.63$, $p = 0.011$, 144h: $F_{1,23} = 4.72$, $p = 0.039$).

sample time(h)	FAST DRY females	FAST DRY males	FAST WET females	FAST WET males	SLOW DRY females	SLOW DRY males	SLOW WET females	SLOW WET males	UNSELECTED females	UNSELECTED males
0	73 10 *4*	67 11 *4*	77 7 *4*	68 14 *4*	65 3 *4*	80 15 *4*	84 11 *4*	87 6 *4*	80 6 *6*	69 4 *6*
24	237 11 *5*	198 41 *4*	352 73 *4*	263 48 *4*	179 33 *5*	141 18 *4*	188 9 *4*	175 6 *4*	221 12 *6*	170 12 *6*
36	1038 191 *3*	813 39 *3*	1132 312 *4*	557 197 *3*	632 128 *4*	355 52 *3*	878 67 *4*	599 151 *4*	936 58 *6*	579 103 *6*
48	2405 224 *4*	1269 183 *4*	1944 570 *4*	1243 31 *4*	2289 682 *4*	693 152 *4*	2594 471 *3*	1350 156 *3*	1770 208 *6*	1334 100 *6*
60	2153 244 *3*	2606 173 *3*	1837 244 *4*	2604 92 *4*	2177 78 *3*	2250 208 *3*	2246 72 *4*	2382 198 *4*	2177 403 *7*	1768 253 *7*
72	1610 364 *5*	2256 178 *4*	1912 361 *4*	2282 246 *4*	2888 405 *4*	2616 442 *4*	1580 237 *3*	2083 120 *4*	1417 304 *6*	1883 331 *5*
96	318 32 *4*	440 39 *4*	502 115 *4*	807 149 *4*	612 119 *3*	790 184 *3*	359 88 *4*	701 159 *4*	291 27 *6*	518 80 *6*
144	96 38 *3*	96 34 *4*	87 35 *3*	131 27 *4*	115 35 *4*	175 21 *5*	59 29 *4*	94 14 *4*	165 31 *6*	190 99 *6*

Table 3.1 Ecdysteroid titers (in ng/ml, with standard errors and *N*) per selection line, sex and sample time at 22.5ºC.

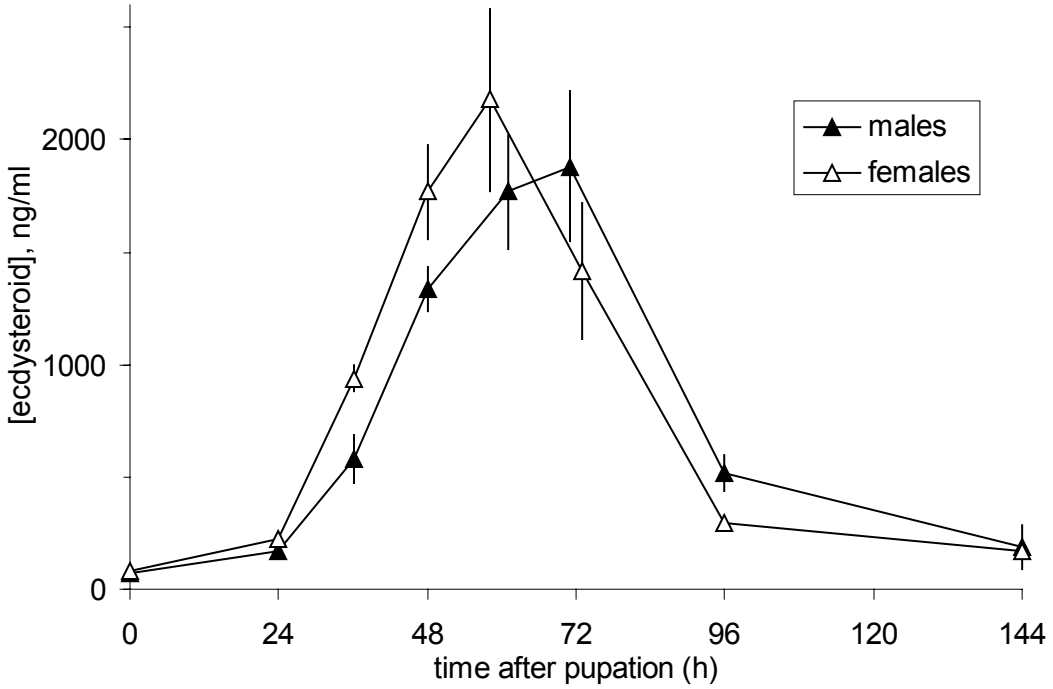

Figure 3.1 Ecdysteroid titers (± standard errors) in hemolymph of the unselected lines, collected at different times after pupation for males (closed triangles) and females (open triangles) at 22.5ºC. Some values are slightly off-set to improve display.

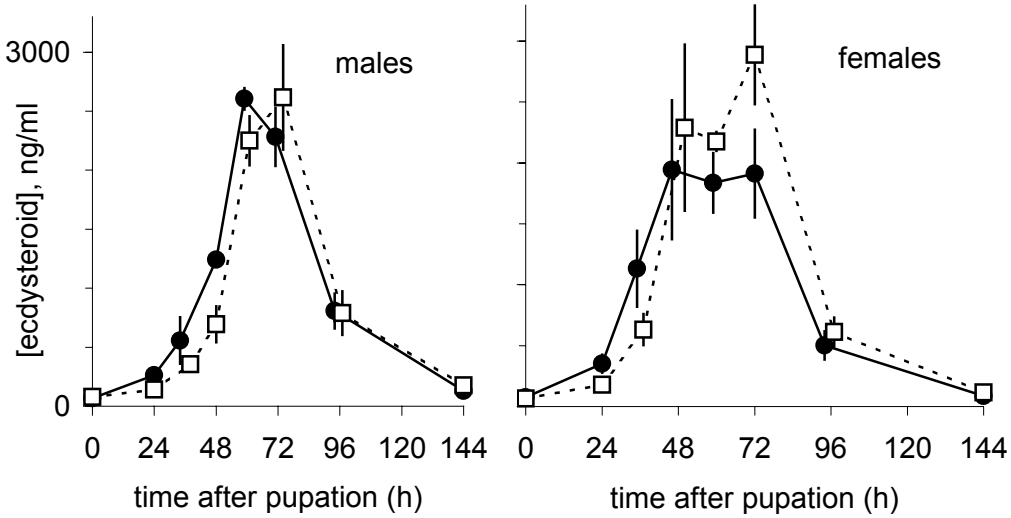

Figure 3.2 Ecdysteroid titers (± standard errors) in hemolymph of the FAST WET (●) and SLOW WET (□) lines, collected at different times after pupation for males (left) and females (right) at 22.5ºC. Some values are slightly off-set to improve display.

sample time (h)	factor sex		selection line	
	p	direction	p	Tukey/trend
0	0.667		0.481	
24	0.017	♀ > ♂	<0.001	FW > [FD, U, SW, SD]
36	<0.001	♀ > ♂	0.074	[FD, FW, U, SW] > SD
48	<0.001	♀ > ♂	0.594	
60	0.564		0.624	
72	0.089	♂ > ♀	0.015	SD ≥ [FW, FD, SW] ≥ U
96	<0.001	♂ > ♀	0.007	SD ≥ [FW, SW] ≥ U, FD
144	0.304		0.232	

Table 3.2 Results of analyses of variance on ecdysteroid titers per sample time, with sex and selection line as factors (F = FAST; S = SLOW; W = WET; D = DRY; U = unselected). When $p < 0.05$ for the selection line factor, the relationships between the lines were based on a Tukey test, otherwise trends are indicated ($p < 0.09$).

Effects of temperature

Both at the low (18ºC) and the high (27ºC) temperature there was a trend for earlier ecdysteroid release after pupation for FAST selected lines (table 3.3). At 18ºC, the agonistic line FAST WET [+ +] had significantly higher hormone levels than its counterpart SLOW DRY [− −], whilst the unselected and antagonistically selected lines had intermediate values. At 27ºC, the two antagonistic selected lines differed significantly from each other; FAST DRY [+ −] had significantly higher levels than SLOW WET [− +]. Unselected and agonistic lines were intermediate, but did not differ significantly from either FAST DRY [+ −] or SLOW WET [− +]. There were no patterns for selection lines at 22.5ºC (cf. time series). When the factor selection line is divided into two factors, a development time and an eyespot size selection component, then at all temperatures FAST lines (tend to) have higher hormone concentrations than SLOW lines (18ºC: $F_{1,49} = 12.82$, $p < 0.001$, 22.5ºC: $F_{1,121} = 3.45$, $p = 0.066$, 27ºC: $F_{1,98} = 6.58$, $p = 0.012$). The wing pattern selection regime was only significant at 27ºC, small eyespot selected lines (DRY) had higher hormone levels than WET-selected animals ($F_{1,98} = 3.95$, $p < 0.05$, cf. Tukey test in table 3.3).

At 22.5ºC, we found a significant difference between males and females, that was not observed in the time series (tables 3.2 and 3.3). At 27ºC, but not at 18ºC, females also had higher hormone concentrations than males (table 3.3). The differences between males and females indicate that the rise in ecdysteroid levels starts earlier for females than for males (also when corrected for sex differences in pupal time).

tempe-rature	sample time (h)	*N*	factor sex		selection line	
			p	direction	p	Tukey
18ºC	96	76	0.404		0.036	FW ≥ [FD, U, SW] ≥ SD
22.5ºC	60	174	0.011	♀ > ♂	0.189	
27ºC	36	143	<0.001	♀ > ♂	0.046	FD ≥ [U, FW, SD] ≥ SW

Table 3.3 Results of analyses of variance on ecdysteroid titers per temperature, with sex and selection line as factors. Pairwise comparisons were done using a Tukey test ($p < 0.05$).

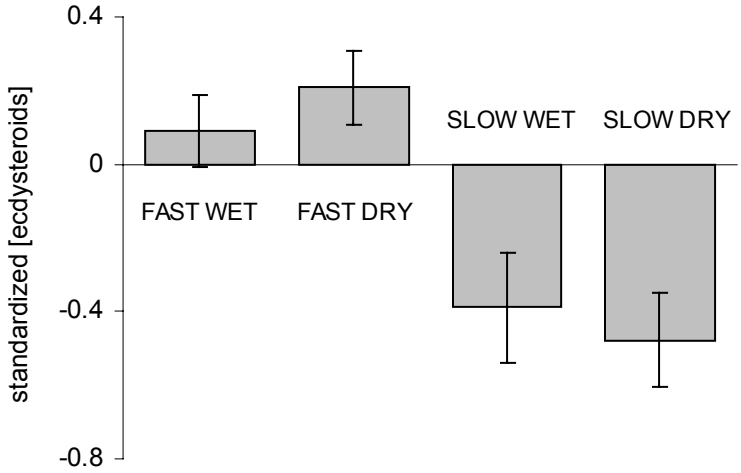

Figure 3.3 Least square means (± standard errors) for standardized ecdysteroid concentrations from three temperatures. Zero on the y-axis represents the unselected mean. See text for more information and other factors in the model.

Comparing hormonal differences across temperatures using standardized hormone levels, a clear difference between FAST and SLOW selected lines was observed (selection line $F_{3,265} = 17.51$, contrast FAST versus SLOW, $F_{1,265} = 22.83$, both $p < 0.0001$, see figure 3.3). The temperature × selection line interaction was also significant ($F_{6,265} = 2.16$, $p < 0.05$) because differences between selection lines were less pronounced at 22.5 ºC. The model with standardized ecdysteroid levels also included a significant sex and sex × temperature interaction ($F_{1,265} = 6.91$ and $F_{2,265} = 5.71$, respectively, both $p < 0.01$), due to the pronounced sex differences at 27ºC (see table 3.3). When the main effect selection line is partitioned into its two components, only the component for development time explains significant variation in standardized ecdysteroid titers ($F_{1,268} = 23.40$, $p < 0.0001$, eyespot size selection: $F_{1,268} = 0.01$, $p = 0.91$).

The main results for hormone titer were thus, that females had an earlier increase in hormone production than males. Both sets of lines selected for FAST development started producing ecdysteroids earlier than SLOW selected lines, whereas selection on eyespot size did not greatly affect hormone dynamics.

Injections

The numbers of pupae used for the hormone injection experiment are given in table 3.4. Pupal survival was significantly lower for 20-hydroxyecdysone (ecdysone from here onwards) injected animals compared to untreated pupae at both extreme temperatures, whilst at 22.5ºC, no difference in pupal survival was observed (table 3.4). Probability of survival increased with increasing pupal weight, which was a highly significant covariate ($p < 0.001$) in the logistic regression at all temperatures. With an increase in pupal weight of 10 mg, survival probability increased by 1.13 at 27ºC, 1.20 at 22.5ºC and 1.26 at 18ºC. There were no interactions between pupal weight and treatment. Pupal survival probability did not differ between the sexes, but the significance levels of the covariate pupal weight were reduced to $p < 0.05$ (females are larger than males). The effects of treatment remained unchanged. Because we did not know the sex of all unhatched pupa, incorporating this factor decreased the power of the test.

Survival of untreated control pupae differs across temperatures ($\chi^2 = 15.3$, df = 2, $p < 0.001$), and is lowest at 18ºC (table 3.4). The other two temperatures do not differ in survival ($\chi^2 = 0.4$, df = 1, $p = 0.53$). We could not incorporate pupal weight, because it differed between temperatures ($F_{2,2540} = 478.1$, $p < 0.0001$; pattern: 22.5ºC > 18ºC > 27ºC).

temp.	injection	*N* emerged	*N* dead	% emerged		odds ratio [95% C.I.]
18ºC	ecdysone	10	36	22	***	0.03 [0.01-0.10]
	injected controls	6	5	55	ns	1.55 [0.27-9.52]
	untreated controls	599	91	87		
22.5ºC	ecdysone	172	16	91	ns	0.54 [0.14-1.63]
	injected controls	62	2	97	ns	3.15 [0.63-36.1]
	untreated controls	1041	88	92		
27ºC	ecdysone	125	55	69	***	0.13 [0.06-0.27]
	injected controls	50	5	91	ns	2.41 [0.77-10.1]
	untreated controls	901	87	91		

Table 3.4 Numbers of pupae used for the hormone injection analysis at the three different temperatures. Survival of the three groups (ecdysone injected, saline injected and untreated) was compared using logistic regression (per temperature) with pupal weight as a covariate; ns: not significant, ***: $p < 0.001$. Odds ratio [95% confidence interval] are for survival probability versus untreated control pupae.

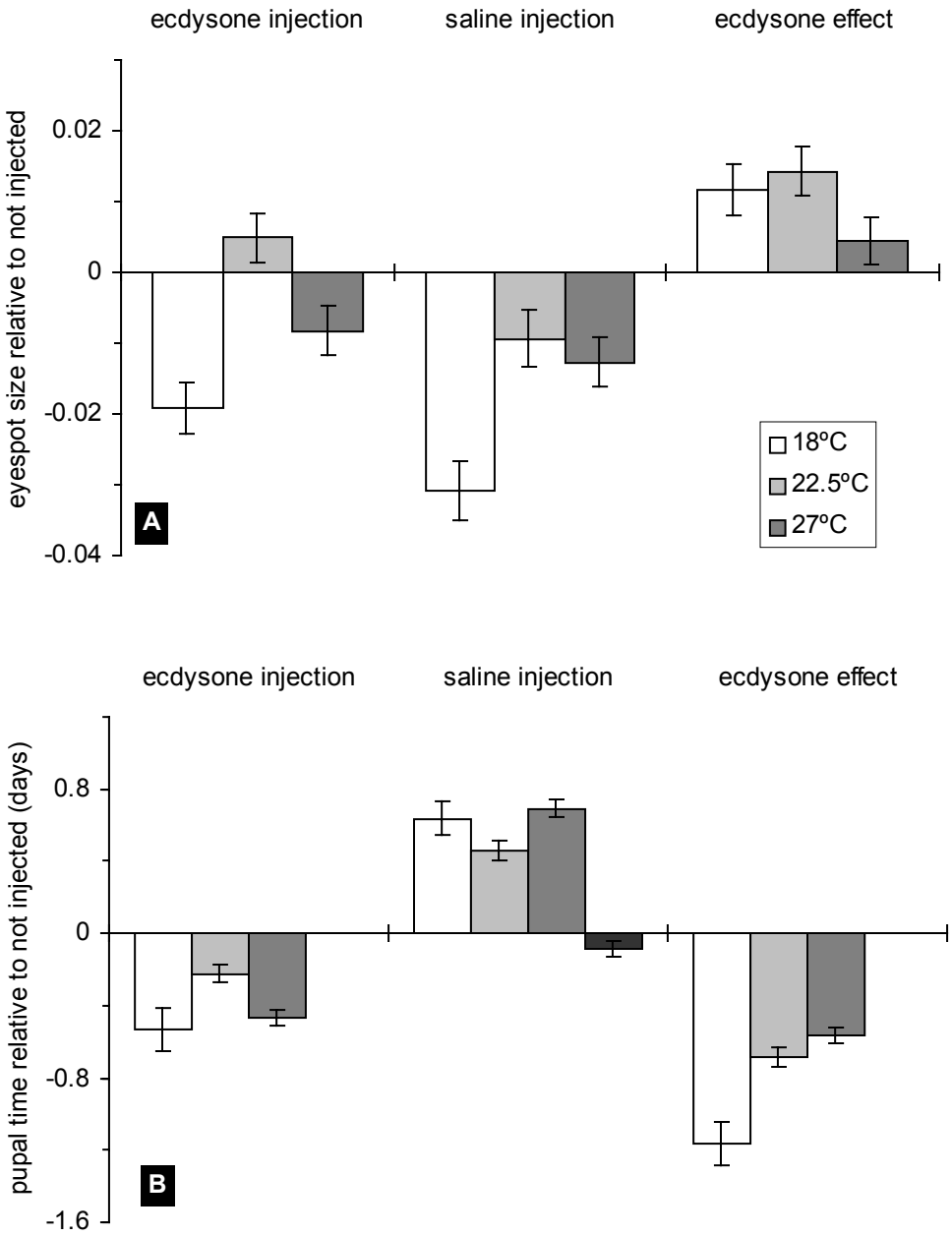

Figure 3.4 Effects of injection of physiological salt or ecdysone on pupal time and eyespot size at different temperatures. Effects of ecdysone are derived by subtracting effects of physiological salt injection from ecdysone injection. At 27ºC, selection lines reacted differently to injection *per se*, hence the two bars (lighter bar for SLOW, see text for more information).

At all temperatures, pupae injected with physiological salt had significantly smaller eyespots than the untreated animals. Pupal time also tended (significant at 22.5ºC only) to be longer for saline injected butterflies (figure 3.4). The effect of injection *per se* might, therefore, counteract potential effects of the hormone implying that ecdysone injection has a twofold effect: one due to the injection treatment *per se*, and one resulting from the hormone. We can disentangle these two effects by subtracting the injection effects, as measured by the effects of physiological salt injection. At 27ºC, selection lines differed in their change in pupal time as a reaction to saline injection (significant line × injection interaction): lines with a SLOW component of selection showed a significant increase in pupal time after physiological salt injection, whilst partly FAST selected and unselected lines did not (figure 3.4). Because genetic background influenced the effect of injection *per se*, we corrected pupal time at 27ºC using separate correction factors for the SLOW lines, and for the FAST and unselected lines, and used those data for all further analyses.

Pupal time significantly decreased for the ecdysone-treated animals at all temperatures (figure 3.4). Although pupal time increased with pupal weight, the mass of a pupa did not affect the effects of ecdysone and/or injection (no significant interaction terms, $p > 0.10$). Only at 22.5ºC was there an interaction between selection line and hormone treatment ($F_{1,1188} = 2.46$, $p = 0.044$; other factors in the model were: line, sex, line × sex, pupal weight and replicate[line]). The decline in pupal time was significantly less for SLOW selected groups than for partly FAST selected lines (contrast, $t = 2.36$, $p = 0.018$); this is clearest for males in figure 3.5. However, the sex by treatment interaction was not significant ($F_{1,1188} = 1.52$, $p = 0.22$).

Eyespot size increased under the influence of ecdysone at 18ºC (trend, $F_{1,287} = 2.89$, $p = 0.09$) and at 22.5ºC ($F_{1,547} = 8.91$, $p = 0.003$), but hormone treatment did not alter eyespot size at 27ºC ($F_{1,454} = 0.08$, $p = 0.77$), see figure 3.4. There were no interactions between eyespot size and hormone treatment.

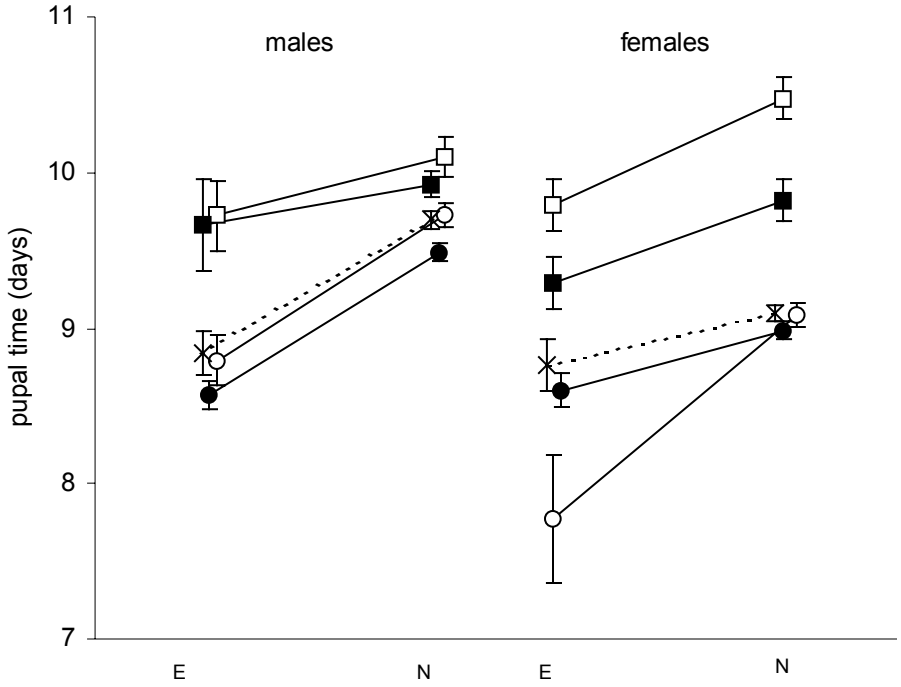

Figure 3.5 Effects of ecdysone, corrected for effect of injection (denoted as E) on pupal time (± standard error) versus not treated animals (N), for males and females at 22.5ºC. Selection lines are: FAST DRY (○), FAST WET (●), SLOW DRY (□), SLOW WET (■), and UNSELECTED (×).

Discussion

We have shown differences in ecdysteroid production and sensitivity between selection lines for development time and wing pattern of *Bicyclus anynana*. Lines which were selected for SLOW development time (especially SLOW DRY) showed a later hormone peak than FAST selected animals. The effects of concurrent wing pattern selection on ecdysteroid titers were less marked and not uniform.

Previous studies in *B. anynana* found an earlier ecdysone peak for FAST or for WET selected animals, compared to SLOW or DRY selected animals, respectively (Koch *et al.*, 1996; Brakefield *et al.*, 1998). Here we found that development time selection was more important in determining the hormone dynamics than eyespot size selection. Thus, the effect of (antagonistic) eyespot size selection on hormone titers was less evident than that arising from the direction of development time selection. In contrast, the response in the trait taregeted by wing pattern selection was much larger than the corresponding response for development time selection (chapter 2). Although selection was only for 8 generations (compared to >25 for the one-trait selected lines), we observed endocrine differences in timing of the ecdysteroid sigmoidal curve after pupation between FAST versus SLOW lines even when individual variation in total pupal time was taken into account. Maximum titers of hormone levels were similar for all lines.

The effects of eyespot size selection on hormone dynamics were much weaker than anticipated from the results of single-trait selection lines. Also, the selection lines showed distictly diverged eyespot sizes, albeit not completely corresponding to the two seasonal forms. Some of the weak hormonal response to wing pattern selection may be due to the small number of generations of selection, and/or the intensity of selection. Nonetheless, the observed changes in ecdysteroid titers could also be partly accounted for by correlated responses on development time during eyespot size selection; wing pattern selection itself apparently did not strongly influence ecdysteroid dynamics. Although ecdysteroids are important in determining wing pattern, as can be seen by the effects of injection, they are more critical for regulating pupal time and overall development. Quantitative variation in wing pattern must also be regulated by other factors that are independent of ecdysteroid levels, because even though concurrent selection on development time created hormone dynamics in conflict with the desired response in ventral eyespot size, there was no apparent constraint to selection. For example, in the FAST DRY line, an earlier increase in ecdysteroids after pupation has arisen because of the FAST selection, that opposes the desired endocrinological pattern for eyespot size. Yet, this line showed markedly smaller eyespots and had the highest realized heritabilities for this trait (chapter 2). Further support for this reasoning is given by the lack of response of the non-plastic dorsal eyespots to hormone injections (Brakefield *et al.*, 1998), although they can respond readily to artificial selection (Monteiro *et al.*, 1994; Beldade *et al.*, 2002b). Developmental mechanisms may play a larger role in determining ventral eyespot size than whole body endocrinology. It appears that at least two different mechanisms (developmental and physiological/endocrine) are involved in changing ventral eyespot size across temperatures (in contrast to the dorsal eyespots where only the developmental mechanism is relevant, because they are non-plastic). Which one is involved in the response to artificial selection, depends on which other traits are under selection at the same time. In our case, the mechanism which changes in response to selection on eyespot size appears to have been primarily that of the developmental mechanism, because the concurrent selection on development time dictated the endocrine dynamics (physiological mechanism). Because of the alternative, non-endocrine means of changing wing pattern, ecdysteroid levels pose less of a constraint on independent evolution of development time and eyespot size than previously thought.

To summarize, there are three ways of genetically changing ventral eyespot size: (*i*) change hormone dynamics, as partially seen for lines selected for larger or smaller ventral eyespot only (Koch *et al.*, 1996; Brakefield *et al.*, 1998), (*ii*) change the developmental pathways in the wing tissue (Monteiro *et al.*, 1994; Beldade *et al.*, 2002a), or (*iii*) a combination of both. Our results here argue for the second explanation, because substantial changes in ventral eyespot size were achieved without any change in hormone dynamics. The observed changes in endocrinology of the single trait selection lines for ventral eyespot could then be due to correlated responses on development time. However, eyespot sizes can be partly shifted by ecdysteroid injections (Koch *et al.*, 1996; Brakefield *et al.*, 1998). In addition, the distribution of ecdysteroid receptors coincides with the final eyespot pattern (P. B. Koch, unpubl. res.) suggesting some direct role for hormones in eyespot size determination.

To compare hormone titers across temperatures, we only sampled at one time point, namely at one-third of pupal time. A possible criticism of this method is that

changes in timing are not accounted for and that not observing differences could be due to the use of an inappropriate time-point. However, we consistently found that FAST selected lines had higher ecdysteroid levels than SLOW selected lines in the ascending phase of the ecdysteroid peak after pupation (see figure 3.3). These differences interacted with temperature, but only because the magnitude of the differences was not constant over temperatures. The direction was the same at all temperatures; FAST selected lines had higher hormone titers than SLOW selected lines. The timing of sampling could explain the differences in absolute titer levels between the temperatures, and sampling at several time points might allow for better comparisons between temperatures.

The differences in hormone dynamics between males and females, combined with the effects of ecdysone microinjection on pupal time (see below), suggest that ecdysteroids regulate the duration of the pupal stage. Although egg-to-adult development time for males is shorter than for females (protandry), the duration of their pupal stage is longer than for females (figure 3.5; Brakefield & Kesbeke, 1997). This can be explained by the earlier ecdysone peak for females compared to males. We found no differences between the sexes in ecdysteroid sensitivity with respect to pupal time.

Previous work on *B. anynana* endocrinology showed that pupal time and wing pattern are affected by 20-hydroxyecdysone administration, but did not compare different genetic backgrounds in their response to ecdysone. In this study we showed that at 22.5ºC, SLOW selected lines have a lower sensitivity for ecdysteroids than FAST selected lines (figure 3.5). Thus, pupal time of SLOW selected lines decreased less under the influence of exogenous ecdysteroids than FAST selected lines. *A priori*, the opposite is expected because untreated FAST and SLOW lines differed significantly in their pupal time (figure 3.5), and the scope for a decrease in pupal time was much greater for SLOW than for FAST lines since the latter are more likely to be near a minimum for time necessary for pupal development. The observation that unselected lines followed the FAST lines in their response to ecdysteroid injection, suggests that the SLOW selected lines have fewer ecdysteroid receptors or ones with a lower binding affinity, or have increased amounts of ecdysteroid degrading compounds. Whatever the mechanistic reason, SLOW lines have a lower receptivity for ecdysteroid signals than FAST lines.

Interestingly enough, we only observed differences in receptivity at 22.5ºC, the temperature at which selection took place. High mortality may have precluded detection at 18ºC, but this was not the case for 27ºC. There are several possible explanations which are not mutually exclusive. Firstly, all (enzymatic) processes occur at faster rates at 27ºC compared to 22.5ºC, so the differences between selection lines are smaller and harder to detect. However, trends do not point in this direction. Another possibility is that because 22.5 ºC is in the steeply ascending part of the plasticity curve, small changes on the x-axis lead to large changes on the other axis (e.g., endocrine sensitivity) (Brakefield *et al.*, 1996). One of the reasons we chose 22.5ºC as the temperature to select at was to rule out any bias in the outcome of selection. Furthermore and related, endocrine differences might not be constant over temperatures, e.g., ecdysteroid receptors may differ in affinity at 22.5ºC but not at 27ºC. A hormonal receptivity by temperature interaction is quite possible.

The high mortality at 18ºC as a result of injection may come about because low temperatures interfere with healing of the injection wound. However, caterpillars reared at 22.5ºC that were transferred to 18ºC upon pupation and injected at that temperature,

did not show significantly lower pupal survival than pupae injected at 22.5ºC (data not shown). Furthermore, it appears that 18ºC is also a less optimal temperature for uninjected pupae, because significantly more pupae died at 18ºC than at either 22.5ºC or 27ºC. This may, therefore, represent an additional factor in making the dry season unfavorable.

In conclusion, we found that development time is tightly linked to ecdysteroid levels after pupation and is also related to changes in hormone sensitivity. SLOW selected lines had a later hormone peak after pupation, and a lower sensitivity for ecdysone. Wing pattern formation was less dependent on endocrine dynamics, although it may be influenced via correlated responses. It therefore seems likely that different mechanisms can influence ventral eyespot size. The response in eyespot size to two-trait selection mainly depends on changes in developmental pathways (e.g., strength of focal signal, morphogen sensitivity), whilst the ecdysteroid physiology is shaped by selection on development time.

Chapter 4

Life history of *Bicyclus anynana* mutants: Can they serve as internal controls?[†]

Abstract

Correction for environmental effects in quantitative genetics experiments is often achieved by use of separate control populations. However, this method may not always correct accurately for environmental fluctuations in rearing conditions. A more suitable approach could be rearing mutants together with experimental groups, provided that these mutants are clearly distinguishable phenotypically and have similar life-histories. We tested whether two mutants (*yellow* and *Spotty*) available for the tropical butterfly *Bicyclus anynana* could serve as such true internal controls. The *yellow* butterflies had lower viability and longer egg-to-adult development time than the wild type animals they were reared with, whereas *Spotty* individuals were equivalent in those respects to their wild type counterparts. Moreover, heritability estimates for development time in the *Spotty* stock were mostly significantly different from zero and fell within the range found for wild type butterflies. Therefore, we conclude that a backcrossed stock homozygous for the *Spotty* mutant of *B. anynana* can reliably serve as an internal control in life-history studies and we discuss potential advantages of this method.

[†] Zijlstra, W. G., Zwaan, B. J. & Brakefield, P. M. (2002) *Entomologia Experimentali et Applicata*, **102**: 87-92.

Introduction

Most quantitative genetics studies are concerned with accurately partitioning phenotypic variation in a trait to genetic and environmental components. Therefore, in laboratory (selection) experiments, the effects of the environment on the studied trait are usually estimated using (unselected) control populations (Hill, 1972; Falconer & Mackay, 1996, p. 197). In most cases, unselected controls are reared in separate cages or vials and are assumed to encounter similar conditions to the experimental groups. Differences between cages/vials are minimized by randomization when possible, but may still exist. Rearing experimental animals and (mutant) controls in the same cage could solve the problem of inter-cage environmental differences, thus creating true internal controls. A source of error can be introduced into selection experiments because the cages housing the various selection lines are inherently different, for example because of different food-plant quality. With the internal control approach, we can control in a very precise manner for such between-cage differences that are very common in rearing systems that have not been, or cannot be, rigidly standardized. In addition, fewer replicates are needed to exclude environmental noise. Furthermore, inadvertent selection on the control populations can be detected when using internal controls.

The most practical implementation for internal controls would involve using phenotypic marker mutants, similar to techniques used in competition experiments (e.g., Bakker, 1961, with *Drosophila*). The mutant must meet two important requirements: (*i*) it must be clearly distinguishable phenotypically from the experimental, wild-type animals, but (*ii*) it should be equivalent or closely similar in all other respects (e.g., life-history traits) to the initial stock population from which the experimental populations (e.g., selection lines) will be derived. Ideally, the only effect of the mutation is to make it possible for the researcher to distinguish the controls from the experimental animals. During the experiment, there is no crossing between the experimental and mutant groups. The groups develop together, but are separated before any mating can occur. Therefore, the genetic background of the mutant is not affected by experimental selection regimes, and is similar to that of the stock used to start the experimental lines, thus enabling the description and quantification of any genetic changes during the course of the (selection) experiments.

Several mutants are available in the tropical butterfly, *Bicyclus anynana*, which have arisen spontaneously in the more than ten years that this animal has been kept in the laboratory (Brakefield & French, 1993; Brakefield *et al.*, 1996). *Spotty* is an autosomal allele with incomplete dominance; homozygotes express two extra eyespots (3 and 4) on the forewing, both ventrally and dorsally (figure 4.1). The autosomal recessive *yellow* mutant causes yellow, instead of green colored pupae, because the synthesis of blue pterobilin pigments is blocked, leaving only yellow carotenoids derived from larval food plants over (Brakefield & Kesbeke, 1997). Both mutants can be easily distinguished from the wild type, either during the pupal stage (*yellow*) or in the adult stage (*Spotty*).

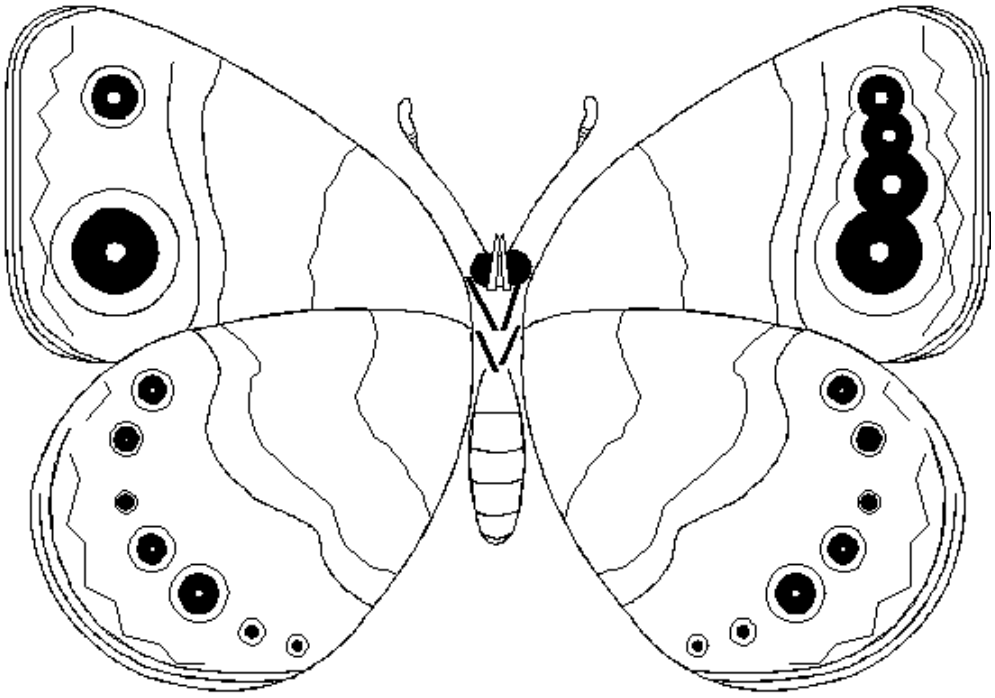

Figure 4.1 Schematic ventral wing surface of *Bicyclus anynana*; the left side shows the wild type phenotype, on the right side the *Spotty* mutant, with additional forewing eyespots.

The only previous work on differential life histories between wild type and mutant *B. anynana* has been done by Van Oosterhout, in a study on genetic divergence in small captive populations (Van Oosterhout, 2000). In 25 polymorphic populations reared over nine generations, he did not observe a significant change in the *Spotty* allele frequency. However, the frequency of the *yellow* allele did decline, indicating a fitness disadvantage for *yellow*, but not for *Spotty*. The main question of the present study is whether these mutants can be used as a true internal control for laboratory studies on development time and other life-history traits. They both meet the first criterion mentioned above, but do they have life histories that match the wild type phenotype closely enough to allow them to be used as internal control tester stocks? To examine this question, we assessed development time and egg-to-adult survival of wild type and mutant *B. anynana* reared together. We also performed an experiment to verify that the heritability of development time in the *Spotty* stock did not differ from that in the outbred stock. If that is the case, it would ensure that the scope of the response to the environment or specific experimental treatment, was the same at the start of the experiment for the mutant as well as for the experimental population.

Materials and Methods

The initial stock population of *Bicyclus anynana* was established in 1988 from about 80 gravid females caught in Malawi. Both the *Spotty* and the *yellow* mutant were isolated

more than ten years ago, during normal lab rearing. The *yellow* mutant may have been present as a rare allele in the founder population and *Spotty*-like *Bicyclus safitza* butterflies occur with significant frequency in Malawi (Brakefield *et al.*, 1996). Pure breeding mutant strains were made after backcrossing to the stock and have since then been maintained at high effective population sizes to avoid inbreeding (Brakefield *et al.*, 2001). We reared (*i*) one population cage (0.5x 0.5 x 0.5m) with 465 wild type eggs and 113 *yellow* eggs, (*ii*) eleven cages with batches of on average 40.1 ± 0.8 *Spotty* eggs together with larger numbers of wild type eggs (range: 156 - 462), and (*iii*) cages with only one genotype, either *Spotty*, *yellow* or wild type. Eggs were collected over a one-day period, all *Spotty* eggs originated from the same, large population and all caterpillars were fed on young maize plants. Emerged adults were marked with day of eclosion, separated according to sex and fed moist banana.

To establish heritability (h^2) for development time and to ensure assortative mating for egg-to-adult development time (to increase power of parent-offspring regression), five mating cages were set up, ranging from a cage containing the fastest males and females to a cage containing the slowest males and females. Each cage contained 16 - 24 males and 18 - 34 females. Copulating pairs were removed and a batch of eggs laid over a 12h-period was reared in a sleeve cage (0.1 x 0.2 x 0.4m, one per family). All experiments were done at an intermediate temperature of 22.5 ± 0.5ºC, with 70 ± 15% r.h., and L12 : D12.

Heritabilities for development time (log transformed to obtain homogeneous variances) were calculated with bootstrapping (1000 runs per estimate) using H2boot (P.C. Philips, http://darkwing.uoregon.edu/~pphil/software.html). Two methods were used: (*i*) (mid)parent-offspring regression on family means, and (*ii*) one-way analysis of variance (ANOVA) among full-sib families. The regressions were weighted by the number of offspring per family. Heritabilities were estimated using both sexes, as well as single sexes. To correct for protandry and possible unequal sex-ratios between families, we first performed an ANOVA with sex as a factor on the full-sib data and used the residuals for the h^2 estimation. Because we used assortative mating, heritability estimates were corrected using the correlation (r) between the development times of the parents; the h^2 based on single parent-offspring regression, was corrected using $h^2 = h^2_0 / (1 + r)$, and estimates based on a one-way ANOVA by the formula $h^2 = [-1 + \sqrt{} (1 + 4rh^2_0)] / 2r$ (where h^2_0 is the uncorrected h^2 estimate) (Falconer & Mackay, 1996, p. 174-177).

Results

Mean egg-to-adult survival percentage of the eleven groups of *Spotty* eggs reared together with wild type eggs was 66% (range: 54% - 82%). Egg hatching percentage for *Spotty* mutants was 79% (determined on four separate egg batches). Mean wild type survival was 37% (range 7% - 65%), resulting in a significantly higher mutant than wild type survival (Mann-Whitney test, $W = 181$, $p < 0.001$). There was no significant relationship between the egg-to-adult survival of the wild type in a particular cage and the survival of the *Spotty* mutant (correlation = −0.445, $N = 11$, $p = 0.17$). When only *Spotty* animals were reared in a population cage, almost half of the eggs developed to adults (236 out of 482 eggs, 49%).

Egg-to-adult development times were also similar for *Spotty* and wild type. However, since some of the wild type eggs originated from lines selected for development time, we will only compare development times of *Spotty* and three unselected lines. In the ANOVA, which also included sex and cage as factors, no significant differences in egg-to-adult development time were found between the mutant and the wild type, $F_{1,463} = 0.58$, $p = 0.485$. Sex (protandry) and cage (nested in genotype) were significant factors: $F_{1,463} = 37.86$, $p < 0.001$ and $F_{4,463} = 25.77$, $p < 0.001$, respectively. Similar analyses for the *yellow* mutant showed that survival was significantly lower and development time was substantially longer compared to the wild type, and thus was unsuitable as an internal control (data not shown).

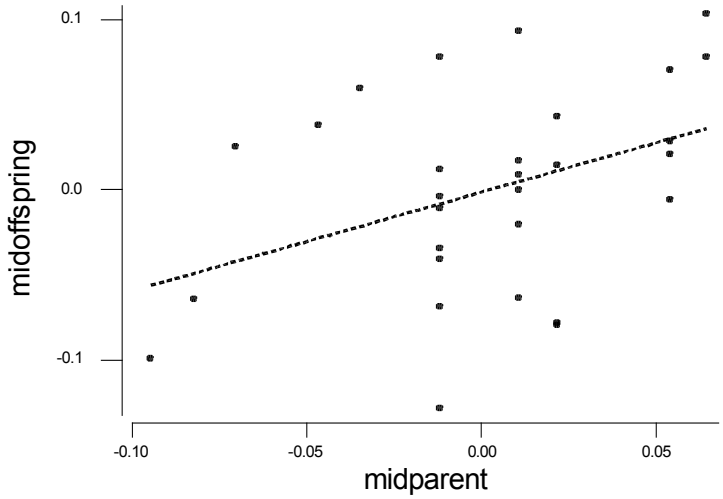

Figure 4.2 Standardized midparent-midoffspring regression of *Spotty* development time (log transformed).

	Parent-offspring regression			Full-sib ANOVA		
	midparent-midoffspring	father-son	mother-daughter	residuals (sexes pooled)	Males	females
h^2 ± s.e.	0.59 ± 0.19 **	0.41 ± 0.31 ns	0.84 ± 0.25 ***	0.43 ± 0.08 ***	0.48 ± 0.11 ***	0.39 ± 0.10 ***
V_P ($\times 10^{-3}$) ± s.e.	3.1 ± 1.0 ***	1.7 ± 0.7 *	1.6 ± 0.5 ***	8.2 ± 0.7 ***	8.5 ± 0.9 ***	8.1 ± 0.7 ***
V_G ($\times 10^{-3}$) ± s.e.	1.8 ± 0.8 **	0.7 ± 0.6 ns	1.3 ± 0.5 **	3.5 ± 0.8 ***	4.1 ± 1.1 ***	3.2 ± 0.9 ***

Table 4.1 Heritability and variance components of *Spotty* development time (log transformed). Residuals were used in the full-sib analysis, to obtain an estimate for both sexes pooled, but corrected for sexual differences (see text). V_P is phenotypic variance, V_G is genetic variance, deviations from zero are denoted as: ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

We reared 33 families with a mean of 13.5 *Spotty* butterflies per family (range: 1 - 39). Mean development times for male and female parents were 43.4 days (range 38 - 47) and 45.7 days (range 42 - 49), respectively. Those for sons and daughters were 52.6 days ($N = 173$) and 55.6 days ($N = 258$), respectively. The differences in development time between parents and offspring are probably due to differences in rearing conditions (e.g., quality of maize plants; an example of the need for internal controls). Heritability estimates for development time (log transformed) calculated with parent-offspring regression and one-way ANOVA full-sib methods are shown in table 4.1. The regression of family means of offspring on midparental development time is shown in figure 4.2.

All heritability estimates, except those for sons on fathers regression, were significantly ($p < 0.01$) different from zero. Although the sons on father ($h^2 = 0.41$) and daughters on mother ($h^2 = 0.84$) regressions did differ to some extent, heritabilities for males and females were never significantly different from each other, nor from the midparent or residual estimate. The genetic variances did differ between full-sib and parent-offspring methods, because the full-sib genetic variance includes common environment effects and non-additive genetic variance. However, when we used the midparent estimate ($h^2 = 0.59$) and approximated breeding value correlation of the parents with their correlation ($r = 0.709$) due to assortative mating (Falconer & Mackay, 1996), then the expected correlation between sibs (t) was 0.50. The observed correlation, derived from the ANOVA on residuals, is actually lower: 0.28. Heritabilities obtained with different methods did not differ significantly from each other.

Discussion

Development time and survival of *Spotty* mutants was at least equal to that of wild type *Bicyclus anynana* butterflies. Therefore, because of their clearly different wing pattern, they could serve as reliable internal control populations. The *yellow* mutants cannot serve this purpose because of a slower development time in comparison to wild type.

Our results are consistent with Van Oosterhout's observations of changes in allele frequency in small, polymorphic populations: *Spotty* is equivalent to the wild type, whilst the *yellow* allele is at a selective disadvantage (Van Oosterhout, 2000, chapter 4). In his set-up, parents within a certain time window were randomly chosen to establish the next generation so that individuals carrying *yellow* allele(s) probably tended to fall outside the time-window because they emerged too late. The competition effect reported above would reinforce this effect.

The heritability estimates found for development time of the *Spotty* mutants fall within the range of values obtained by Wijngaarden for wild type butterflies (Wijngaarden, 2000, chapter 4). His estimates, obtained at 24ºC with the full-sib method, range from 0.15 - 1.19 ± 0.27 (average s.e.) for males and 0.27 - 1.30 ± 0.21 for females. The *Spotty* mutants do not deviate in this respect of their life history from the wild type. Apparently, the backcrossing of the original *Spotty* mutation to outbred stock has effectively homogenized the genetic background of the mutation. This outbred stock is always used as the basis for new selection lines. Were we to use another stock (e.g., a selection line for development time) as the basis for a new selection experiment, the equality of that stock and the *Spotty* mutant would have to be assessed. The main point, therefore, is that the *Spotty* mutant of *B. anynana* does not differ from patterns seen for the wild type stock animals, and could therefore be used as a reliable internal control for life-history experiments. Mutant and wild type may differ in other, unmeasured traits (e.g., longevity) but these are less relevant to the proposed application of this technique: control for environmental fluctuations during development, and measurement of larval competitive ability. Not only is the mean development time similar, but also the variance around that mean is comparable between mutant and wild type, as indicated by the heritability estimates. This is an advantage of using backcrossed mutant lines over inbred lines that might possess the same trait mean but not variance. The scope of the response to the environment will be similar for the mutant and the experimental population. The assumption that there are no interactions between the genotypes in the exploitation of resources is probably warranted in the *Bicyclus* system. There is no *a priori* reason why certain selection lines would interact differently with the *Spotty* mutant. Although such interactions have been reported for *Drosophila* (Lewontin, 1955) and *Musca* (McIntyre & Gooding, 2000), those are likely to be accounted for by the reliance of both these species on initial medium conditioning by newly hatched larvae.

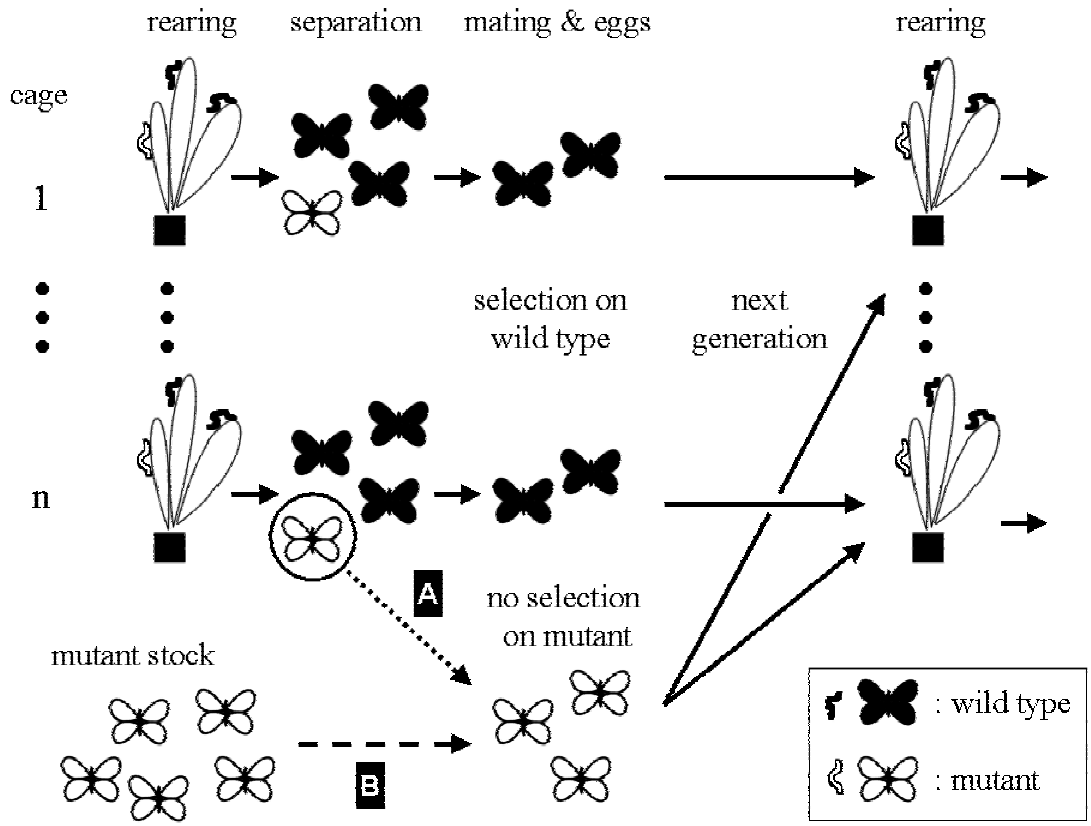

Figure 4.3 Diagram illustrating the application of mutants as an internal control over a standard generation of rearing. Mutants and wild type animals are reared together, but are mated separately, and selection is only applied to wild type animals. Two possible sources of the mutants used in the next generation are indicated: (A) using the progeny of all mutant individuals from the previous generation, or (B) from an independent mutant stock (see text for more explanation). Eggs from both the selected line and from the mutant are then pooled to set up the rearing of the next generation.

We know of no previous studies that have used backcrossed mutant lines as an internal control. Mutants are widely used in the context of competition experiments (e.g., Bakker, 1961, with *Drosophila*) but not in the context of artificial selection experiments. Using mutants as internal controls has several advantages over using additional replicate cages: (*i*) competitive ability of the experimental lines can be monitored during selection (see below); (*ii*) the total number of cages that can be maintained during a generation is limited, but because using this technique fewer replicates per line are necessary, the scope and power of selection experiments can be increased (i.e. more different selection criteria); and (*iii*) not all rearing systems can be fully standardized, and possess inherent environmental noise (e.g., the use of maize plants in the *Bicyclus* system). Obviously, this will average out with enough replicates, but this is inefficient for most experimental systems in terms of costs, time and space; using mutants will be more effective and

reliable. Therefore, especially for larger bodied model systems such as *Bicyclus*, the use of internal controls can be very valuable.

The technique can be used in several ways, depending on the nature of the experiments (figure 4.3). One possibility is to rear each experimental generation together with mutants (path A in figure 4.3). Emerging experimental and mutant animals are separated, and all (virgin) mutants are pooled and mated with each other. The offspring from this large pool of mutants is randomly distributed over the different cages with progeny from experimental lines. Not only can the environmental circumstances be corrected for with internal controls (and more accurate quantitative genetic estimates obtained), also a measure of changing competitive ability during selection can be obtained. Because there is no selection in the mutant control (mutants and experimental animals are separated before any mating can occur, figure 4.3) and the two populations are initially the same, changes in competitive ability relative to the control can be monitored during selection. Alternatively (B in figure 4.3), mutants from a base stock could be used at regular intervals, for example in every third generation. In this way, any inadvertent selection on the mutant tester stock during the artificial selection experiment would be avoided.

Acknowledgements

We thank Els Schlatmann, Bert de Winter and Wil van der Hoeven for maize cultivation, and two anonymous referees for helpful comments. This research was supported by the Netherlands Organisation for Scientific Research (NWO), grant 805-36-033 to WGZ and grant PULS-805-48-002 to BJZ.

Chapter 5

Protandry in the butterfly *Bicyclus anynana*[†]

Abstract

Protandry is the earlier adult emergence of males than females. This is predicted from sexual selection theory because under certain circumstances earlier emergence of males will maximize their mating opportunities. The tropical butterfly *Bicyclus anynana* is adapted to highly seasonal environments and selection pressures for protandry may vary across seasons. To examine such effects, we compared protandry across a wide range of rearing temperatures in the laboratory which match those occurring in the different seasons. The absolute amount of protandry (in days) remained constant at intermediate and high temperatures (wet season), but increased with temperature at lower temperatures (dry season). Nevertheless, average male development time as a percentage of female development time remained constant. We also compared lines established by artificial selection on development time and pupal weight across temperatures to assess the impact of different selection pressures on protandry. Data for the pupal weight selected lines were inconclusive. The male to female development time ratio did not change across temperatures for the development time selected lines. However, females were relatively slower than males in the slow selected lines (lower male : female development time ratio) than in either the fast lines or the stock. This indicates that sex-specific components to development time are present in *B. anynana*, and thus that there is scope for a response of protandry to selection.

[†] Zijlstra, W. G., Kesbeke, F., Zwaan, B. J. & Brakefield, P. M., *Evolutionary Ecology Research*, submitted.

Introduction

Darwin already observed that "throughout the great class of insects the males almost always emerge from the pupal state before the other sex" (Darwin, 1871, p. 260). Interest in this phenomenon, called protandry, has been rekindled by Wiklund and Fagerström (Wiklund & Fagerström, 1977). Two hypotheses to account for protandry have been postulated: (1) sexual selection acts on protandry itself, called the 'adaptive' explanation by Wiklund & Solbreck (1982), or (2) protandry is a side effect of natural selection working differently on the sexes ('incidental' explanation).

In the sexual selection hypothesis, males that emerge before females are favored, because this maximizes the number of encounters with females willing to mate, and thus their reproductive success. Especially in (near) monandrous species it is important to be the first to mate, and the amount of protandry is expected to decline with the degree of polyandry (e.g., Zonneveld, 1996). A prerequisite is some discreteness of generations, because were receptive females continuously available, earlier eclosion of males would not be favored (Singer, 1982). Protandry can also be considered a female reproductive strategy because it will minimize the period females are unmated, thus decreasing the risk of death before reproduction (Fagerström & Wiklund, 1982). An extra factor shaping the degree of protandry is temporal variation in female quality. A lower fecundity for slower developing females would increase selection in favor of an earlier emergence of males (Kleckner *et al.*, 1995; Carvalho *et al.*, 1998). Thus in this 'adaptive' explanation (Wiklund & Solbreck, 1982), the optimal difference between male and female emergence only depends on conditions in the adult environment, such as adult mortality and mating structure. If the adult life history does not change, the optimal protandry does not change, and different larval conditions should still lead to the same amount of protandry.

An alternative ('incidental', Wiklund & Solbreck, 1982) explanation of protandry is based on natural selection. If females could increase their reproductive output by an increase in size, but males could not, and a larger size means a longer period of development, then males are expected to emerge before females. In this case it is not primarily that selection is favoring males to emerge earlier, but rather that females emerge later to maximize their size and subsequent fecundity (Thornhill & Alcock, 1983). Moreover, when protandry is selected for *per se*, we expect males to be smaller than females, because, given equal growth rates, body size at maturity trades off with development time (Singer, 1982).

Most of the evidence to date points to sexual selection being the driving force in shaping protandry (e.g., Nylin *et al.*, 1993, using a comparative approach). Nylin and co-workers found a largely constant amount of protandry in Swedish and English populations of the butterfly *Pararge aegeria* reared at different temperatures, in line with predictions from the sexual selection theory. Protandry was absent in Madeiran populations where seasonality is absent and generations are not discrete (Nylin *et al.*, 1993).

The tropical butterfly *Bicyclus anynana* occurs in environments with two pronounced seasons and exhibits seasonal polyphenism. During the warm (>23°C), wet season there is ample food available for caterpillars. Those nearing pupation at these temperatures will develop conspicuous wing patterns that function to deflect attacks of predators away from the vulnerable body (Brakefield & Larsen, 1984). In the dry season,

temperatures drop (<20°C) and foodplants are not available. Butterflies in this season have no conspicuous wing patterns and rely on camouflage for survival. They must survive many months before egg laying can begin in the the early wet season. Some mating may happen at the beginning of the dry season, but most occurs shortly before the rains. This seasonal polyphenism also affects other (life-history) traits including fat reserves and oviposition behaviour (Brakefield & Reitsma, 1991). *B. anynana* can have two to three generations in the wet season, but only one in the dry season (Windig *et al.*, 1994). In the wet season there may be some overlap in generations, but this will then be synchronized by the harsh conditions in the dry season. The different life histories during the different seasons may also have repercussions for protandry. Selection pressures regarding protandry will vary across the two seasons. In the wet (warm) season, there is strong (sexual) selection for protandry, and the emphasis in this season is on fast reproduction to exploit the available resources for caterpillars and to perhaps establish an extra generation before the dry season arrives. Earlier emergence is predicted to ensure a higher probability of mating for males. At lower temperatures, in the dry season, survival is more important and early emergence is unlikely to confer fitness advantages. In contrast, it could decrease survival chances because of lower body weight and/or fat reserves.

In this study we investigate the effects on protandry of divergent selection in the field by measuring protandry at different rearing temperatures in the laboratory corresponding to the different seasons. Does the amount of protandry fluctuate within and across environments and/or does the development time of males relative to females remain constant? To further investigate which selection pressures are important in the different seasons, we compared protandry in lines artificially selected for fast or slow development time, and also in lines selected for high or low pupal weight. These lines were highly divergent for these selected traits that are essential in shaping patterns of protandry. Our main aim in this paper is to quantify and compare the amount of protandry at different temperatures (corresponding to the different seasons), and with genetically different lines of *B. anynana* to obtain more information about the causes and consequences of the different selection pressures that occur across sexes and environments.

Materials and Methods

Butterflies

The base stock of *Bicyclus anynana* was established in 1988 from approximately 80 gravid females caught at a single locality in Malawi. This stock has been kept in climate-controlled chambers with high humidity at the laboratory in Leiden with population census sizes of >500 individuals. It has retained substantial genetic variation (Saccheri & Bruford, 1993). Caterpillars feed on young maize plants, adults on moist banana.

Stock butterflies at different temperatures

From three different generations (in February and April 1998, and March 2000) of the base stock population we collected eggs over a 12h period. Two of these egg collections (February and April 1998) were reared at an intermediate temperature of 22ºC, in fifteen and seven replicate population cages, respectively. The third collection (March 2000) was reared at 25ºC (high, wet seasonal temperature) in six replicate cages. Each population cage contained approximately 500 eggs. In addition, for protandry comparisons of the stock across a wider range of temperatures, we used sleeve cages (containing ~100 eggs each). Eggs from a large group of stock females (March 1999) were randomly allocated to be reared at 18°C (low), 22°C (intermediate) and 27°C (high temperature) in three to four sleeve cages at each temperature.

Selection lines for development time and pupal weight

The development time selection lines have been selected for at least 30 generations and have diverged markedly from the stock (Brakefield & Kesbeke, 1997). Some of these lines were selected at 20°C, and some at 27°C. Because the selection lines from different temperatures behaved similarly, we reclassified them as FAST (decreased development time) and SLOW (increased development time). Twenty-one sleeve cages per selection regime, and seven sleeve cages with stock animals were reared at both 20°C and 27°C. The selection lines for pupal weight have been selected at 27ºC for 14 generations for either increased pupal weight (LARGE), or decreased pupal weight (SMALL), and also showed a large response to selection (B. J. Zwaan, unpubl. res.). We reared these lines in ten sleeve cages each, at both 20°C and 27°C, together with ten sleeves with unselected CONTROLs that had been propagated and kept under similar conditions throughout the pupal size selection experiment. The two sets of selection lines were reared in different periods.

Statistics

Statistical analyses were performed using JMP 4.0.2 and MINITAB 13. In analyses of (co)variance (AN(C)OVAs), cage was always a random factor, and temperature was treated as a discrete factor. Groups were post hoc compared using Tukey comparisons or contrasts. The absolute amount of protandry was calculated by subtracting the mean development time of males from the mean development time of females. Using bootstrapping, i.e. repeatedly taking the difference between a randomly drawn male and female, yielded similar values. A relative measure of protandry (independent of development time) was derived by taking values for male development time as a percentage of female development time. This relative measure was normally distributed and always had equal variances (Levene's test, $p > 0.05$). Therefore we used ANOVAs to analyze these data. The variable overall development time was calculated by averaging the mean values of male and female development time of a cage, to capture development time of a cage in one value. Furthermore, we also used orthogonal regressions to test if

the relationships between male and female development time remained constant over a range of environments and subsequent development times (in which case the slope is unity). We used orthogonal regression to adjust for variability in both traits, and assumed equal variances.

Results

Stock butterflies at intermediate/high temperature

In all 28 population cages, males developed faster than females (ANOVA, $F_{1,10602}$ = 1640.52, $p < 0.0001$, figure 5.1). Egg batches ($F_{2,10602} = 53.92$, $p < 0.0001$) and cages which were nested within batch ($F_{25,10602} = 79.16$, $p < 0.0001$) differed from each other. Mean protandry (female − male development time, in days ± standard errors) was 1.73 ± 0.08 for 22ºC$_A$, 1.63 ± 0.16 for 22ºC$_B$, and 1.75 ± 0.15 at 25ºC. The two collections at 22ºC also differed from each other in development time (Tukey, $p < 0.05$). Presumably, 22ºC$_A$ was raised at a slightly lower average temperature leading to generally slower development. Furthermore, in an ANCOVA on protandry, there was no interaction between collection and the covariate of overall development time ($F_{2,22} = 2.02$, $p = 0.16$), and overall development time (mean of male and female development time) did not explain variance in protandry ($F_{1,24} = 1.40$, $p = 0.25$). To further test whether protandry changed with development time, we performed an orthogonal regression on male and female development time. The slope of the regression was 1.02, with the lower 95% confidence limit at 0.95 (correlation = 0.985, figure 5.1). Because the slope is not significantly different from unity, there is no change in the relationship at these intermediate to high temperatures between male development time and female development time with increasing overall development time. Although development times varied considerably between the different cages and egg batches (figure 5.1), the amount of protandry remained constant at these intermediate to high temperatures (wet seasonal conditions).

Stock butterflies over the whole range of temperatures

At all three temperatures spanning the relevant range, egg-to-adult development time of males was shorter than for females (ANOVA, 18ºC: $F_{1,113} = 50.01$, 22ºC: $F_{1,356} = 60.31$, and 27ºC: $F_{1,218} = 16.79$, all $p < 0.0001$), but females had a significantly shorter pupal phase than males (18ºC: $F_{1,113} = 15.54$, 22ºC: $F_{1,356} = 58.69$, and 27ºC: $F_{1,218} = 16.61$, all $p < 0.0001$). Data on differences in length of (components of) development time for the two sexes are shown in table 5.1. Protandry significantly increased with decreasing temperature ($F_{2,28} = 21.54$, $p < 0.0001$). Differences in larval time between the sexes were significant between temperatures ($F_{2,28} = 29.29$, $p < 0.0001$), but differences in pupal time were not ($F_{2,28} = 3.26$, $p = 0.054$). Tukey comparisons showed that differences between the sexes were always larger at 18ºC than at either 22ºC or 27ºC (table 5.1). Addition of the covariate overall development time did not increase the explanatory power of the model, and at no temperature was there a significant relationship between protandry and

development time (regressions, $p > 0.30$). Although the difference in development time increased with decreasing temperature, male development time as percentage of female development time showed no change across temperatures ($F_{2,28} = 0.79$, $p = 0.47$). This was true for each component of development time (larval time: $F_{2,28} = 0.61$, pupal time: $F_{2,28} = 0.53$, both $p > 0.50$, table 5.1). Thus, the absolute amount of protandry (in days) was significantly larger at 18ºC, a temperature representative for the dry season, than at intermediate or high temperatures, whilst the relative difference between male and female development time did not change across temperatures.

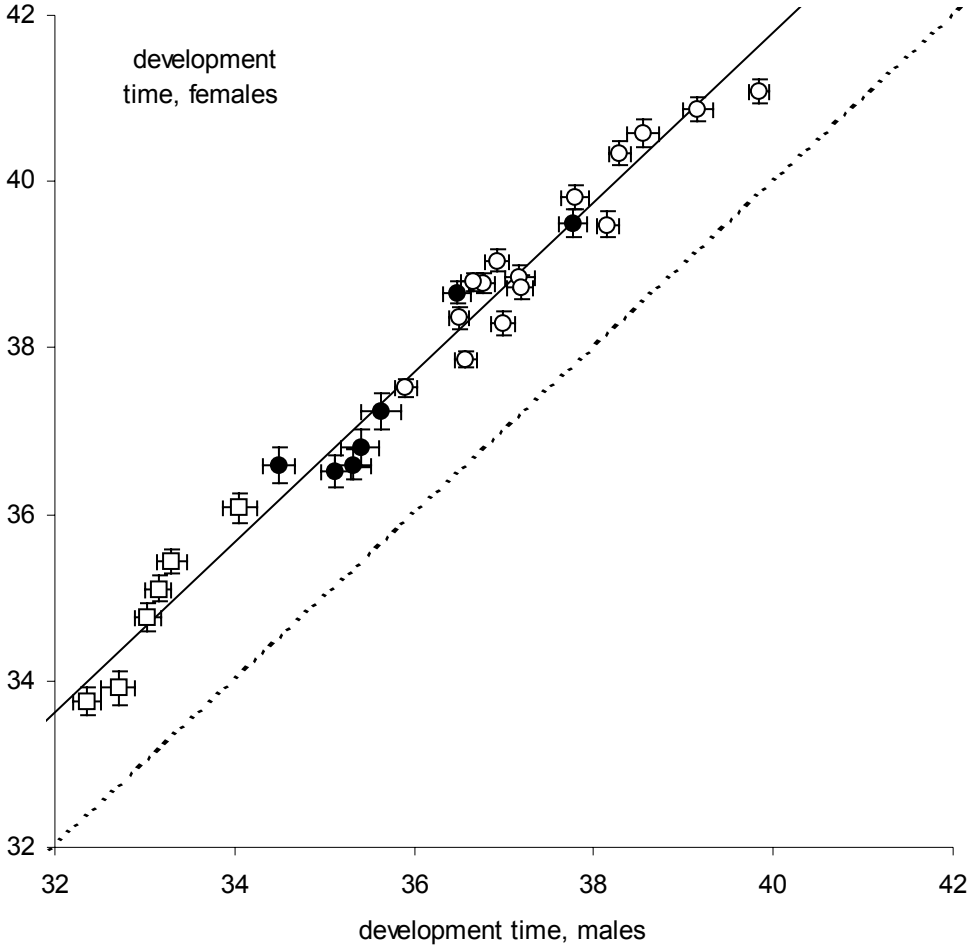

Figure 5.1 Development time (in days) for population cages ± standard errors. Three different egg collections were used: 22ºC$_A$ (○), 22ºC$_B$ (●), and 25ºC (□). Dashed line: development times of males and females are equal, solid line: fitted orthogonal regression line (female development time = 0.96 + 1.02 × male development time)

temperature	*N*	development time	larval time	pupal time
18°C	8	6.37 ± 0.99 *a*	6.85 ± 0.74 *a*	−0.92 ± 0.23 *a*
		93.4% ± 0.9	90.2% ± 1.2	104.1% ± 1.0
22°C	12	2.20 ± 0.26 *b*	2.50 ± 0.14 *b*	−0.59 ± 0.10 *b*
		94.9% ± 0.9	91.8% ± 0.6	106.5% ± 1.1
27°C	11	1.79 ± 0.20 *b*	1.84 ± 0.30 *b*	−0.36 ± 0.13 *b*
		94.4% ± 0.9	91.5% ± 1.2	105.6% ± 2.1

Table 5.1 Differences between Stock females and males in (components of) development time, in days ± standard error. *N*: number of cages used. Percentages refer to the male (component of) development time as percentage of female development time. Identical letters indicate no significant differences in a Tukey comparison ($p > 0.05$). Larval and pupal time do not sum to development time because of pupal mortality.

Selection lines

The protandry data for the different selection lines at 20ºC and 27ºC are shown in table 5.2. The control groups for the two different selection regimes, development time and pupal weight, differed significantly from each other in protandry ($F_{1,30} = 4.72$, $p = 0.038$). Therefore, we will examine development time and pupal weight selection lines separately.

Protandry in selection lines for development time differed between temperatures and between selection lines (temperature: $F_{1,92} = 6.58$, $p = 0.012$; line: $F_{2,92} = 12.82$, $p < 0.0001$, table 5.2). The interaction between temperature and selection line was also significant ($F_{2,92} = 4.78$, $p = 0.011$). Protandry for the SLOW line increased much more from 27ºC to 20ºC, compared to the FAST line and the stock (contrast, $t = 2.81$, $p = 0.006$, table 5.2).

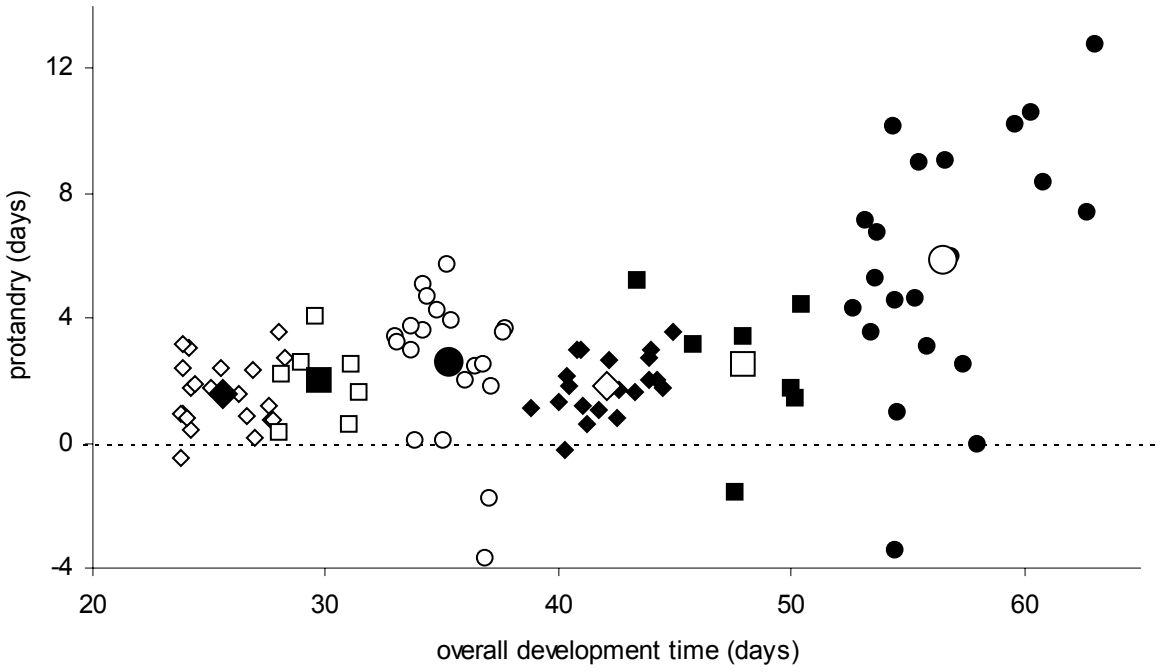

Figure 5.2 Protandry (female development time − male development time) versus overall development time (average of male and female development time) at 20ºC (closed small symbols) and 27ºC (open small symbols) for the development time selection lines FAST (◇), SLOW (○) and for stock (□). Large symbols represent the means per selection line and temperature (closed symbols for 27ºC, open symbols for 20ºC).

Alternatively, we can analyze protandry using overall development time. Overall development time ($F_{1,94}$ = 21.75, $p < 0.0001$) and temperature ($F_{1,94}$ = 4.71, $p = 0.033$) were significant, as well as their interaction ($F_{1,94}$ = 7.57, $p = 0.007$); protandry increased more with development time at 20ºC than at 27ºC (figure 5.2). None of the slopes of orthogonal regressions on male and female development time were significantly different from unity at 27ºC (slope of all lines combined = 0.92; upper 95% confidence limit [CL] = 1.03). At 20°C, however, the slopes for both FAST and SLOW were significantly lower than one, 0.77 (upper 95% CL = 0.99) and 0.43 (upper 95% CL = 0.93), respectively. The slope for all lines combined was 0.73, upper 95%CL = 0.82 (see figure 5.2). This implies that at 20°C, females became relatively slower compared to males with increasing development times of both sexes.

Male development time as a percentage of female development time did not differ across temperatures, but was significantly different between selection lines (ANOVA, $F_{2,95}$ = 4.64, $p = 0.012$). This was confirmed by a Kruskall-Wallis test (H = 13.84, df = 2, $p = 0.001$). Tukey comparisons with temperatures pooled ($p < 0.05$) showed that male development time as percentage of female development time was significantly larger for FAST, compared to SLOW lines. That is, the difference between the sexes is less for the FAST lines (closer to equal development times for males and females). Stock was

intermediate and did not differ from either selection line (table 5.2, Tukey results not indicated).

The pupal weight selected lines only differed in the amount of protandry between temperatures. At 20°C, the difference between males and females in development time was larger than at 27°C (ANOVA, $F_{1,56} = 10.01$, $p = 0.003$, table 5.2). Neither selection line, nor line × temperature were significant factors ($F_{2,56} = 0.01$, $p = 0.99$, and $F_{2,54} = 1.39$, $p = 0.26$, respectively). Furthermore, the control and the SMALL population did not show significant protandry at 27°C (table 5.2). When overall development time is used, protandry of the pupal weight selected lines differed between temperatures ($F_{1,57} = 6.07$, $p = 0.017$), and protandry decreased with increasing development time ($F_{1,57} = 4.44$, $p = 0.040$). The interaction was not significant ($F_{1,55} = 0.42$, $p = 0.52$). The decrease in protandry with increasing development time was not observed in the orthogonal regression analyses. At both temperatures, the orthogonal regression slope did not differ significantly from unity (20°C, slope = 2.03, lower 95%CL = 0.85; 27°C slope = 1.30, lower 95%CL = 0.69).

		20°C		27°C	
	N	protandry	%	protandry	%
Development time selection					
FAST	21	1.85 ± 0.20 *a*	95.8 ± 0.4	1.58 ± 0.23 *a*	94.1 ± 0.9
SLOW	21	5.86 ± 0.86 *b*	90.4 ± 1.4	2.59 ± 0.49 *a*	93.0 ± 1.4
STOCK	7	2.56 ± 0.86 *a*	94.8 ± 1.8	1.99 ± 0.48 *a*	93.6 ± 1.5
Pupal weight selection					
LARGE	10	1.29 ± 0.67 *ef*	97.8 ± 1.1	0.82 ± 0.36 *ef*	97.6 ± 1.0
SMALL	10	2.18 ± 0.71 *f*	96.2 ± 1.2	−0.06 ± 0.34 *e* †	100.1 ± 1.0 †
CONTROL	10	1.73 ± 0.62 *ef*	97.0 ± 1.1	0.28 ± 0.34 *ef* †	99.2 ± 1.0 †

Table 5.2 Protandry (mean female development time – mean male development time) in days ± standard error, for different selection lines at 20°C and 27°C. *N* represents the number of cages used, and % refers to the mean male development time as percentage of mean female development time. Same letters denote no differences after ANOVA on either development time or pupal weight selected lines at both temperatures (Tukey, $p > 0.05$). †: does not differ significantly from zero, or 100%.

Pupal weight selection lines did not differ in male development time as a percentage of female development time, but this ratio was significantly lower at 20ºC than 27ºC ($F_{1,58} = 5.07$, $p = 0.028$). The interaction was not significant ($F_{2,54} = 1.86$, $p = 0.17$). The non-parametric Mann-Whitney test confirmed the differences in ratio between temperatures ($W = 1064.0$, $p = 0.028$). The ratio between male and female development time was similar for the stock used together with the development time selection lines

(mean = 94.2%, table 5.2) and the stock butterflies measured across temperatures (mean = 94.7%, cf table 5.1). However, male development time as percentage of female development time was significantly higher for the controls in the pupal weight selected lines (mean = 98.1%, table 5.2) compared to either stock population.

The main findings were thus that development time of stock males relative to females remained constant over temperatures, but that the value of this ratio was significantly lower for SLOW selected lines. Pupal weight selected lines differed across temperatures in this relative measure (ratio between male and female development time).

Discussion

Protandry at intermediate/high temperatures

Protandry (in absolute terms, i.e. days) in stock *Bicyclus anynana* did not differ across intermediate and high temperatures (22ºC to 25ºC). Even though mean development times varied from 30 to 41 days over this temperature range (figure 5.1), the difference in development time between females and males was fairly constant at around two days. This constancy is consistent with sexual selection being the primary force shaping the degree of protandry in these (wet-seasonal) conditions, because this selective factor is expected to be largely environment-independent. The difference in development time between the sexes is probably the outcome of balancing two selective factors in the adult stage: males are selected to increase the difference between the sexes because it increases the number of mating opportunities, but this is opposed by the increase in pre-reproductive mortality (assuming adults have higher mortality) (Wiklund & Fagerström, 1977). It is beneficial to be the first male to mate with a female, since in the field about one-third of the *B. anynana* females remate (Brakefield & Reitsma, 1991), and in the lab about one-quarter of females remate, without strict last male sperm precedence (Brakefield *et al.*, 2001). The two-day difference between males and females in these circumstances presumably represents the optimal balance between these two selective pressures. Furthermore, there may be a premium on rapid reproduction rather than long-term survival in the wet season, because an extra generation confers large fitness benefits in an environment rich in larval food plants (grasses).

Protandry from low to high temperatures

In the experiment where we examined a wider range of temperatures (from 18ºC to 27ºC), we did find changes in protandry. At 18ºC (dry season conditions), females emerged much later (approximately 6 days) relative to males, than at >22ºC temperatures (protandry is around 2 days, table 5.1). However because of longer development, the ratio between male development time and female development time remains constant across temperatures (at ~95%, table 5.1). Thus we have two measures of the relationship between male and female development time: a relative one (ratio between males and females) and an absolute measure (difference between males and females) that seem at odds with each other. The 'adaptive' explanation of protandry (based on sexual selection)

predicts a fixed absolute difference and a varying relative measure under different larval conditions (assuming adult conditions remain constant). The 'incidental' explanation predicts variation in the absolute amount of protandry and a fixed ratio between male and female development time across temperatures. Here, we found arguments for both explanations; absolute protandry remains constant at intermediate and high temperatures (see above; figure 5.1), yet, the relative measure also remains constant across wider temperatures. Further research is needed to determine which of these two findings should be given the most weight, and under what (natural) conditions.

In figure 5.3, results for stock butterflies (from table 5.1) and ballpark predictions (values only have illustrative meaning) for the sexual selection and the natural selection hypotheses are shown. It is assumed that adult circumstances are similar at different temperatures. The sexual selection explanation states that protandry is independent of environment, male and female development time are only contingent on each other, and therefore, protandry should remain constant across temperatures. The natural selection hypothesis would predict a fixed relationship between male and female development time. Because of non-parallel reaction norms across temperatures, the difference between males and females increases with increasing development time (as a result of decreasing temperature). The consistency of protandry values we obtained at intermediate to high temperatures (>22ºC) favors the sexual selection hypothesis (also see figure 5.1). However, at low, dry season temperatures, the amount of protandry increases. There are several possible reasons why protandry is so much higher at 18ºC than at intermediate - high temperatures (22ºC - 27ºC):

(a) Natural selection (the 'incidental' explanation) is much more important at 18ºC than the sexual selection hypothesis (the 'adaptive' explanation);
(b) Adult circumstances are different at 18ºC, therefore the optimal difference between male and female development time is different (this is in accordance with the 'adaptive' explanation);
(c) In both environmental circumstances, there is selection for protandry of ~2 days. However, the physiological mechanism is constrained such that the same difference cannot be produced at different temperatures;
(d) There is no selection in favor of protandry at 18ºC and the observed difference is a by-product of the fine-tuned physiological mechanism to produce a ~2 day difference at high temperatures.

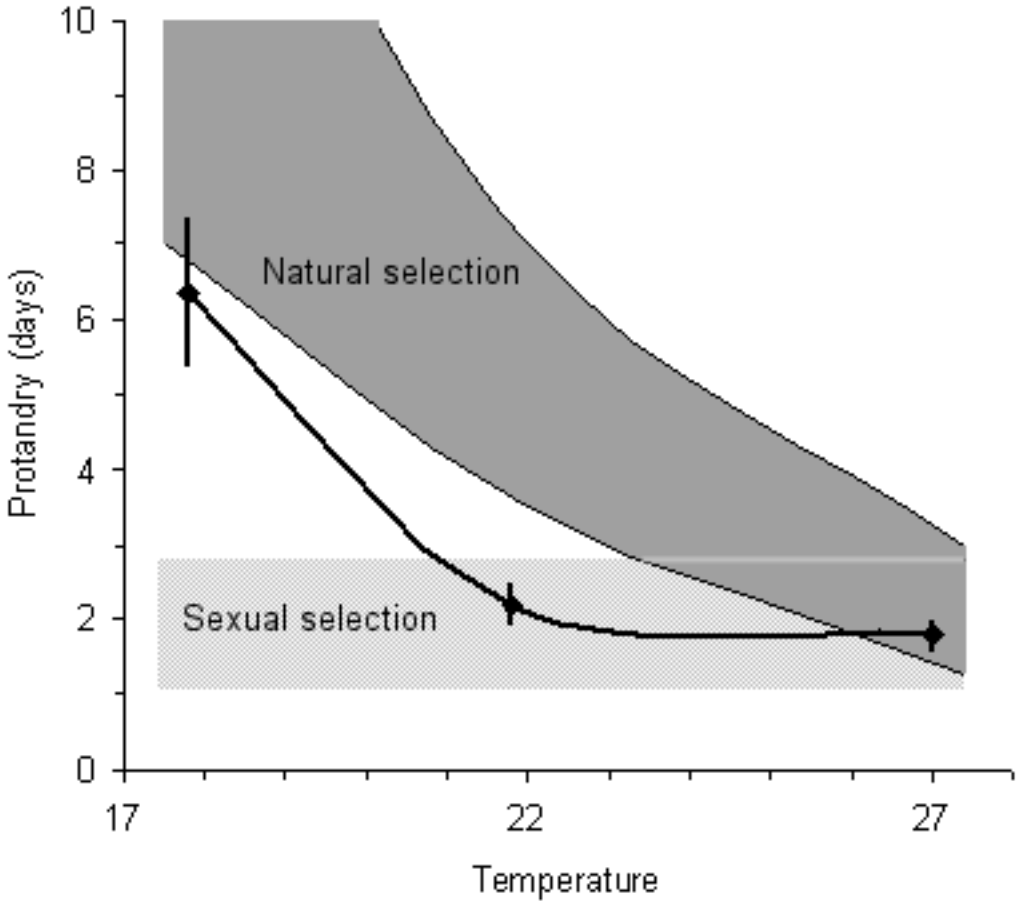

Figure 5.3 Protandry patterns across different environments (represented by temperature), as predicted by the sexual selection ('adaptive' explanation; light gray area) and the natural selection hypothesis ('incidental' explanation; dark gray). Adult circumstances are assumed not to change across temperatures. Values for stock butterflies are connected by a solid line (from table 5.1). Note that hypothesized areas and values only serve an illustrative purpose. See text for more information.

No matings occur in the main, mid-period of the dry season, and males have to wait until just before the onset of the wet season to become reproductively active (Brakefield & Reitsma, 1991; N. Reitsma, pers. comm.). Hence, protandry may not present any fitness benefits in the dry season, and this season can effectively be regarded as an 'adult diapause'. However, there could be some selection on protandry in the early stages of the dry season by males mating with dry season females followed by sperm storage before aestivation. During the dry season, the emphasis is more on survival than on reproduction, since dry season form females have larger fat bodies, fewer mature eggs in storage, and have longer delays before oviposition than wet seasonal forms (Brakefield & Reitsma, 1991). Butterflies of the same family in Australia exhibit various degrees of adult reproductive dormancy during the dry season, ranging from complete reproductive

diapause during the whole season, to a decline in reproductive activity as the dry season progresses (Jones, 1987; Braby, 1995).

Protandry in selection lines

The development time selection lines of *B. anynana* showed that both the absolute and relative difference between male and female development time had changed at least in the SLOW selected lines. This agrees with work on the pitcher-plant mosquito, where protandry also increased for lines selected for slow development (Bradshaw *et al.*, 1997). The differences in protandry were especially clear at 20ºC; the difference between SLOW males and females was about 4 days more than for the FAST lines or the stock (table 5.2, figure 5.2). Furthermore, development time of SLOW males only constituted ~92% of female development time, compared to >94% for the other lines. Although this does not appear to be a wide difference, it does imply that protandry for SLOW lines is 1.33 times that of the other lines. The response to selection for a decreased development time seems to have been greater in females, presumably because of a contribution of sex-specific components of development time. Such components must have been present at some stage for protandry to arise during evolution. This also suggests that development time can be manipulated independently within a single sex and that selection on the trait protandry itself is possible. We will specifically test this by selection experiments on protandry *per se* (chapter 6). The converse did not occur; the relation between male and female development time was similar for FAST and stock, although FAST lines tended to have a slightly higher ratio. For all development time selection lines, male development time as a percentage of female development time was constant over temperatures, analogous to the stock butterflies (compare tables 5.1 and 5.2). This consistent relationship across temperatures (reaction norm) has been observed previously for reaction norms in this species. For example, Wijngaarden and Brakefield succeeded in changing the elevation of reaction norms for wing pattern via artificial selection, but not change the shape of the reaction norm (Wijngaarden & Brakefield, 2001; Wijngaarden *et al.*, 2002; see also chapter 2).

At 20ºC, we observed orthogonal regressions which were not unity, but these may be a by-product of the fact that the range of developmental times is wider at 20ºC (figure 5.2), in combination with a constant relationship between male and female development time. The main conclusion from the development time selection lines is that the relationship between male and female development time can be changed (cf. SLOW with FAST and unselected), but that this relationship remains consistent across temperatures.

We have no explanation for why the pupal weight selection lines should be sensitive to temperature in their relationship between male and female development. The change in male development time as a percentage of female development time for unselected controls (from 94-95% to 98%, tables 5.1, 5.2) perhaps indicates some inadvertent selection during the selection experiment, but it is unlikely that it caused such a major shift. Differential environmental circumstances is also an improbable explanation, because the male : female development time relation is very conserved and environment-independent (see above, figure 5.1). Pupal weight selection did not affect the sexes differently, the relation between male and female development time remained

unaltered between the selection lines. There was a trend for the SMALL selection line to be more temperature sensitive than the control, and the LARGE line to be less sensitive (table 5.2). This might be due to physiological processes being more or less buffered from temperature in large or small pupae, respectively.

In conclusion, the absolute amount of protandry is constant over a range of intermediate and high temperatures (figure 5.1), which correspond to the characteristics of the wet season and supports the sexual selection hypothesis for protandry. More research is needed to determine how this can be reconciled with the finding that the relative relationship between male and female development time isconserved across all temperatures.Only artificial selection for slow development time has changed the value of this ratio in such a manner that females have become even more slowly developing compared to males. This underlines the fact that development time of a sex can be changed independently of the other sex in *B. anynana*, and suggests that potential is retained for a response to selection on protandry.

Acknowledgements

We thank Els Schlatmann, Bert de Winter, and Wil van der Hoeven for maize cultivation. This research was supported by the Netherlands Organisation for Scientific Research (NWO), grant 805-36-033 to WGZ and grant PULS-805-48-002 to BJZ.

Chapter 6

Artificial selection on protandry and development time in the butterfly *Bicyclus anynana*[†]

Abstract

The earlier mean adult emergence between males and females, protandry, has been well studied mathematically and in comparative studies. However, quantitative and evolutionary genetics work on protandry is scarce. The butterfly, *Bicyclus anynana*, exhibits protandry. Here, we selected for each of the different combinations of male and female development time (i.e. + +, − −, + −, and − +) in this species, thus including direct selection on protandry. We found no response in lines selected for a change in protandry, but lines where both sexes were selected in the same direction did respond (heritability of divergence between fast and slow lines is 0.11 for males and 0.12 for females). Although genetic variation was present for development time, the lack of response in protandry indicates that the genetic co-variance across sexes is near unity. Any sex-specific genetic variation, necessary for protandry to arise, appears to have been eroded, or buffered by developmental mechanisms. Interestingly, lines selected for decreased protandry (slow males and fast females) had lower egg-to-adult survival, and broods from these lines had lower rates of egg hatching. This suggests that interactions with fertility constrain certain directions of change in patterns of protandry.

[†] Zijlstra, W. G., Pijpe, J., Brakefield, P. M. & Zwaan, B. J. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, submitted.

Introduction

Some aspects of protandry, an earlier adult emergence of males than females, have been well studied. Theoretical studies predict that protandry can be advantageous for males because it increases their probability of mating (Wiklund & Fagerström, 1977), and that this will be balanced by an increased chance of pre-reproductive death. Models suggest that these two selective forces shape the distribution of male and female development time, and that the distribution for males should be truncated at a point determined by pre- and post-emergence mortality (Bulmer, 1983; Iwasa *et al.*, 1983). When female emergence patterns are hard to predict, the male emergence curve should broaden (Iwasa & Haccou, 1994). Several features of the mating system are pivotal to the sexual selection hypothesis that males emerge earlier than females to maximize their mating success. Males must be able to mate multiple times. The first male to mate with a female should gain reproductive benefits because of monandry or sperm competition, and overlap between generations should be minimal (Wiklund & Fagerström, 1977; Singer, 1982; Zonneveld, 1992). 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 pre-reproductive mortality (Fagerström & Wiklund, 1982; Zonneveld & Metz, 1991). 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). An alternative 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. Hence, protandry is then viewed as a by-product of asymmetric fitness benefits to the sexes (Thornhill & Alcock, 1983).

In the past decades, the sexual selection hypothesis (males maximize mating opportunity) versus natural selection (protandry as a side effect) debate has been resolved to a large extent by comparative work on butterflies (e.g., Nylin *et al.*, 1993). Sexual selection seems to be the major selection force, although some argue for a combination of natural and sexual selection (e.g., Kleckner *et al.*, 1995; Bradshaw *et al.*, 1997). However, the quantitative and evolutionary genetics of protandry have not been well studied. The implications for life history and other correlated characters are unknown, and also the issue of how these contrasting demands on the two sexes have been integrated into a single genome remains open. The main question of this 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 studied the African butterfly, *Bicyclus anynana*, which is consistently protandrous (chapter 5). 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 were relatively longer in development than males, compared to unselected or fast selected lines (chapter 5). This suggested that it might be possible to change the relationship of development times between the sexes.

To address the question of how integrated or coupled male and female development time are, we selected for male and female development time in each of the different combinations (i.e. + +, − −, + −, and − +). 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 elucidate the evolutionary genetics of protandry. Is it possible to alter established patterns of protandry?

Materials and Methods

Butterflies

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

Protandry selection

From the base stock we established selection lines at 25ºC 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 **F**ast **M**ale, and **S**low **F**emale development time) were selected for an increased amount of protandry, the SMFF (**S**low **M**ale, **F**ast **F**emale) lines for decreased protandry. All selection lines were replicated twice. We attempted to set up 500 eggs per replicate per generation (eggs were always counted) and the mean number of emerged butterflies per replicate cage was approximately 250. We selected the 30-40 males and females with the most (appropriate) extreme development time for eight generations at 25ºC. Virgin adults were kept at a lower temperature (18ºC) prior to selection and mating. The FAST lines were reared independently after the first generation and had gained one generation at generation 5 relative to the other lines (i.e. generation 6 for FAST). Lines were then again reared concurrently.

In two generations we employed specific techniques to account for environmental differences. Multiple sleeve cages were used in generation 5 (four per replicate of a selection regime), and an internal mutant control (*Spotty*) in generation 8 (see chapter 4 for methods).

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 originated from different selection lines, 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 6. Only males from lines diametrically opposite to each other competed with each other, i.e. FAST versus SLOW and FMSF vs SMFF. Five virgin females from one line were put in a cylindrical hanging cage (0.3m diameter) with five unmated males from both the same selection line and the other, opposing line. 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 25ºC and pupal weight was measured one day after pupation. Mating tests also occurred at this temperature.

Statistics

In analyses of (co)variance, the factors replicate and sleeve were always random factors. Non-significant terms were removed from the model. Realized heritabilities were calculated by regressing the response on the cumulative selection differential. To obtain estimates of realized heritability for protandry we used the FMSF and SMFF lines, for development time we used the difference between FAST and SLOW lines (divergence; Falconer & Mackay, 1996). Fertility (percentage of hatching eggs) was analyzed using logistic regression, and the Likelihood-Ratio (L-R) χ^2 has 1 degree of freedom, unless otherwise stated.

Results

Response to selection

Protandry did not change over the generations when comparisons are made between the selection lines (figure 6.1). 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 did not differ significantly from zero (table 6.1).

Heritable additive genetic variation was present for development time. Differences in environmental conditions between generations made it difficult to estimate realized heritabilities directly (figure 6.2). However, we can use the difference between FAST and SLOW selected lines (divergence) to obtain realized heritability for development time: males: $h^2 = 0.111 \pm 0.033$, females $h^2 = 0.120 \pm 0.040$ (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 $h^2 = 0.116 \pm 0.037$. Only generations were used where FAST and SLOW were reared concurrently, although including the other generations did not alter the results.

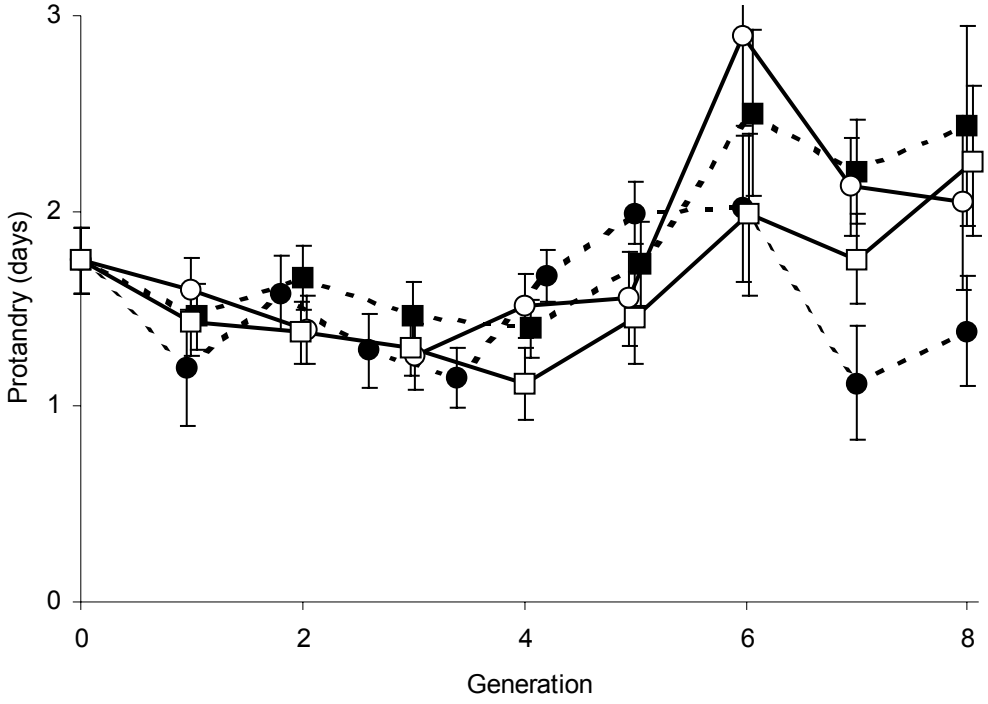

Figure 6.1 Protandry (difference between mean female and male development time) at 25ºC for the different selection lines (replicates pooled, ± standard errors). Selection lines are: SLOW (■), FMSF (□), SMFF (○) and FAST (●). Note that the FAST lines have an extra generation between generation 1 and 5. Thus generation 5 is in fact the sixth generation for FAST.

	replicate	realized h^2	replicates pooled
FMSF (more protandry)	1	−0.012 ± 0.010	−0.014 ± 0.007
	2	−0.017 ± 0.009	
SMFF (less protandry)	1	0.034 ± 0.030	0.034 ± 0.032
	2	0.027 ± 0.043	

Table 6.1 Realized heritabilities for protandry based on eight generations of selection at 25ºC. None of the estimates is significantly different from zero.

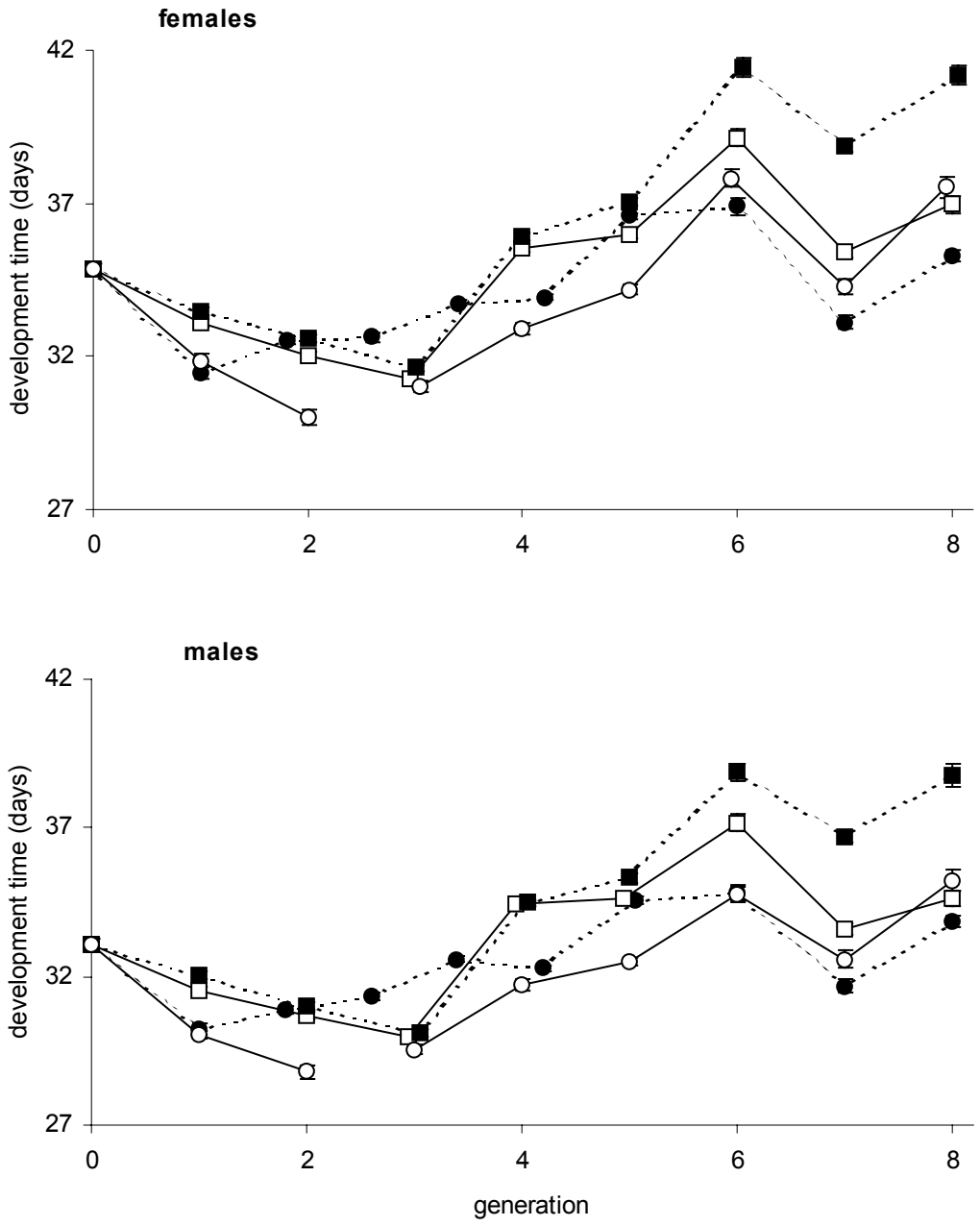

Figure 6.2 Development times (± s.e.) at 25ºC for the different selection lines for females (top) and males. Lines are: SLOW (■), FMSF (□), SMFF (○) and FAST (●). The FAST lines have an extra generation between generation 1 and 5, and the SMFF lines were re-established from the stock, effectively making generation 3 the second generation 0 for these lines.

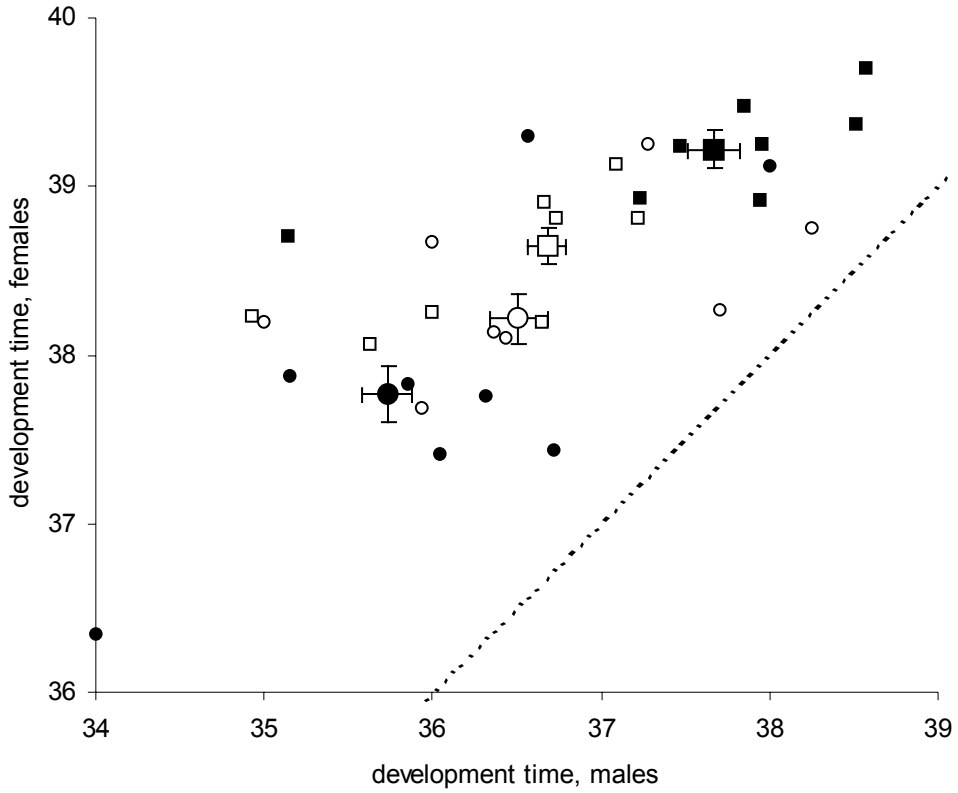

Figure 6.3 Female development time in days plotted against male development time at 25ºC for generation 5 (generation 6 for FAST). Small symbols are means for single sleeve cages, large symbols (± s.e.) are selection line means; SLOW (■), FMSF (□), SMFF (○) and FAST (●). Dotted line depicts equal development times of the sexes.

Four sleeve cages were reared per replicate selection line in generation 5 (generation 6 for FAST) thus enabling a comparison of development time. Sleeve cages (nested in 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 (see figure 6.3).

In the final, eighth generation of selection we used *Spotty* mutants as an internal control to correct for environmental differences between lines (chapter 3). Development times differed significantly between *Spotty*-corrected selection lines ($F_{3,4} = 51.27$, $p = 0.0012$). Tukey comparisons revealed the following pattern: FAST < [SMFF = FMSF] < SLOW (see figure 6.2). Replicate, nested in line, was also significant ($F_{4,1319} = 3.37$, $p = 0.0093$), mainly because of differences between replicates for SMFF. Furthermore, males were consistently faster than females ($F_{1,1319} = 105.09$, $p < 0.0001$).

Slow male - fast female incompatibility

During the selection procedure, we had to restart the SMFF lines because of low numbers in generation 2 (figure 6.4). Egg-to-adult survival percentage was significantly lower for SMFF than for the other lines (logistic regression, 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 × 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).

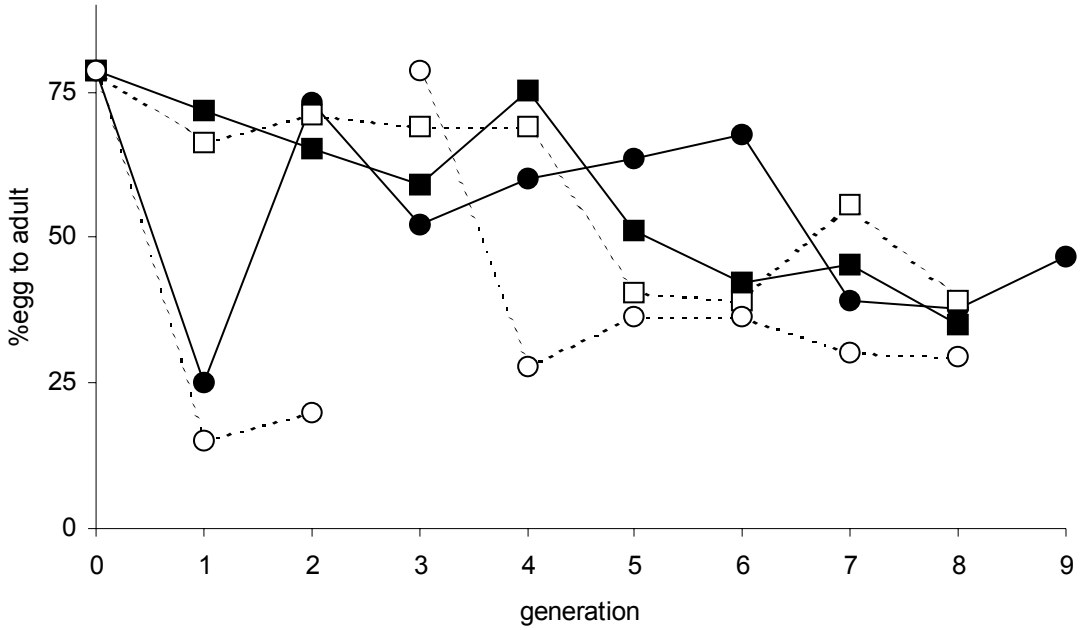

Figure 6.4 Egg-to-adult survival during protandry selection for SLOW (■), FMSF (□), SMFF (○) and FAST (●) lines. The SMFF lines were re-established from the stock in generation 3.

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. 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 6.5). 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 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 6.5). 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 (cf. low egg-to-adult survival for SMFF). However, interaction complicates matters (figure 6.5). 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 6.5). 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 $\chi^2 = 1.90$, $p = 0.17$). A small number of sterile broods were included in our analyses (4 out of 85 broods only contained unfertilized eggs), but the results did not change when they were omitted.

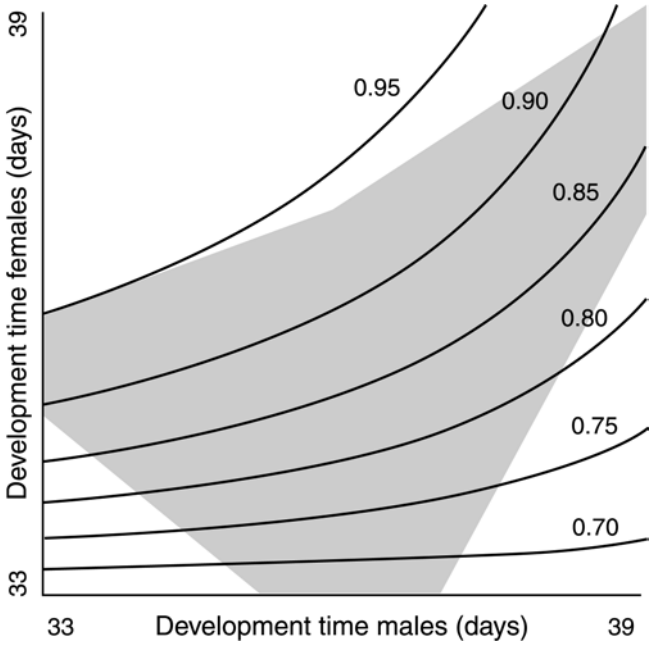

Figure 6.5 Logistic regression model for fertility. Lines connect combinations of parental development times with the same predicted proportion of hatching eggs. For the other effects in the model, fecundity and male weight, mean values were used (22.5 eggs per day, and 172.5 mg, respectively). Gray area outlines those combinations of male and female development time actually used to produce the data set.

No differences in mating success were observed in the mate choice experiments with FAST and SLOW lines. 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).

In summary, offspring from a male with a long development time and a fast developing female has a lower chance of egg-hatching and of survival to adult. Furthermore, males from lines selected for such a combination of parental development times (i.e. SMFF) have lower mating success when competing against those of the converse combination (FMSF).

Discussion

No response for more or less protandry was observed after eight generations of selection, although selection for increased or decreased development time in both sexes (either fast or slow) was successful. Heritability estimates for development time were similar in each sex. The genetic architecture across the sexes for development time might be too tightly integrated, with the genetic covariances near unity, to obtain opposite responses in the sexes (slow males and fast females or vice versa). The sex-specific component of development time that must have been present at one time in order for protandry to arise is either too small to give a significant sex-specific reaction to development time selection, or has been eroded.

However, the ratio of male to female development time did show a change for other slow lines selected for a longer period (>30 generations), suggesting that some form of sex-specific genetic variance for development time still exists (chapter 5). In this shorter term study we did not detect the effect of such variance. The genetic correlation of development time across the sexes appears to be very close to unity. Alternatively, changes in protandry may have been buffered by changes in either growth rate and/or pupal weight. Alterations in development time could be strongly limited by changes in these two other components that form a tightly interconnected triangle with development time.

The result of both scenarios would be that protandry is highly buffered from environmental influences. The same difference between male and female development time is observed, regardless of microenvironmental variations (i.e. small temperature fluctuations between cages and/or generations). Macroenvironmental variation, the difference between low temperatures of the dry season and the warm wet season, does lead to changes in absolute protandry (difference in days), but only because development time changes with temperature, whilst male development time as a percentage of female development time remains relatively constant (~94%, chapter 5).

Selection lines for decreased protandry (SMFF lines) had to be restarted because of low numbers, due to low egg-to-adult survival (figure 6.4). This obviously decreased the selection intensity. However, the incompatibility found between slow males and fast females, is very interesting and acts as an additional factor maintaining or even increasing 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 6.5). 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 shaping protandry.

The positive effect of paternal weight on brood fertility could be accounted for by the transfer of a richer nuptial gift (e.g., Karlsson, 1998). Thus, larger males might contribute a larger spermatophore of higher quality, increasing egg hatching, but little is known about paternal gifts in this butterfly. However, the absence of paternal weight in explaining total number of eggs laid counters the nuptial gift argument, but the nuptial gift may influence remating of the inseminated female.

Previous work on differential female fecundity in other insects has shown that protandry is favored because early females lay more eggs than later females (Kleckner *et al.*, 1995; Carvalho *et al.*, 1998). In both cases (the mosquito *Aedes sierrensis*, and the tropical butterfly *Brassolis sophorae*, respectively), later emerging females had a smaller size and lower fecundity in the field. This pattern is identical to that found here: fecundity in *B. anynana* declined with development time and increased with weight. However, in our study, 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 weights (chapter 7). Therefore, variation in female quality will result in selection on protandry that lies somewhere between directional selection for increased protandry and stabilizing selection.

Furthermore, males from the protandry-decreasing combination of parents mated less successfully when in competition with those from the conversely selected lines. This could be another factor working in the wild against a decrease in protandry, if males from such combinations have lower mating success. In our selection experiment this factor was less important because all competing males had the same selective history. Possibly, slow males even have a slight competitive advantage because they were younger at the time of mating in our experiment. It remains to be investigated what is the relative importance in nature of these selective factors (differential fertility based on male and female development time, differential fecundity based on female development time), compared to other factors shaping protandry.

To summarize: (*i*) heritable genetic variation is present for development time in *B. anynana*; (*ii*) no response to short-term selection for protandry was found; (*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 favor of protandry. Our results suggest that once substantial protandry has evolved in a species like *B. anynana* it may require long periods of time and mutational input to produce further evolutionary change.

Chapter 7

Correlated responses to selection on protandry and development time in *Bicyclus anynana*

Abstract

Males of the butterfly *Bicyclus anynana* have a shorter average development time than females (protandry). Previously, we observed that selection on the difference in timing of emergence between the sexes failed to yield any short term response. Lines where both sexes were selected in the same direction for egg-to-adult development time did show a response. In this study we examine correlated responses in these selection lines at two temperatures. Patterns of protandry across temperatures were inconclusive. There were no differences in egg weights between selection lines. In all lines, individuals with intermediate larval time (egg-to-pupation) had the highest pupal weights, and this relationship was less pronounced for lines selected for a slow development time. Pupal development time of females was equal for all selection lines, but differed for the males, in concordance with the selection; that is, fast selected males had the shortest pupal times, slow males the longest, and males from lines selected for a change in protandry had intermediate pupal times. This suggests sex-specific factors for at least this component of development time. However, protandry selected lines did not differ in pupal weight or growth rate, making it unlikely that changes in protandry were buffered by these traits.

Introduction

Development time is a key trait in the life history of insects, which is closely interrelated with growth rate and body weight. Genetic and plastic variation is present for this triangle of traits and its connections, despite the fact that it is strongly influenced by temperature (Nylin & Gotthard, 1998). All three traits in this interconnected web of trade-offs can show (adaptive) variation; growth rate is not necessarily the passive resultant of development time and body size (e.g., Nylin, 1992). An extra level of complexity is added by sexual differences in optimal body size and development.

As Darwin (1871) noted, males tend to eclose before females, a phenomenon called protandry. Models predict that, given certain characteristics of the mating system, males are sexually selected to emerge before females to increase their number of matings (Wiklund & Fagerström, 1977; Bulmer, 1983; Iwasa *et al.*, 1983), and females are selected to minimize their pre-reproductive time as adults (Fagerström & Wiklund, 1982; Zonneveld & Metz, 1991). Alternatively or concordantly, females, but not males, are selected for larger body size (and thus increased development time) because this increases fecundity. Hence, natural selection will work asymmetrically on the sexes, giving rise to protandry (Thornhill & Alcock, 1983). Comparative studies on butterflies by Nylin and co-workers singled out sexual selection as the main cause of protandry (Nylin *et al.*, 1993). Other studies, however, argue that natural and sexual selection both play a role, and that their relative importance depends on specific circumstances (e.g., Bradshaw *et al.*, 1997). To substantiate protandry theory we not only need comparative studies, but also experimental approaches.

In an effort to investigate the quantitative and evolutionary genetics of protandry, we established selection lines in the tropical butterfly *Bicyclus anynana* for all possible directions of change in male and female development time. We found significant heritable variation for development time, but not for protandry (chapter 6). Here, we examine whether other traits were affected during protandry selection, perhaps buffering against a response to protandry selection. Furthermore, by studying correlated responses in pupal weight and growth rate in fast and slow selected lines, we might, in an indirect manner, gain more insight into the complex interplay between growth rate, development time and pupal weight relevant to understanding both the causes and consequences of protandry.

In addition, we compared two temperatures, corresponding to the wet (25ºC) and the dry (20ºC) seasons this butterfly experiences in its natural habitat. In the warm, wet season, there are many food-plants available to caterpillars and development is rapid. Butterflies have two generations during this season, but a third generation could be achieved as well, if development is rapid enough (Windig *et al.*, 1994). With the strong emphasis on fast reproduction that characterizes the wet season, small differences in reproductive success early in the wet season will be magnified as the population grows. The cold, dry season, on the other hand, is unfavorable to the species, as no food-plants are available. In this season, butterflies are effectively in a reproductive diapause and survival is paramount (Brakefield, 1997). In the field, fat bodies are much larger in the dry season and the number of mature eggs in the ovaries of female butterflies is reduced (Brakefield & Reitsma, 1991). These divergent selection pressures have repercussions for protandry selection; in the wet season, there is selection for rapid development, whilst in

the dry season selection will favor a larger investment in the (fat) body, probably associated with an increased development time.

In this study, we ask how selection on male and female development time at a wet season temperature (25ºC) shaped this trait when butterflies are reared in dry seasonal conditions (20ºC). How does protandry of the different lines change between temperatures? Furthermore, we investigated correlated responses in pupal weight (at both temperatures), growth rate and egg weight.

Materials and Methods

The stock population of *Bicyclus anynana* has been kept in the Leiden laboratory for over ten years. From this stock, selection lines were established for all combinations of male and female development time, resulting in the following lines (each replicated twice): FAST, SLOW, FMSF (fast males, slow females; selected for an increase in protandry), and SMFF (slow males, fast females, decreased protandry). The exact selection procedure is described elsewhere (chapter 6). We assessed the following correlated responses for these lines after 5 generations of selection (6 generations for FAST lines):

Egg weight and size – Five eggs from 68 females (4-23 females per selection regime) were weighed to the nearest 0.001 mg. Although originating from different development time selected lines, we used mothers for this part of the experiment that did not differ in development time or age. Pupal weight (to the nearest 0.01mg) and development time of both parents were known. Furthermore, egg size (cross-sectional diameter) based on 10 eggs was assessed for 111-141 females per selection regime using image analysis. Egg size and weight are highly correlated (Van Oosterhout *et al.*, 1993, Fischer *et al.*, 2002).

Pupal time, pupal weight and growth rate at 25ºC – Four sleeve cages per replicate per selection line were reared, and each day checked for pupae. These were weighed one day after pupation and allowed to emerge individually. Thus, pupal time, from pupation to adult eclosion, was known. Pupal weight and larval time (time from egg hatching to pupation) were used together with average egg weight to calculate growth rate: log (growth rate) = (log (pupal weight) − log (egg weight)) / larval time (Nylin, 1992).

Development time, protandry, pupal weight and growth rate at 20ºC – We also reared progeny at 20ºC that originated from the same set of parents as the offspring reared at 25ºC. Again we used four replicate cages per replicate of a selection line. We measured overall development time and one-day pupal weight. Protandry was calculated as the difference between mean female development time and mean male development time, and growth rate as above. Male development time as a percentage of female development time was calculated as a relative measure of gender differences. Despite its proportional nature, this measure was normally distributed with equal variances. Adults did not emerge individually at 20ºC but were pooled at the sleeve cage level, and, therefore, we did not know the sex of the weighed pupae. The sex ratio of the emerging butterflies did not differ from one-to-one.

Statistics

Replicates as a factor in analyses of (co)variance (AN(C)OVA) never differed from each other, and were pooled. Sleeve cages were nested within the factor selection lines and treated as a random factor. Nonsignificant interaction terms were removed from the model. Post-hoc comparisons between levels of nominal factors were made with Tukey tests, slopes and nominal factors in significant interaction terms were compared with contrasts. Comparisons across temperatures were made with temperature as a discrete factor.

Results

Egg size and weight

Egg weight did not differ between selection lines (ANCOVA, $F_{3,48} = 1.65$, $p = 0.19$). The only significant factor affecting egg weight was maternal development time (figure 7.1). Neither paternal development time, nor parental pupal weight significantly influenced egg weight. A second test, with increased sample size also detected no significant differences in egg sizes (diameter) between selection lines (ANOVA, $F_{3,480} = 0.25$, $p = 0.86$).

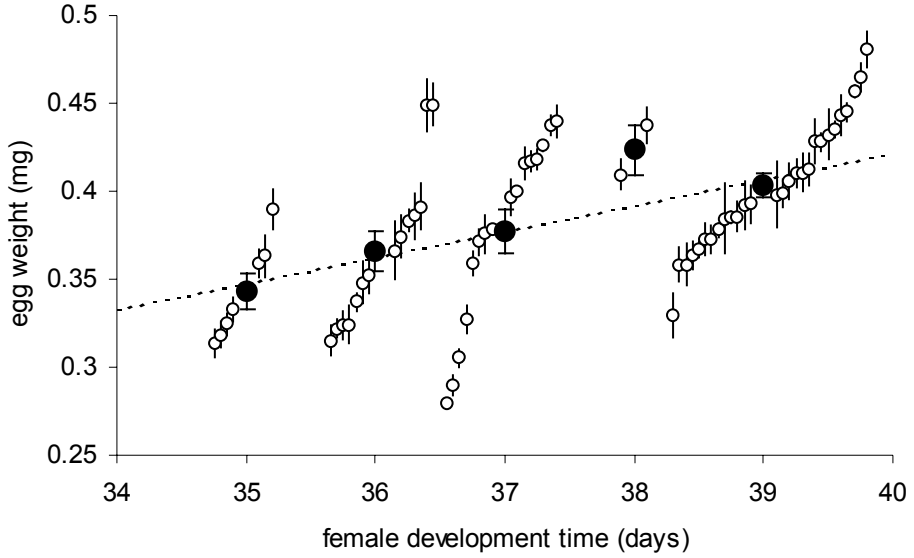

Figure 7.1 Female development time at 25ºC versus egg weight. Each open symbol represents the mean and s.e. for five eggs of one female, with values ranked per day of development time. Large, closed circles are means per eclosion day. Regression (dotted line): egg weight = −0.17 + 0.015 × female development time ($r^2 = 0.19$, $p < 0.0001$). Note that there are five classes of female development time.

Pupal time at 25ºC

Pupal time, the time from pupation to adult eclosion, increased with increasing pupal weight ($F_{1,1459} = 7.94$, $p < 0.01$). Furthermore, despite protandry, females always had a shorter mean pupal time than males ($F_{1,1459} = 140.66$, $p < 0.0001$, figure 7.2). There was a significant interaction between selection line and sex ($F_{3,1459} = 20.73$, $p < 0.0001$); males of the different selection lines differed in pupal time (contrast, $F_{3,1459} = 11.56$, $p < 0.0001$, FAST < [FMSF = SMFF] < SLOW), but females did not (contrast, $F_{3,1459} = 0.75$, $p = 0.52$; see figure 7.2). Sleeve cage, nested in selection line, was also significant ($F_{12,1459} = 2.78$, $p < 0.0001$).

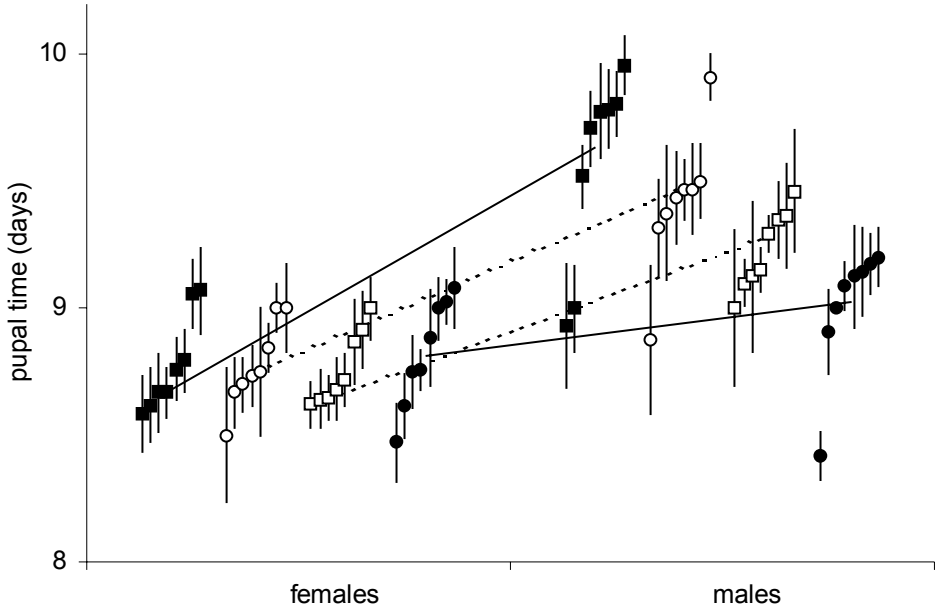

Figure 7.2 Pupal times for males and females of the different selection lines at 25ºC (■: SLOW □: FMSF ○: SMFF ●: FAST). Each point (± s.e.) represents the mean for a sleeve cage with values ranked per group. Lines (solid for SLOW and FAST, dotted for SMFF and FMSF) connect least-square means of the two sexes.

Pupal weight and growth rate at 25ºC

Pupal weight is lower for males than for females ($F_{1,1465} = 1398.41$, $p < 0.0001$), and is highest at an intermediate larval time (larval time × larval time component, $F_{1,1465} = 84.45$, $p < 0.0001$). This quadratic larval time component is stronger for females than for males ($F_{1,1465} = 18.54$, $p < 0.0001$; figure 7.3). Furthermore, selection lines differed significantly in pupal weight, ($F_{3,1465} = 12.48$, $p < 0.0001$; Tukey pattern: [FAST = SMFF = FMSF] < SLOW, figure 7.3), and also in the shape of the weight - development time relation (larval time × larval time × line: $F_{3,1465} = 3.71$, $p < 0.05$, with significantly less curvature for SLOW, $t = 2.15$, $p < 0.05$, figure 7.3a). The sleeve cages did not differ from one another.

Selection lines differed significantly in growth rates, $F_{3,12} = 4.13$, $p < 0.05$, Tukey comparison: FAST > [SMFF = FMSF] > SLOW. Males have higher growth rates than females, 31.6% mean daily weight gain per day for males versus 29.4% per day for females ($F_{1,1463} = 209.74$, $p < 0.0001$). Furthermore, significant differences were observed between sleeves ($F_{12,1463} = 3.76$, $p < 0.0001$).

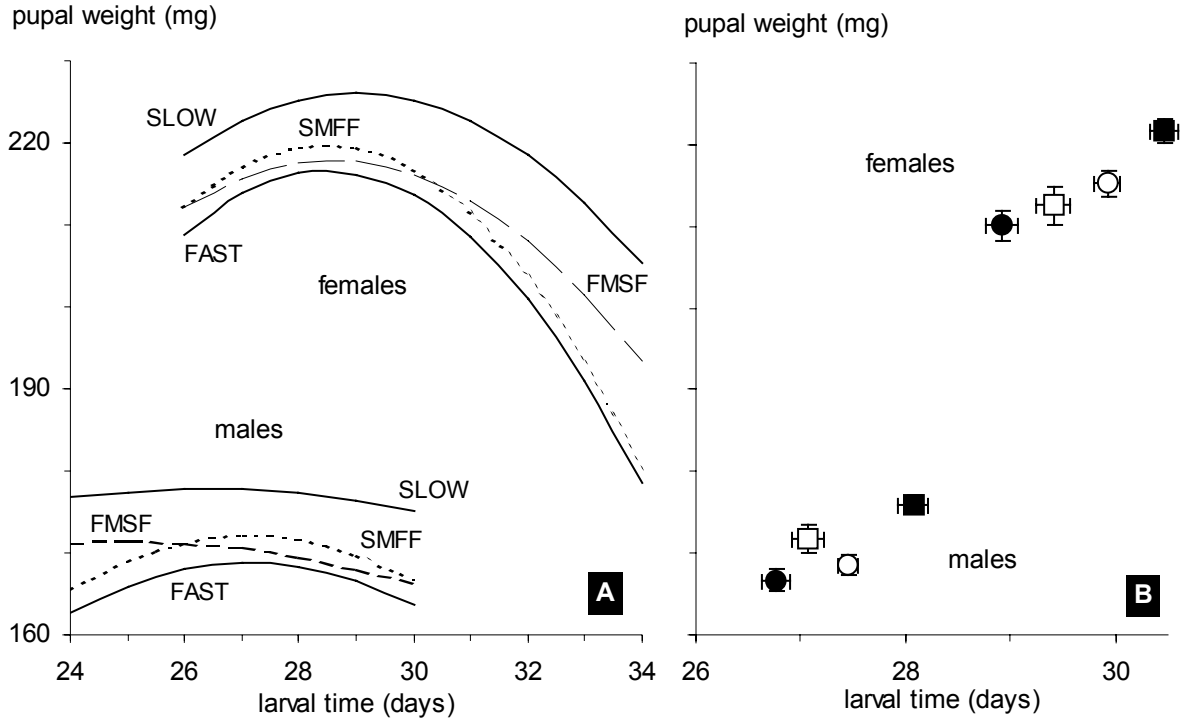

Figure 7.3 A: Predicted relationships between larval time and pupal weight at 25ºC for the different sexes and selection lines (dotted lines are SMFF, dashed lines are FMSF), see text for complete model. B: Larval time and pupal weight means (± s.e.) for males and females of the selection lines at 25ºC (■: SLOW □: FMSF ○: SMFF ●: FAST).

Development time at 20ºC

Egg-to-adult development time is longer at 20ºC than at 25ºC (compare figure 7.4 with figure 6.3). Development time of sleeve cages at 20ºC differed significantly from each other ($F_{12,812} = 7.01$, $p < 0.0001$) and females were slower than males ($F_{1,812} = 72.25$, $p < 0.0001$). Selection lines did not differ from each other ($F_{3,12} = 0.75$, $p = 0.55$), but the sex × line interaction approached significance ($F_{3,812} = 2.39$, $p = 0.068$), suggesting that the difference between male and female development time is smaller for the SMFF line than for the other lines (contrast, $t = 2.34$, $p < 0.05$; see figure 7.4).

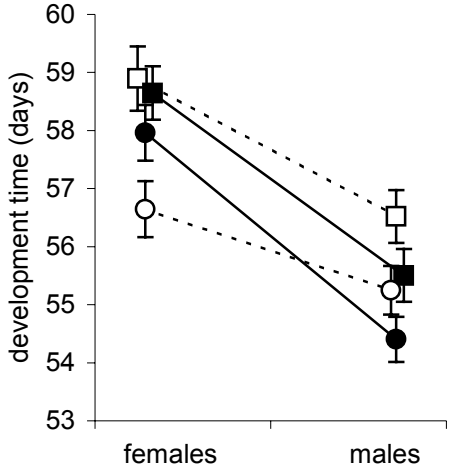

Figure 7.4 Development time for males and females (± s.e.) at 20ºC. Symbols represent selection lines SLOW (■), FMSF (□), SMFF (○), and FAST(●).

Protandry at 20ºC

Protandry is more marked at 20ºC than at 25ºC ($F_{1,54} = 8.51$, $p < 0.01$), and temperature interacts significantly with selection line ($F_{3,54} = 3.50$, $p < 0.05$); protandry for the SLOW line is significantly larger at 20ºC than at 25ºC (Tukey comparison, $p < 0.05$, figure 7.5), whilst the other lines (including FAST) do not differ in protandry between temperatures. As expected for a relative measure, temperature is no longer a significant factor for the male development time as a percentage of female development time ($F_{1,54} = 0.01$, $p = 0.92$). However, the line × temperature interaction remained ($F_{3,54} = 3.17$, $p < 0.05$). Tukey comparisons were not significant, but patterns were similar to the protandry analysis (figure 7.5).

Pupal weight and growth rate at 20ºC

For pupal weight at 20ºC, we only have data for the pooled sexes. Pupae were significantly smaller at 20ºC than at 25ºC ($F_{1,2627} = 372.20$, $p < 0.0001$). Selection lines differed significantly from each other at 20ºC ($F_{3,1049} = 22.24$, $p < 0.0001$), with the same pattern as at 25ºC; the SLOW line had heavier pupae than the other lines (Tukey, $p < 0.05$). Both larval time and its quadratic term were significant at 20ºC, indicating highest pupal weights at intermediate larval times ($F_{1,1049} = 58.53$ and 19.68, respectively, both $p < 0.0001$). Growth rates at 20ºC did not differ between the selection lines ($F_{3,12} = 0.50$, $p = 0.69$) but were lower than at 25ºC (~18% mean daily increase per day at 20ºC versus ~30% at 25ºC).

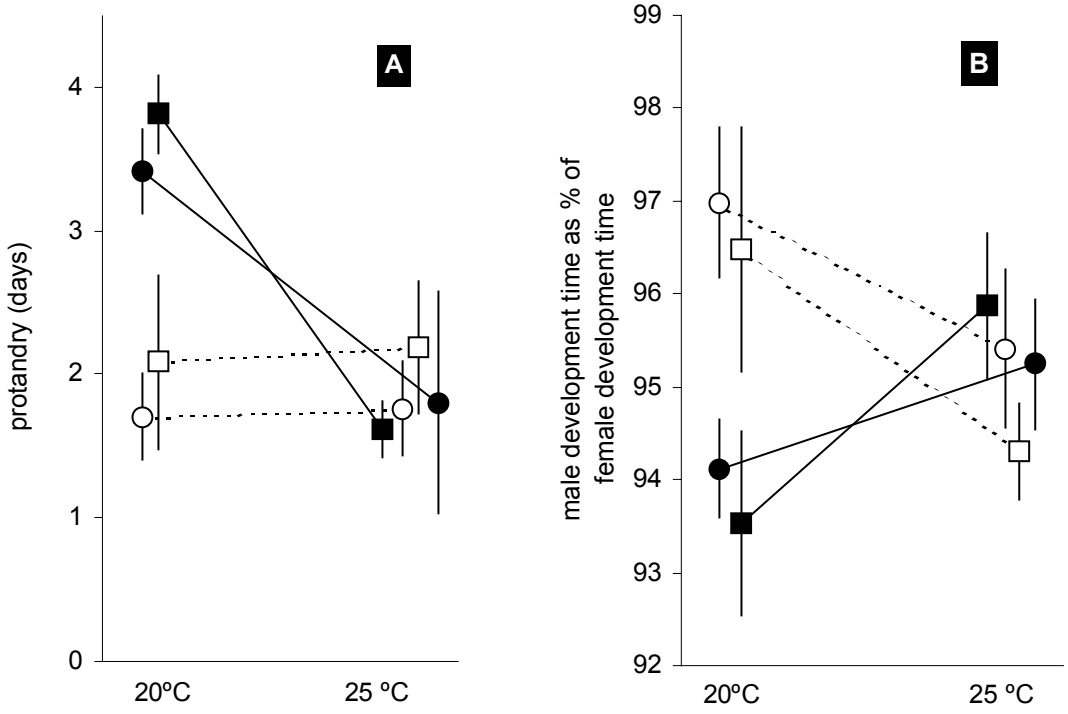

Figure 7.5 A: Protandry (means ± s.e.) comparison between 20ºC and 25ºC for selection lines SLOW (■), FMSF (□), SMFF (○), and FAST (●). B: comparison of male development time as a percentage of female development at both temperatures.

Discussion

Egg size and weight

Longer developing females laid heavier eggs which suggests that selection would favor later female eclosion and increase protandry if heavier eggs increase maternal and/or offspring fitness. However, previous studies on *B. anynana* (Van Oosterhout *et al.*, 1993) and other satyrids (Wiklund & Karlsson, 1984) were unable to correlate egg weight to offspring fitness (but see Fischer *et al.*, 2002). Furthermore, egg weight did not differ between selection lines, although we previously showed that lines selected for an decrease in protandry (SMFF) had lower fertilities and egg-to-adult survival than other lines (chapter 5). Perhaps a bias in the window of development times used to assess egg weights precluded finding differences between lines. Currently, selection lines for egg size are running and it will be interesting to determine the correlated response in development time and protandry. As it stands, egg size variation is unlikely to be a major factor in shaping protandry.

Pupal time at 25ºC

Pupal time of males reacted in a similar manner to selection as egg-to-adult development time selection; FAST males had shortest, and SLOW males had the longest pupal time (figure 7.2). Females, however, did not show line differences in pupal time. Sex differences in pupal time were opposite to the overall pattern of protandry; females have a shorter average pupal development than males. This is not a peculiar side-effect of our selection procedure, but also occurs in the stock. Possibly, males complete parts of their development as pupae that females realize as larvae. Pupal times of *Polygonia c-album* butterflies did not significantly differ between the sexes (Nylin *et al.*, 1993). So this component of development time did not add to differences found in overall development time, in a similar way to females in this study. Males of our *B. anynana* selection lines, however, did differ significantly in pupal time between lines (figure 7.2). As males and females react similarly to development time selection (comparable heritability estimates, chapter 6), this indicates that changes are achieved at different life-stages for the sexes. Development time of females is only altered by changes in larval development, whilst males also gain or lose time during pupal development. Perhaps females have reached the minimum amount of time necessary for pupal development. But that does not explain why pupal time of females did not increase for the SLOW lines.

The sexual differences in the correlated response in pupal time suggest that a response to selection for protandry should be possible. Applying selection solely on the rate of pupal development could alter adult emergence patterns because female pupal time will remain the same while males will respond. However, this assumes that development in the larval stage will remain constant. If larval time and pupal time are connected through some sort of negative feedback mechanism, whereby changes in one trait will be counterbalanced by an opposing change in the other trait, this could then lead to no change in overall development time.

Pupal weight and growth rate at 25ºC

SLOW selected lines had both a lower growth rate and an increased pupal weight contributing to a longer development time. However, the trade-off between development time and body weight was not a linearly increasing one, but had an intermediate optimum (figure 7.3a). In the short term, selection for an increase in development time can be (as seen for SLOW) associated with an increase in pupal weight, but after the intermediate maximum, pupal weight will decrease with increasing larval time. Possibly the first, ascending part of the curve represents a trade-off, whilst the latter, descending phase is more a result of environmental variables. The parabolic relationship would suggest that obtaining a longer development period in the long term can only be achieved by decreasing growth rate, and will be associated with a decrease in pupal weight. However, the interaction between the quadratic larval time component and selection line points to the fact that the development time - weight relation could change for SLOW selected lines (compare the curvature of the selection lines in figure 7.3a, the SLOW line is much flatter). FAST lines achieved their faster development via a higher growth rate, but had equal pupal weight to FMSF and SMFF lines. Presumably, this higher growth rate has come at a cost in terms of some other component of fitness (Nylin, 1994). Alternatively, the protandry selection lines may have decreased pupal weights because their genetic architecture has been disrupted.

The change in growth rate accounting for the changes in development time, and the non-linear relationship between larval time and pupal weight underline the importance of growth rate in life-history evolution. The straightforward time - weight trade-off which is often assumed would in this case lead to erroneous conclusions. Conclusions about trade-offs and in particular with respect to protandry, are further complicated by the fact that fertility and fecundity do not relate in a straightforward fashion to development time and body size (chapter 6).

In figure 7.6, the relationships between development time, pupal weight, and growth rate (inverted for easier comparison) are shown conceptually within and across sexes. In theory, all connections in this figure can vary under specific circumstances, but the important connection with regard to protandry is the line connecting male and female development time (the line between the two apexes). We have been unable to change this relationship (chapter 6), which may perhaps arise as a result of changes in other connections between life-history traits across the sexes (assuming constancy of the triangle within one sex). For example, selection for a decreased amount of protandry, that is, for slow males and fast females, might not have yielded a response because of changes in growth rate or pupal weight (visualised as the dotted triangles). However, the protandry selected lines showed intermediate growth rates and pupal weights that did not differ from patterns expected for stock animals. This refutes the hypothesis that changes in protandry are buffered by changes in either growth rate or pupal weight (see below). In other words, the absence of a response to selection for protandry cannot be accounted for by changes in pupal weight and/or growth rate.

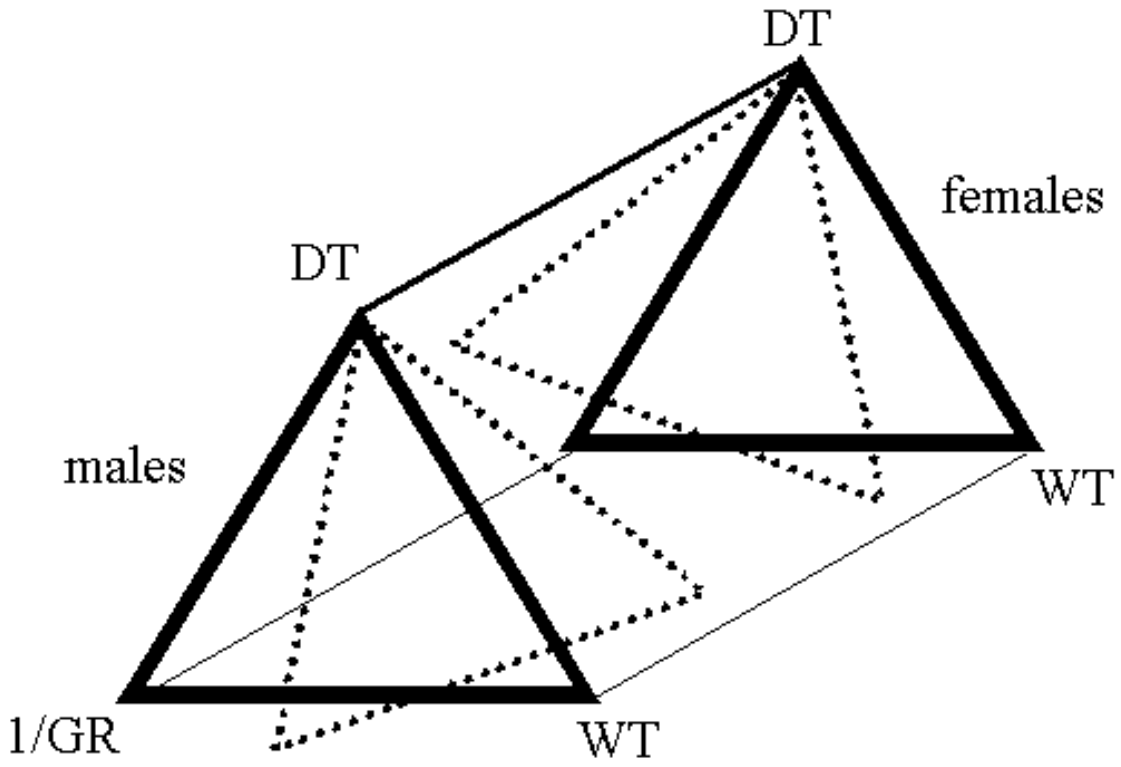

Figure 7.6 Conceptual figure of the triangle development time (DT), body weight (WT), and the inverse of growth rate (1/GR) for males and females. Dotted figures show how response in development time of selection on slow males and fast females might be buffered against by changes in body weight (increased for males, decreased for females) and growth rate. See text for more details.

Development time and protandry at 20ºC

The absolute measure of protandry (difference in days) was larger at lower temperatures, but the relative measure (male : female ratio in development time) did not differ between 20ºC and 25ºC. More importantly, both measurements showed significant line by temperature interactions. The trend towards a smaller difference between males and females in development time for the line selected for less protandry (SMFF) points to some response to protandry selection. However, this trend is in no way supported by the response to selection at 25ºC, the temperature where selection took place (e.g., see figure 6.1). The pattern observed at 20ºC for FMSF, lines selected for an increase in protandry, is opposite to expectation. There is no clear cut explanation for the altered patterns of the protandry selected lines at 20ºC. The relative measure of protandry (male development time expressed as a proportion of female development time, figure 7.5) remained constant at different temperatures. This agrees with previous studies on protandry in the stock of this butterfly (chapter 5). However, the male : female ratio in development time had changed for lines selected over 30 generations for slow development (chapter 5). Our slow selected lines did not show this tendency, perhaps because they had not yet reached

the stage where such a response occurred. The significant line by temperature interaction is just as puzzling as for the absolute measure of protandry (see above).

For growth rate and pupal weight we can only draw tentative conclusions because we could not account for sex which is an important determinant (see results at 25ºC). Both pupal weight and growth rate are significantly lower at 20ºC compared to 25ºC. At the dry season temperature we see the same parabolic relation between larval time and pupal weight as at 25ºC. Pupal weights also differed in the same manner as at the wet season temperature.

The patterns observed for growth rate and pupal weight at both temperatures do not support the hypothesis that a response to protandry selection was buffered by changes in either pupal weight and/or growth rate. The protandry selected lines did not show markedly different growth rates or pupal weights to substantiate this hypothesis. Therefore, it seems more likely that the current genetic arrangement of development time across the sexes is too tightly linked to respond to selection, precluding any short term response to selection on protandry (chapter 6). Perhaps changes in protandry would only be possible with selection over a long period of time coupled with mutational input. It will be interesting to compare the levels of protandry in species of *Bicyclus* from non-seasonal environments where generations are unlikely to be discrete.

Samenvatting

Beperkingen op evolutie bij de vlinder *Bicyclus anynana*

Selectie en beperking

Volgens doctor Pangloss, de huisleraar van Candide uit Voltaires boek *Candide, of het optimisme*, leven we in de best mogelijke wereld van alle werelden en is alles gemaakt met een doel. In de evolutiebiologie heeft deze doctor furore gemaakt omdat zijn naam gekoppeld werd aan het adaptatieprogramma waartegen Stephen Jay Gould en Richard Lewontin in 1979 ageerden. Zij stelden dat Darwins natuurlijke selectie ten onrechte gezien wordt als de enige factor die de uitkomst van evolutie bepaalt. Alles zou ontstaan zijn als aanpassing ten gevolge van natuurlijke selectie.

Natuurlijke selectie volgens Darwin werkt als volgt: levende organismen vermenigvuldigen zich in principe exponentieel en bij oneindige middelen tot in het oneindige. In de echte wereld zijn middelen (voedsel, zonlicht, etc.) beperkt, waardoor er competitie is. Sommige organismen overleven en planten zich beter voort dan anderen. Als deze variatie (deels) erfelijk bepaald is, dan hebben hun nakomelingen ook de eigenschappen waardoor ze zich beter handhaven en vermenigvuldigen dan anderen, en zal hun relatieve aantal toenemen. Op den duur blijven alleen de best aangepaste combinaties van erfelijke eigenschappen over. Door bijvoorbeeld mutaties ontstaan nieuwe (combinaties van) erfelijke eigenschappen die al dan niet beter aangepast zijn.

Wat de adaptionisten verweten wordt, is dat zij alleen selectie en adaptatie als oorzaak van evolutie zien. Volgens Gould en Lewontin spelen ook beperkende factoren (*constraints*) een rol en zijn niet alle eigenschappen en biologische structuren adaptief. Stephen Jay Gould bepleit zelfs een actieve rol voor deze *constraints*. Er zijn verschillende voorbeelden van *constraints* te geven. Zo zouden extra ogen in het achterhoofd van een konijn best voordelig zijn; het konijn ziet altijd de vos aankomen en is op tijd weg. Toch zal deze innovatie waarschijnlijk niet plaatsvinden. Er zijn meer

innovaties te bedenken die op zich wel voordeel bieden, maar toch niet zullen voorkomen, bijvoorbeeld door beperkingen tijdens de ontwikkeling en groei, of doordat er voor die eigenschap geen variatie aanwezig is in het erfelijk materiaal. Als een bepaalde eigenschap nooit voorkomt, dan kan er ook geen selectie op plaatsvinden.

De koppeling van twee eigenschappen kan ook beperkend op evolutie zijn. Als bijvoorbeeld een groeihormoon de lengte van zowel de armen als de benen bepaalt, dan variëren die twee eigenschappen niet onafhankelijk van elkaar. Iemand met veel groeihormoon heeft lange armen én lange benen. Maar om de combinatie lange armen en korte benen te krijgen, zou je grote hoeveelheden voor de armen nodig hebben en een klein beetje hormoon voor de benen. Door de afhankelijkheid van beide eigenschappen van hetzelfde hormoon ontstaan mogelijke beperkingen op evolutie.

Mijn onderzoek

In dit proefschrift heb ik vooral gekeken naar het relatieve belang van *constraints* en selectie. Kunnen we door streng te selecteren in het laboratorium alle mogelijke combinaties van eigenschappen krijgen of zijn bepaalde combinaties niet mogelijk? Om dit te onderzoeken heb ik gebruik gemaakt van de tropische vlinder *Bicyclus anynana* (‘tropisch zandoogje’). Ik heb twee grote selectie-experimenten gedaan waarbij ik geprobeerd heb twee gekoppelde eigenschappen los te koppelen. Als *constraints* belangrijker zijn dan selectie, dan zal selectie geen veranderingen opleveren wanneer beide eigenschappen in tegengestelde richting geselecteerd worden. Reageren de antagonistische (tegengestelde, bijvoorbeeld lange armen en korte benen) selectielijnen relatief net zo goed als de agonistische (beide eigenschappen in dezelfde richting, bijvoorbeeld lange armen en lange benen), dan lijkt alles mogelijk via selectie.

De hoofdstukken

De twee eigenschappen voor mijn eerste selectie-experiment waren ontwikkelingstijd en oogvlekgrootte. Vlinders die zich snel ontwikkeld hebben ('snelle' vlinders) hebben grotere oogvlekken dan 'langzame' vlinders (die meer tijd nodig hadden om van ei tot vlinder te volgroeien, zie bijvoorbeeld figuur 1.2). In **hoofdstuk 2** beschrijf ik de uitkomsten van het selectie-experiment met deze twee eigenschappen. De koppeling tussen deze twee eigenschappen blijkt niet remmend te werken op het resultaat van selectie: lijnen waarbij ik geselecteerd heb voor 'snelle' vlinders met kleine oogvlekken (tegengestelde selectie) reageerden relatief net zo goed als selectielijnen voor bijvoorbeeld 'snelle' vlinders met grote oogvlekken.

Dit roept de vraag op hoe dat hormonaal werkt. Verschillen in zowel ontwikkelingstijd als oogvlekgrootte zijn gerelateerd aan hormoonspiegels van het hormoon ecdyson. Vlinders die geselecteerd zijn voor een snelle ontwikkeling hebben een eerdere hormoonpiek na verpopping dan 'langzame' vlinders. Selectie voor grotere oogvlekgrootte resulteert in een eerdere hormoonpiek in vergelijking met vlinders die geselecteerd zijn voor kleinere oogvlekgrootte. Hoe is dat opgelost in selectielijnen met tegengestelde selectie, waarbij de hormoonpiek twee verschillende kanten wordt uitgetrokken? Nu blijkt (**hoofdstuk 3**) dat het hormoon ecdyson belangrijker is voor ontwikkelingstijd dan voor oogvlekgrootte. De hormoonpiek die ik gemeten heb voor tegengesteld geselecteerde lijnen leek meer op wat je zou verwachten voor ontwikkelingstijdselectie, dan voor oogvlekselectie. Dat wil zeggen dat de piek voor 'snelle' vlinders eerder is en later voor 'langzame' vlinders. Waarschijnlijk ontstaan de verschillen in oogvlekgrootte die wel aanwezig zijn in mijn selectielijnen via een ander mechanisme, bijvoorbeeld doordat de cellen in de vleugels (genetisch) meer of minder gevoelig worden voor het signaal om een andere kleur aan te nemen, en zo een oogvlek te vormen.

Hoofdstuk 4 is een methodologisch stuk. Hier heb ik een techniek onderzocht die het mogelijk maakt ontwikkelingstijd te corrigeren voor omgevingsinvloeden. We zijn het meest geïnteresseerd in erfelijke verschillen in ontwikkelingstijd, niet zozeer in de effecten van bijvoorbeeld kleine temperatuur- of voedselkwaliteitverschillen tussen

kooien. Door bepaalde mutanten als ijkpunt te gebruiken kunnen we de experimentele dieren beter vergelijken. Een korte, schematische weergave van deze methode is te zien in figuur 4.3.

De laatste drie hoofdstukken gaan over protandrie, het eerder uitkomen van mannetjes. In **hoofdstuk 5** heb ik naar het verschil in ontwikkelingstijd tussen mannetjes en vrouwtjes bij verschillende temperaturen gekeken. Ook vergelijk ik protandriepatronen van selectielijnen voor ontwikkelingstijd of voor popgewicht. Bij een hogere opgroeitemperatuur (>23 ºC) was het verschil in gemiddelde uitkomst tussen mannetjes en vrouwtjes onveranderlijk twee dagen. Bij een lagere temperatuur nam het verschil toe, en met name bij lijnen geselecteerd voor langzame ontwikkeling was het verschil groot. Dit impliceert dat het mogelijk zou moeten zijn om de sekses onafhankelijk van elkaar te selecteren voor ontwikkelingstijd. Dat is precies wat in **hoofdstuk 6** beschreven wordt. Ik heb alle mogelijke combinaties van ontwikkelingstijd van mannetjes en vrouwtjes geselecteerd. Om bijvoorbeeld protandrie te verminderen heb ik steeds de langzaamste mannetjes met de snelste vrouwtjes laten paren. Is het mogelijk om de ontwikkelingstijd van mannetjes te veranderen in een richting die tegenovergesteld is aan die van vrouwtjes en zo bijvoorbeeld lijnen te krijgen waar mannetjes en vrouwtjes gemiddeld even snel zijn? Na acht generaties van selectie waren er geen verschillen in protandrie tussen de selectielijnen en was het ook niet anders dan de protandrie van de ouders aan het begin van het experiment. Dit ligt niet aan het feit dat ontwikkelingstijd niet reageert: lijnen waarbij zowel mannetjes als vrouwtjes voor snellere, danwel langzamere ontwikkeling geselecteerd waren, zijn beduidend sneller of langzamer. De relatie tussen mannelijke en vrouwelijke ontwikkelingssnelheid was echter onveranderd. Ooit moet er wel sekse-specifieke genetische aanleg voor ontwikkelingstijd aanwezig geweest zijn, anders had protandrie nooit kunnen ontstaan. In dit geval lijkt het er dus op dat selectie niet omnipotent is. Op de korte termijn konden we de relatie tussen de sekses met betrekking tot ontwikkelingstijd niet veranderen.

In **hoofdstuk 7** kijk ik naar gecorreleerde responsen, dat wil zeggen, veranderingen in eigenschappen waar niet direct op geselecteerd is, maar waar de selectielijnen wel in verschillen. Vlinders die zich snel ontwikkelen hebben bijvoorbeeld een lager popgewicht dan zich langzaam ontwikkelende vlinders. Mogelijk bleven de

protandriepatronen onveranderd door veranderingen in popgewicht, die als een soort buffer werkten. Lijnen geselecteerd voor meer of minder protandrie (groter of kleiner gemiddeld verschil tussen mannetjes en vrouwtjes) vertoonden echter geen afwijkende popgewichten of groeisnelheden.

Wie wint?

De hamvraag in dit proefschrift was: wat is belangrijker, *constraint* of selectie? Mijn twee selectie-experimenten geven twee verschillende antwoorden. Voor oogvlekgrootte en ontwikkelingstijd lijkt de koppeling niet uit te maken. Door selectie kun je alle mogelijke combinaties bereiken. Maar het protandrieselectie-experiment toont aan dat niet alles mogelijk is door selectie.

De waarheid ligt ergens in het midden.

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Nawoord

Dit proefschrift zou niet tot stand gekomen zijn zonder de medewerking van veel mensen. Hongerige rupsen moesten gevoed worden en zelfs in de grootste mais-recessies wisten Els c.s. (en later Niels c.s.) wel planten te leveren. Die moesten dan nog wel in kooien gezet worden, iets waar Fanja en anderen altijd wel voor te porren waren tijdens vakanties of hectische periodes. Daarnaast vond (en vind) ik de algehele relaxte atmosfeer bij evolutiebiologie zeer stimulerend.

Als gidsen in de wondere wereld der endocrinologie fungeerden Bernd Koch en Marga Lenz. Tijdens een verblijf in Ulm werd ik wegwijs gemaakt in het meten van ecdysteroiden concentraties en de edele kunst van de Wiederholungen.

Nogal wat experimenten zouden onmogelijk geweest zijn zonder de hulp van enkele studenten. Marc Steigenga en ik hebben samen veel werk verzet, waar hoofdstukken 2 en 3 slechts een kleine afspiegeling van zijn. Datzelfde geldt voor de laatste twee hoofdstukken, waarvoor de samenwerking met Jeroen Pijpe onontbeerlijk was. Ook Linda de Kooter heeft hier haar steentje aan bijgedragen.

Kortom, veel ondersteuning. Maar één iemand kan, mag en wil ik niet ongenoemd laten en dat is Joanne, zonder wie ik nu een verhongerend emotioneel wrak zou zijn, levend in een kartonnen doos.

Curriculum Vitae

Wilte Zijlstra werd op 23 april 1973 in Groningen geboren. Na zijn eindexamen in 1991 aan het Praedinius Gymnasium te Groningen, deed hij een jaar high school in de Verenigde Staten, in Peotone, Illinois. In 1992 begon hij aan zijn studie Biologie aan de Universiteit Leiden, waar een jaar later *cum laude* de propedeuse werd behaald.

Tijdens zijn studie volgde hij verschillende stages in binnen- en buitenland. In 1994 deed hij onderzoek naar circadiane ritmes aan de Kent State University in Kent, Ohio (Verenigde Staten) onder leiding van David Glass en Huaming Shen. Het jaar daarop begon hij aan een stage bij de vakgroep evolutiebiologie. Onder begeleiding van Cock van Oosterhout en Paul Brakefield bestudeerde hij de gevolgen van inteelt bij de vlinder *Bicyclus anynana*. Aan het Leiden/Amsterdam Center for Drug Research deed hij in 1996 onderzoek onder leiding van Menno Kruk naar de relatie tussen neurale signalen in de hypothalamus en gedrag van ratten. In 1997 vertrok hij naar Barcelona (Spanje) om samen met Manuel Puigcerver en José Domingo Rodríguez Teijeiro veldwerk naar kwartels (*Coturnix coturnix coturnix*) te doen, met name naar vocale communicatie en het paarsysteem. In datzelfde jaar studeerde hij *cum laude* af met als specialisatie evolutiebiologie.

In december 1997 begon hij aan een door NWO gefinancierd promotie-onderzoek (ALW, 805-36-033) bij de vakgroep evolutiebiologie van de Universiteit Leiden met Paul Brakefield als promotor en Bas Zwaan als co-promotor. Dit proefschrift is daar de weerslag van. Hij ging op werkbezoeken bij Bernd Koch (Universität Ulm), en bij Linda Partridge en Dave Clancy (University College London). Daarnaast presenteerde hij zijn resultaten op internationale congressen in Barcelona (1999), Sheffield (2001), Aarhus (2001) en Noordwijkerhout (2002), en op congressen van de Nederlands Entomologische Vereniging (1998-2001).

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