Evolutionary and proximate constraints on egg size in butterflies

Dissertation

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Contents

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Introduction

Life-history evolution – constraints and trade-offs

One of the most astonishing features of the living world is its tremendous variation in life cycles that are composed of relatively few, yet diverse components, the life-history traits (Stearns 1992). These include – amongst others – growth trajectories, age and size at maturity, number, size and sex ratio of offspring, age- and size-specific reproductive investment as well as mortality schedules (Stearns 1992). All of these are usually closely correlated to fitness and involved in various trade-offs among each other (see below; Gotthard 1999; Stearns 2000). In more general terms an individual's life-history is the allocation pattern of time and energy to various fundamental activities (Freeman and Herron 2001).

Life-history theory relates variation in life-history traits to variation in fitness and attempts to explain differences in development, growth and reproduction among populations and individuals (Stearns 1992; 2000; Roff 2002). One central assumption is that such differences have been shaped by natural selection (Stearns 1992; Roff 2002), favouring the evolution of mechanisms and traits that maximize fitness (Stearns 1992; Barnes and Partridge 2003), thus resulting in optimization / adaptation to the prevailing environmental conditions (Pigliucci 2003).

While selective forces have long been considered to be almost omnipotent in shaping phenotypes, the evolution of certain traits or trait combinations may only be partially realised due to counteracting mechanisms that limit or channel responses to selective pressures (i.e. constraints and trade-offs Stearns 1992; Schlichting and Pigliucci 1998; Roff 2002; Barnes and Partridge 2003). Therefore, it is by now general consent that life histories must involve compromises between what selection can achieve (adaptation) and what selection is prevented from achieving (constraints; Barnes and Partridge 2003).

Constraints have been identified on various functional levels (e.g. historical, morphological and developmental constraints; see also Cockburn 1995; Pigliucci 2003; Brakefield 2006), however, their relative importance as compared to natural selection in shaping life histories is still a matter of a controversial debate (e.g. Schlichting and Pigliucci 1998; Pigliucci and Kaplan 2000; Beldade et al. 2002). This is partly a result of a decidedly poor amount of empirical data investigating constraints directly (Beldade and Brakefield 2003) and the still ongoing discussion

about appropriate experimental approaches (Stearns 1992; Roff 2002; Beldade and Brakefield 2003), leaving the empirical assessment of constraints still a challenge.

Likewise, the measurement of trade-offs has attracted controversy (Stearns 1992), but nevertheless trade-offs have become a central concept in life-history theory (e.g. Angilletta et al. 2003). Trade-offs are linkages between two (or more) traits that constrain independent trait evolution. Thus, a trade-off can be defined as a negative correlation between (typically) two fitness-related traits: a change in one trait that increases fitness is linked to a change in another trait that decreases fitness (Stearns 1989; 1992; Fox and Czesak 2000; Roff 2002). Trade-offs have a genetic and a phenotypic component and both can be important in shaping the evolutionary trajectory (Stearns 1992; Roff 2002; Stearns and Magwene 2003). Thus, an organism's life-history can be viewed as a whole suit of traits adapted to characteristics of the environment and adapted to each other, thereby forming a complex strategy based on co-adapted traits (Nylin and Gotthard 1998; Roff 2002).

The analysis of trade-offs in life-history evolution is dominated by the idea that resources required for the expression of life-history traits are environmentally limited (Barnes and Partridge 2003). Life histories have been divided into three categories: 'growth', 'maintenance' and 'reproduction', each of which is considered to compete with the others for resources (Fox and Czesak 2000; Barnes and Partridge 2003) and each of which can in turn be subdivided into competing sections. Of these the fundamental trade-off between egg size and number as introduced by Smith and Fretwell (1974) has long received an almost axiomatic status (see also Fox and Czesak 2000).

Butterfly reproduction – why study offspring size?

A large amount of work done on arthropod reproduction has been developed around the concept of a trade-off between egg size and number. The pivotal point is that the size of offspring can only be increased at the expense of reduced offspring numbers (Smith and Fretwell 1974; Fox and Czesak 2000). Thus, for any fixed parental allocation to reproduction, progeny size is believed to be under balancing selection (Fischer et al. 2006). *Progeny fitness* usually increases with increasing parental investment per offspring, thus favouring the production of large-sized progeny (see also Azevedo et al. 1997; Fox and Czesak 2000). For instance, larger offspring were frequently found to mature earlier, to have improved ability to

withstand competition, or to survive better in stressful environments as compared to small offspring (Azevedo et al. 1997; Fox and Czesak 2000; Czesak and Fox 2003; Roff 2002; Fischer et al. 2003; Fischer et al. 2006). Note however, that a couple of studies were not able to correlate large offspring size to fitness benefits in various species (e.g. Wiklund and Persson 1983; Karlsson and Wiklund 1984). On the other hand, *maternal fitness* increases with increasing progeny numbers, thus favouring the production of small-sized offspring – within the limits posed by offspring viability (Azevedo et al. 1997; Fox and Czesak 2000). This parent-offspring-conflict is predicted to result in the evolution of an optimal egg size balancing maternal and offspring fitness, with mothers expected to have the upper hand in this conflict (Smith and Fretwell 1974; Einum and Fleming 2000). However, the concept of an optimal offspring size has been found to be insufficient to explain the evolution of progeny size (Bernardo 1996), as any environmental variable that affects the relationship between investment per progeny and progeny fitness should also affect optimal progeny size (e.g. Bernardo 1996; Fox and Mousseau 1996; Fox et al. 1997; 1999).

These considerations clearly demonstrate that egg size is simultaneously a maternal and a progeny character rendering egg size a particularly interesting trait in life-history evolution. Further, egg and thus progeny size exhibits a tremendous variation among species, populations within species and females within populations. Even among the progeny produced by a single female egg size may vary considerably (Reavey 1992; Mousseau and Fox 1998; Forbes 1999; Fox and Czesak 2000; Fischer et al. 2002). The high variation found is caused by a complex set of interacting proximate and evolutionary factors (including non-adaptive mechanisms e.g. due to constraints, and variation in direction and / or magnitude of selective pressures; cf. Azevedo et al. 1996; Schwarzkopf et al. 1999), but despite increasing effort such interactive effects are still only partially resolved (Azevedo et al. 1996; Bernardo 1996; Fox and Czesak 2000; Fischer et al. 2002; Olsson et al. 2002; Fischer et al. 2006). Thus, research on the evolution of reproductive characters is still a challenge and the puzzle is far from being complete (Fox and Czesak 2000).

Rationale of this thesis

Using butterflies as model organisms, this study focuses on two main themes that are assumed to strongly affect variation in offspring size and number: **maternal size** and **maternal nutrition**. Both are assumed to channel variation in offspring traits, thereby affecting maternal and offspring fitness (Azevedo et al. 1997; Fox and Czesak 2000; Czesak and Fox 2003; Roff 2002; Fischer et al. 2003; 2006; but also note Karlsson and Wiklund 1984).

Maternal size is generally assumed to be linked to a variety of female reproductive traits and is assumed to act as a morphological and an evolutionary constraint on variation in egg size, resulting from a positive covariance between both traits. Yet, the general validity of this underlying pattern is challenged by recent studies (compare chapters 5.1 and 5.2). This thesis investigates whether maternal size does indeed impose constraints on variation in and evolution of egg size (and other reproductive traits), and whether common wisdom needs to be revised:

Is there evidence for maternal size acting as a morphological constraint on egg size within butterfly species? **Chapter 5.1**

Is there evidence for maternal size acting as an evolutionary constraint on egg size thereby preventing the evolution of certain phenotypes in the butterfly *Bicyclus anynana* (Butler, 1879)? **Chapter 5.2**

Reproduction is a nutrient-limited process, triggered only if sufficient nourishment is available (Wheeler 1996). Availability of sufficient nourishment is referred to as a 'proximate constraint' here. For holometabolous butterflies the importance of adult feeding for reproductive success is still under debate, as Lepidopterans are thought to rely primarily on resources stored during the herbivorous larval stage for egg production (Telang et al. 2001; Mevi-Schütz and Erhardt 2003). Therefore, this study encompasses both a quantitative and qualitative approach to assess the importance of nourishment in the butterfly *B. anynana*:

What are the effects of food limitation during the herbivorous larval stage versus the frugivorous adult stage? Does additional crowding affect reproductive success? Chapter 6.1

What role does adult feeding play in female reproduction and what is the relative importance of different nutritional components? Chapters 6.2 and 6.3

Study organism – the butterfly *Bicyclus anynana*

This study uses butterflies as model organisms. While the first of the above questions will be addressed using various central-European butterfly species from various butterfly families (for further details see chapter 5.1), all others will be addressed using the butterfly *Bicyclus anynana* (Lepidoptera: Nymphalidae).

B. anynana is a tropical, fruit-feeding butterfly ranging from Southern Africa to Ethiopia inhabiting sub-Saharan, highly seasonal environments such as savannahs and dry forests (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen et al. 2004). Reproduction is essentially confined to the warmer wet season when oviposition plants are abundantly available, and where 2-3 generations occur. During the colder dry season reproduction ceases and butterflies do not mate before the first rains at the beginning of the next wet season (Brakefield 1997; Windig et al. 1994). Morphs are gradually replaced during seasonal transitions. Thus, both phenotypes may occur simultaneously (Brakefield and Reitsma 1991). Apart from the lack of conspicuous eyespots, dry season morphs show several differences from the wet season morphs including increased egg and body size, larger fat bodies and a reproductive diapause in order to survive the unfavourable dry season (Brakefield and Reitsma 1991).

A laboratory stock population was established at Bayreuth University, Germany, in 2003 from several hundred eggs derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from 80 gravid females caught at a single locality in Malawi. In each generation several hundred individuals are reared maintaining high levels of heterozygosity at

neutral loci (Van't Hof et al. 2005). For this study butterflies from the Bayreuth stock population were used.

2

Synopsis

2.1 Maternal body size and butterfly reproduction

Maternal body size as a morphological constraint on egg size

In butterflies, there is evidence from a broad-based interspecific comparison for a non-adaptive scaling relationship between egg size and female body size (García-Barros 1994; 2000). Scaling of organs and morphological character traits is common when animals vary in size (e.g. Schmidt-Nielsen 1984), suggesting that egg size is at least partially subject to morphological constraints imposed by female size and morphology (see also Berrigan 1991; Fox and Czesak 2000). Assuming that there is indeed a tight correlation between both traits, i.e. a causal relationship, a corresponding relationship is expected within species (Fischer et al. 2002). So far, however, studies yielded largely inconclusive results (e.g. Blau 1981; Wiklund and Karlsson 1984; Boggs 1986; Svärd and Wiklund 1988), possibly because of low sample sizes and non-standardised conditions (e.g. using field caught females). To assess the generality and biological importance of maternal size for egg size variation, I included representative species from various butterfly families in a study employing highly standardised conditions and high sample sizes (for further details compare chapter 5.1).

Despite a strong positive correlation between maternal size and egg size found at an *interspecific* level (García-Barros 1994; 2000), at an *intraspecific level* phenotypic patterns linking maternal size to egg size varied strongly across and even within butterfly species: the correlation between both traits was not significant in *Pararge aegeria*, *Lycaena tityrus* and *Pieris napi* (in one generation), significantly positive in *P. napi* (in another, the parental generation of the aforementioned stock) and *Papilio machaon*, and significantly negative in *Araschnia levana* (Fig. 1). The high variation found suggests that within species there is no general morphological constraint on egg size imposed by female size, and that the role of such constraints arising from some sort of non-adaptive baseline allometry has been previously overestimated. Rather, such variation seems to be primarily shaped by selective pressures, physiological features (such as feeding patterns) and environmental cues (Parker and Begon 1986; Bernardo 1996; Klingenberg and Spence 1997). For instance, for capital breeders, relying mostly or entirely on resources acquired during larval development for egg production, a positive correlation between maternal size and reproductive traits is expected (Marshall 1990; Boggs 1992). This relation should become less important with an increasing dependence on adult resources (income

breeding; Tammaru and Haukioja 1996). The species used in this study are neither pure capital nor income breeders, but line up along the continuum between these extremes, with *P. machaon* (eclosing with the majority of eggs mature; cf. Wiklund and Karlsson 1984) being closest to a capital breeder. As could be expected, the strongest correlation between maternal size and egg size was found here.

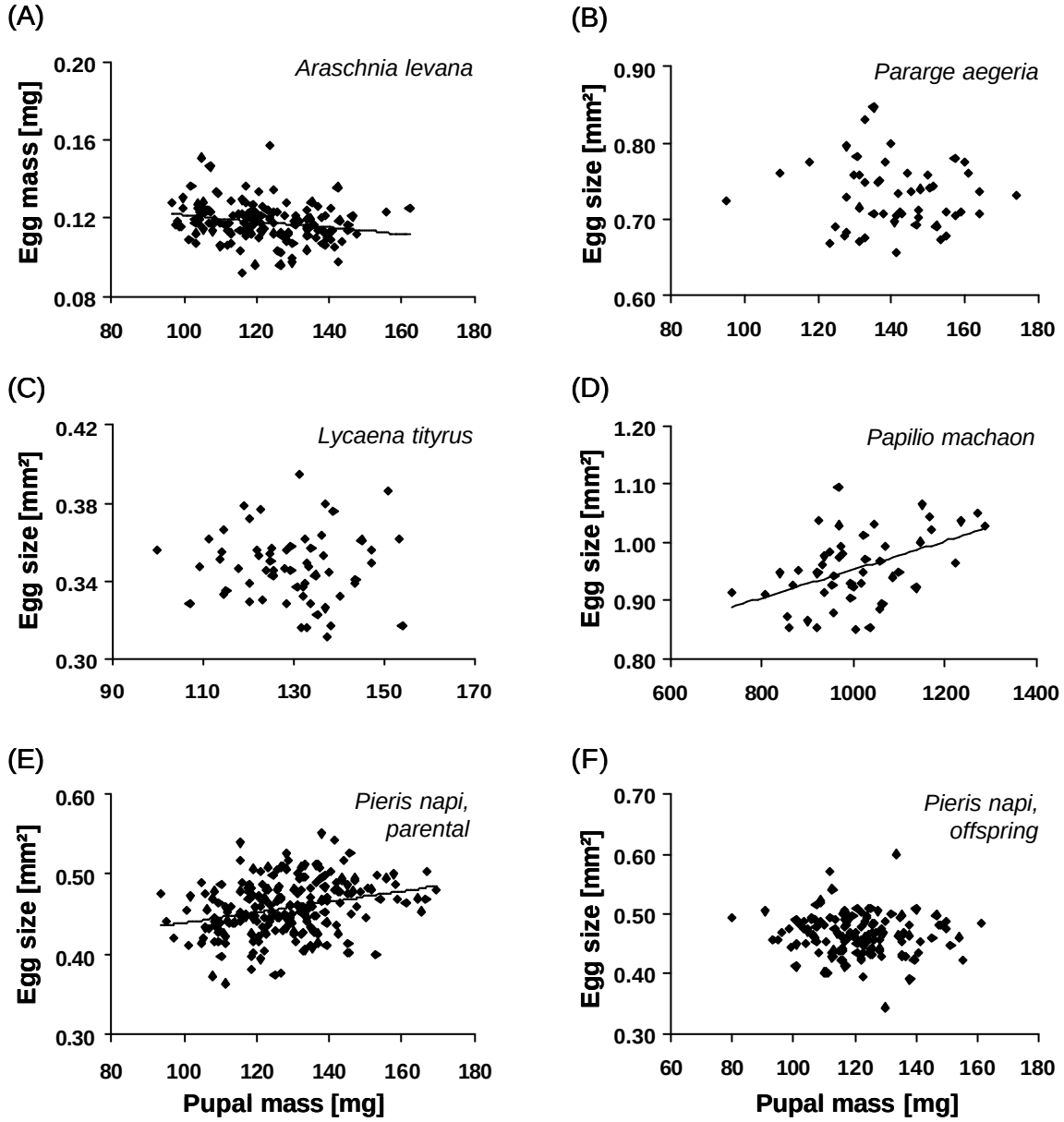

Fig. 1. Correlations between egg size and pupal mass for individual females of (A) *Araschnia levana*, (B) *Pararge aegeria*, (C) *Lycaena tityrus*, (D) *Papilio machaon*, (E) *Pieris napi* (parental generation) and (F) *Pieris napi* (offspring generation); regression lines are indicated if $p < 0.05$; for further details see chapter 5.1.

This study clearly demonstrates that one should be extremely careful when trying to predict patterns within species based upon interrelations found on an interspecific level. Intra- and interspecific allometric relationships do not need to share the same slope of correlation (Kaplan and Salthe 1979; Fischer et al. 2002). Within a population the major source of variability derives from the reproductive adaptations of individuals interacting with age structure and environmental variation, while between species it will largely result from accumulated genetic differences (Kaplan and Salthe 1979; Wickman and Karlsson 1989).

Maternal body size as an evolutionary constraint on egg size

Despite the rather limited importance of maternal body size acting as a *morphological* constraint on egg size within populations (see above; Fischer et al. 2002), maternal size may still bias or limit evolutionary changes in egg size due to a genetic correlation between both traits (i.e. a *developmental* constraint). Phenotypic correlations between body size and reproductive traits may yield only limited evidence of genetic correlations, although both approaches often tend to be similar when sample sizes are large and estimates are reliable (Rose and Charlesworth 1981; Reznick 1985). Thus, the lack of phenotypic correlation does not necessarily imply that maternal size does not bias or limit evolutionary changes in egg size. Genetic correlations have been widely documented for many morphological and life-history traits, and are generally accepted to describe developmental constraints that can bias or limit the evolutionary independence of coupled traits (Falconer and Mackay 1996; Roff 1997; Brakefield 2003).

Indeed, for the tropical butterfly *Bicyclus anynana* (Fischer et al. 2002; 2006; 2007) as well as many other insects there is evidence for a positive genetic correlation between egg size and maternal body size, which may bias or limit the independent evolution of both traits (Parsons 1964; Fox and Czesak 2000; Czesak and Fox 2003). This leads to the expectation that concerted changes of both traits should be more readily produced than ones opposing the positive genetic coupling (see Zijlstra et al. 2003; Brakefield 2003). To validate this hypothesis, a two-trait artificial selection experiment, targeted at simultaneous changes in maternal size and egg size in the butterfly *B. anynana*, was conducted (for further details compare chapter 5.2). Selection was in the same (synergistic) and the opposite (antagonistic) direction as the presumed positive genetic correlation between both traits (Fischer et

al. 2002; 2006; 2007). Although antagonistic selection experiments have been proven to be a powerful tool to unravel the constraining power of genetic covariances, they have been rarely used thus far as opposed to single-trait selection experiments (Zijlstra et al. 2003; Beldade et al. 2002a; Brakefield 2003; Frankino et al. 2005; Fuller et al. 2005; Brakefield 2006). Further, most existing examples focused on morphological traits, such that exploring more complex and integrated life history traits is still a largely unexplored task. Thus, this study considerably extends the scope of previous ones, by using antagonistic two-trait selection and focussing on life-history traits. The results of such experiments are likely to gain new insights into the genetic and developmental coupling respectively independency of life history traits in general, and thereby on the evolution of life histories.

The results of this experiment show that all target phenotypes (i.e. those in the synergistic and in the antagonistic direction) – albeit to varying extents – could be achieved even within a short evolutionary time frame (Fig. 2). Thus, there was no evidence for a strong genetic constraint, i.e. no absolute constraint in terms of 'forbidden' morphologies. Responses to selection and realised heritabilities varied across selection regimes. The most extreme phenotypes for pupal mass, accompanied by higher realised heritabilities, were found in the synergistic selection directions, while in the antagonistic direction trait values were less extreme and realised heritabilities were in parts non-significant. On the other hand, the strongest response and highest realised heritabilities in egg size were found in combination with small female size suggesting that body size may constrain variation in egg size.

Generally, realised heritabilities of egg size exceeded those of female size. This may suggest that body size is more closely related to fitness than is egg size, and / or that body size is more intensely subject to environmentally induced variation than is egg size (e.g. Teuschl et al. 2007 and references therein). The asymmetric pattern found is in agreement with earlier results from single-trait selection experiments using *B. anynana*, showing that selection on body size yields a large correlated response in egg size, while selection on egg size affects body size to a much smaller extent (Fischer et al. 2002, 2006, 2007). These results support the notion that genetic responses to divergent selection may not follow the simple paradigm of diverging frequencies of alleles at the same set of genes; rather, different loci may contribute to the responses in different selection regimes (Fuller et al. 2005). In addition, pleiotropy, linkage disequilibrium or epistatic genetic effects may be involved in

mediating these patterns (Gromko 1995; Fuller et al. 2005). Overall, results suggest, as do studies on butterfly eye-spot patterns (Beldade et al. 2002a; 2002b), that the importance of genetic correlations between traits in shaping evolutionary trajectories may have been overemphasized.

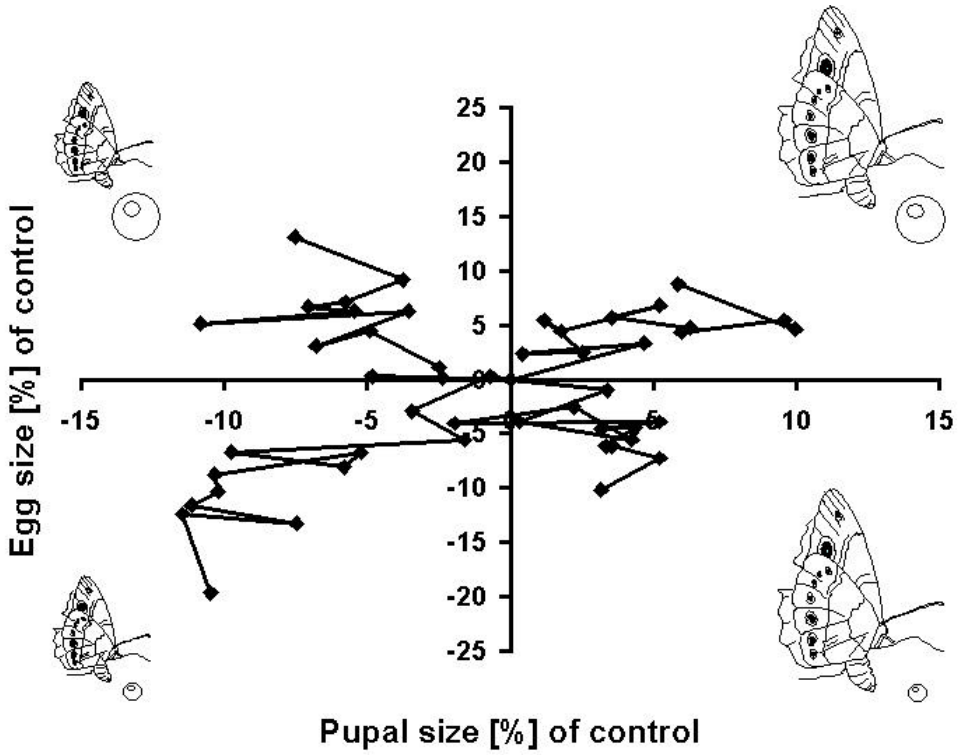

Fig. 2. Response to selection on pupal mass and egg size (for first eggs laid) relative to controls over 12 generations of artificial synergistic and antagonistic selection in *Bicyclus anynana* (pooled across replicate lines; for further detailes see chapter 5.2); all target phenotypes could be produced, yet, responses to selection differed between selection regimes. Drawings by Thorin Geister.

My results suggest the existence of a relative evolutionary constraint, as defined by a bias in the production of certain phenotypes (Beldade et al. 2002a; Brakefield 2006). Although such a bias seems to exist, there were significant responses in all directions of selection. Therefore, it does not prevent evolution towards novel phenotypes, given that enough time is available and that natural selection is strong (cf. Brakefield et al. 2006). However, lowering the speed of adaptive evolution will already impact on the success of any given phenotype, especially so in rapidly changing environments (Beldade et al. 2002b and references therein).

The applied selection regime on divergent female and egg sizes resulted in a variety of correlated responses in almost any of the life-history traits investigated including embryonic, larval and pupal development times (for further details see chapter 5.2). Surprisingly, there was hardly any evidence for positive genetic correlations between female body size and fecundity or reproductive output across selection regimes, although selection targeted at diverging maternal sizes and egg sizes, which are both assumed to be closely connected to fecundity and reproductive investment (e.g. Leather 1988; Honek 1993). Conclusions are twofold: (1) the generality of the assumed positive correlation between female body size and reproduction needs to be reconsidered (e.g. Leather 1988; Honek 1993; Tammaru et al. 1996; Tammaru et al. 2002). The lack of correlation may at least partly be due to the fact that *B. anynana* is highly dependent on adult feeding for egg production (income breeding; compare below) indicating that maternal size may be of only subordinate importance in shaping reproductive traits, while female longevity may gain in relevance (Tammaru and Haukioja 1996). (2) Large females invest relatively less into reproduction, and concomitantly allocate relatively more resources to survival and general maintenance than small females. However, this does not translate into enhanced lifespans (see Reim et al. 2006 and references therein). This is in contrast to the prediction that larger individuals should do better in terms of reproduction and survival as their accumulated energy should last longer (see Reim et al. 2006 and references therein). This again is likely related to the strong dependence of *B. anynana* on adult income (Fischer et al. 2004). Thus, in this species, being large – despite providing a higher amount of resources – will not necessarily entail benefits for the females, at least under the benign feeding conditions used in this study.

In conclusion, the importance of maternal size in shaping variation in egg size seems to be limited, both in the sense of a morphological as well as an evolutionary constraint (also Fischer et al. 2002). As body size seems to be of subordinate importance in determining egg size variation (Fischer et al. 2002; chapter 5), it remains to be solved what is causing the large intra-populational variation in egg size and other reproductive traits.

2.2 Maternal nutrition and butterfly reproduction

The subordinate role of maternal body size as a constraint on egg size strongly suggests that other factors are likely to be more important. Amongst others, these include species-specific feeding patterns (Bernardo 1996; Klingenberg and Spence 1997) that are likely to considerably affect reproductive traits. Yet note that the relative importances of maternal size and maternal nutrition in shaping reproductive traits are linked to each other. For capital breeders, relying mostly or entirely on resources acquired during larval development for egg production, a positive correlation between maternal size and reproductive traits is expected, and was repeatedly found (Boggs 1992; Tammaru and Haukioja 1996; Tammaru et al. 1996). This relation should become less important with an increasing dependence on adult resources (income breeding, Boggs 1992; Tammaru and Haukioja 1996).

The impact of food limitation and crowding on reproduction

The holometabolous life cycle of butterflies causes striking differences in larval and adult diet (relatively protein-rich larval diet versus typically carbohydrate-rich, but protein-poor adult diet), suggesting that shortages during either life stage may have different implications for reproductive resource allocation (e.g. in income versus capital breeders, Tammaru and Haukioja 1996). Food shortage during juvenile development was repeatedly found to increase development time, and to decrease growth rates, body size and reproductive output later in live (e.g. Briegel 1990; Berrigan and Charnov 1994; Blanckenhorn 1998; Fischer and Fiedler 2001a). Likewise, limited food access in the adult stage was found to diminish performance in various ways (e.g. Boggs and Ross 1993; Braby and Jones 1995; Fischer and Fiedler 2001b). Among the most obvious effects are those on reproductive output, as reproduction is a nutrient-limited process, triggered only if sufficient nourishment is available (Braby and Jones 1995; Wheeler 1996; Boggs 1997a). Studying the allocation of limited resources to reproduction is particularly interesting in holometabolous insects because diets and energetic needs change between life stages (e.g. between the herbivorous larval and the usually nectarivorous adult stage in butterflies).

Resources allocated to reproduction may be derived to varying degrees from stored reserves or current feeding, with pure capital or income breeders being exceedingly rare (e.g. Sibly and Calow 1984; Wheeler 1996; Boggs 1997a; O'Brien

et al. 2004). In insects different resource types (e.g. carbohydrates, lipids and amino acids) may also be differentially drawn from reserves and current feeding (e.g. Boggs and Ross 1993; Boggs 1997b). Given the need for resource congruence (the use of nutrient types in a specified ratio; Bazzaz 1996), storage, foraging and reproduction are linked strategies in these animals. Understanding the relative importance of stored (usually larval-derived) and current (usually adult-derived) resources for reproduction is thus a critical element to understanding the functional basis of reproductive patterns. Despite an obvious qualitative difference between larval and adult diet in butterflies, only few studies have investigated nutritional effects across metamorphic boundaries (e.g. Boggs 1997; O'Brien et al. 2002; 2003; 2004; Fischer et al. 2004).

This thesis addressed the impact of food limitations during both the larval and adult stage on female reproductive output in the tropical, fruit-feeding butterfly *B. anynana* (for further details see chapter 6.1). While focusing on dietary effects, crowding may substantially bias results that are attributed to changes in the availability of food and need to be controlled for. This is because crowding in itself may cause differences in life-history traits (e.g. due to chemical or physical cues; Burns 1995), independent of the availability of food. Therefore, this study covers effects of larval and adult crowding in concert with larval and adult food limitations in order to clearly separate between both.

Effects of crowding on reproductive traits have been reported for several insect species, with higher population densities generally resulting in a reduced reproductive output (e.g. Hawley 1985; Credland et al. 1986; Partridge et al. 1987; Fox and Czesak 2000; Agnew et al. 2002). For instance, egg size generally decreases as population densities increase, although theoretical models usually predict an increase to enhance competitive ability (Fox and Czesak 2000). Yet, whether such effects are caused by density itself (i.e. chemical or physical cues; Burns 1995), by food shortage affecting size parameters directly or by food shortage affecting some correlated traits remains largely unclear. In the butterfly *B. anynana*, high larval rearing density had, contrary to common wisdom, very little impact on body size, but reduced larval development time through increased growth rates (Berrigan and Charnov 1994; Gotthard and Nylin 1995; Arendt 1997; Blanckenhorn 1999). Thus, the pattern differs strikingly from the effects of food limitation that cause a prolonged larval development time and decreased growth rates (compare below).

This has several important implications. (1) Growth rates are not maximized under 'normal' conditions, as has been traditionally postulated, but may vary according to environmental cues (Abrams et al. 1996; Arendt 1997). (2) Accelerated growth is assumed to be an adaptation to high densities, driven by the risk of larval food resources becoming exhausted before reaching metamorphosis (Blanckenhorn 1999). While larval crowding significantly affected growth trajectories, neither larval nor adult crowding during oviposition had detectable effects on female reproduction.

In *B. anynana,* restrictions in the amount of available food exerted strong impacts on growth trajectories and reproductive traits. Larval food stress prolonged larval development time and reduced larval growth rate and body size, a result believed to be universal and is generally predicted by life-history models (Berrigan and Charnov 1994; Blanckenhorn 1999). Despite a prolonged larval development time larvae could not fully compensate for the applied starvation regime (starvation for 24h in the last larval instar; for further details see chapter 6.1) suggesting that development time and body size are generally important fitness parameters (Yampolski and Scheiner 1996). If only one of these traits were of prime importance, either a full compensation in body size (by further increasing larval time) or no change in larval time at all (at the expense of an even smaller body size) would be expected.

Larval food limitation had negative effects on fecundity and reproductive investment (mediated through a reduction in body size). Additional negative effects of adult food stress on fecundity were largely confined to females being fed as larvae *ad libitum*, while those being previously starved showed reduced performance regardless of adult income (Fig. 3). Thus, butterflies cannot take advantage of abundant adult resources when storage reserves are reduced, suggesting that the use of adult resources is limited by the availability of nutrients accumulated during the larval stage (likely representing nitrogenous compounds). Yet note that when abundantly fed during the larval stage a limitation of adult resources reduced reproductive output, proving the need for adult feeding in *B. anynana* for egg production (income breeding; Fischer et al. 2004). Thus, for *B. anynana* it is not possible to state that either larval or adult resources are ultimately limiting to reproduction, as they are both: adult income is essential for maturing eggs (Fischer et al. 2004), but adult intake cannot compensate for reduced storage reserves.

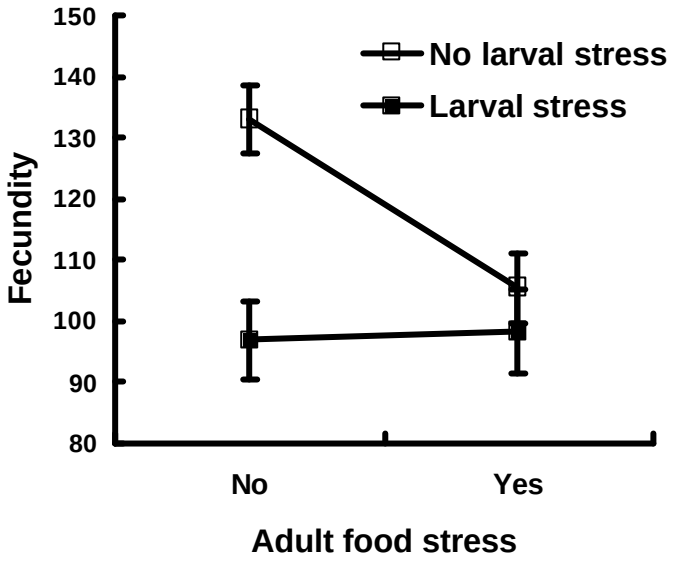

Fig. 3. Effects of adult and larval food stress on fecundity (means ± 1 SE) in *Bicyclus anynana;* adult food stress reduced fecundity only when females were fed as larvae *ad libitum*, while those being previously starved showed reduced performance regardless of adult income.

Therefore, this study clearly demonstrates that restricted food access in different developmental stages sets qualitatively different limits to reproduction, either posed by shortage of larval-derived storage reserves (i.e. nitrogenous compounds) or adult income (i.e. carbohydrates). Consequently, restrictions in both, larval- and adult-derived resources, may limit reproduction in holometabolous insects (see also Boggs and Ross 1993). Similar situations are likely to apply to many arthropods, with, if anything, resource congruence being ultimately limiting. To fully understand the functional basis of reproductive patterns more studies elucidating the relative importance of stored and current resources are needed.

The importance of food quality for reproduction

Apart from differences in food quantity, dietary quality is of profound importance for all life-history traits, as both incoming adult nutrients ('income') and stored larval nutrients ('capital reserves') contribute to reproduction, survival and dispersal in the adult stage (Boggs and Freeman 2005). It was generally believed that butterflies and other holometabolous insects rely primarily on reserves accumulated during the larval stage for reproduction (Leather 1995; Telang et al. 2001; Mevi-Schütz and Erhardt 2003). Recent studies, however, highlight the often fundamental importance

of adult nutrition to realize the full reproductive potential (e.g. Gilbert 1972; Boggs and Jackson 1991; Braby and Jones 1995; Smedley and Eisner 1996; Rusterholz and Erhardt 2000). The general picture simplifies the importance of larval and adult dietary quality to a matter of larval-derived nitrogenous compounds versus adult-derived carbohydrates. While the often crucial importance of adult-derived carbohydrates for female butterfly reproduction has been reported for various butterfly species and is fairly well understood (e.g. Murphy et al. 1983; Leather 1984; Wei et al. 1998; O'Brien et al. 2000; Fischer et al. 2004; O'Brien et al. 2004; Bauerfeind and Fischer 2005), the role of most other adult-derived substances is still controversial, only partially resolved or largely unknown (e.g. amino acids: Dunlap-Pianka et al. 1977; Murphy et al. 1983; Moore and Singer 1987; Hill and Pierce 1989; O'Brien et al. 2000; minerals: Boggs and Jackson 1991; Smedley and Eisner 1995; Beck et al. 1999). Our restricted knowledge is particularly evident in species that feed on fruits that are characterised by a complex nutritional composition (cf. University of Hohenheim 1996). Given these shortcomings, the relative importance of different nutritional components in the adult diet for female reproductive traits was investigated in the fruit-feeding butterfly *B. anynana* (for further details see chapters 6.2 and 6.3).

My results confirm that carbohydrates are the most important nutrients affecting reproduction in *B. anynana* and are essential for egg production. However, other substances – apart from carbohydrates – appear to play a decisive role as *B. anynana* females fed on fruit (banana) showed a significantly higher reproductive output than females fed on a pure sucrose solution (Fig. 4).

Among these, proteins / amino acids and lipids are likely to be of key importance for insect reproduction, as they are major constituents of the oocyte dry mass (Engelmann 1999; Ziegler and Van Antwerpen 2006). Amino acids are generally scarce in the sugar-rich adult diet of butterflies (O'Brien et al. 2002). Nonessential amino acids have been shown to be extensively synthesised by females using adult-derived carbon and endogenous nitrogen-sources (O'Brien et al. 2002), whereas essential amino acids are entirely larval in origin and therefore likely ultimately limiting to reproduction. Hence, the role of amino acids seems to be particularly relevant for the understanding of nutritional constraints on insect reproduction (O'Brien et al. 2002). Lipids serve various functions including their role as the main energy source for the developing embryo (Stanley-Samuelson and Loher 1983; Stanley-Samuelson et al. 1988; Kawooya and Law 1988; Svoboda 1999). As most

insects are neither able to synthesise long-chain polyunsaturated fatty acids (but Blomquist et al. 1982; Beenakkers et al. 1985) nor the tetracyclic steroid nucleus required for the synthesis of sterols (Behmer and Nes 2003) *de novo*, they depend on exogenous sources for successful development and reproduction (Al-Izzi and Hopkins 1982; Beenakkers et al. 1985; Turunen 1990; Behmer and Grebenok 1998; Svoboda 1999; Mondy and Corio-Costet 2000). As in case of essential amino acids, lipid requirements are thought to be mainly covered by larval-derived storage depots in the fat body (Behmer and Grebenok 1998; Arrese et al. 2001). Fruit-feeding butterflies, though, may have access to noticeable amounts of adult-derived amino acids and lipids, whose importance, in contrast to larval-derived ones, is currently only little understood or completely unknown, respectively. Further, adult-derived micronutrients such as minerals and vitamins appear to affect egg production in at least some insect species (e.g. Pappas and Fraenkel 1977; Engelmann 1999), however, studies on the importance of adult-derived micronutrients for somatic maintenance and reproductive output are still scarce (e.g. Boggs and Jackson 1991; Smedley and Eisner 1996).

Contrary to initial expectations, I could not pinpoint a single pivotal substance (in addition to sucrose) that was able to elicit a comparably high reproductive performance as did banana, although I tested all major substances known to be involved in insect egg production (amino acids, cholesterol, polyunsaturated fatty acids) and the micronutrients being most abundantly available in banana in the feeding experiments (cf. University of Hohenheim; minerals: potassium and magnesium chloride; a mixture of vitamins and a combination of both; Fig. 4; see chapters 6.2 and 6.3 for more details).

As it could be largely ruled out that amino acids, lipids or micro-nutrients such as minerals or vitamins play a decisive role, processes associated with the decay of fruit rather than the composition of the fruit itself may account for the increased reproductive output found in banana-fed females. Fruits support the growth of microorganisms and fungi (associated with the production of fermentative products like organic acids and alcohols; Morais et al. 1995; Molleman et al. 2005), providing access to additional resources. Among these yeast is of special concern, providing protein and other substances to insect frugivores (Rattray et al. 1975; Morais et al. 1995; Brown et al. 1996; Good and Tatar 2001), and has been shown to dramatically

increase egg production in various insect species (Chippindale et al. 1993; McEwen and Kidd 1995; Simmons and Bradley 1997; Good and Tatar 2001).

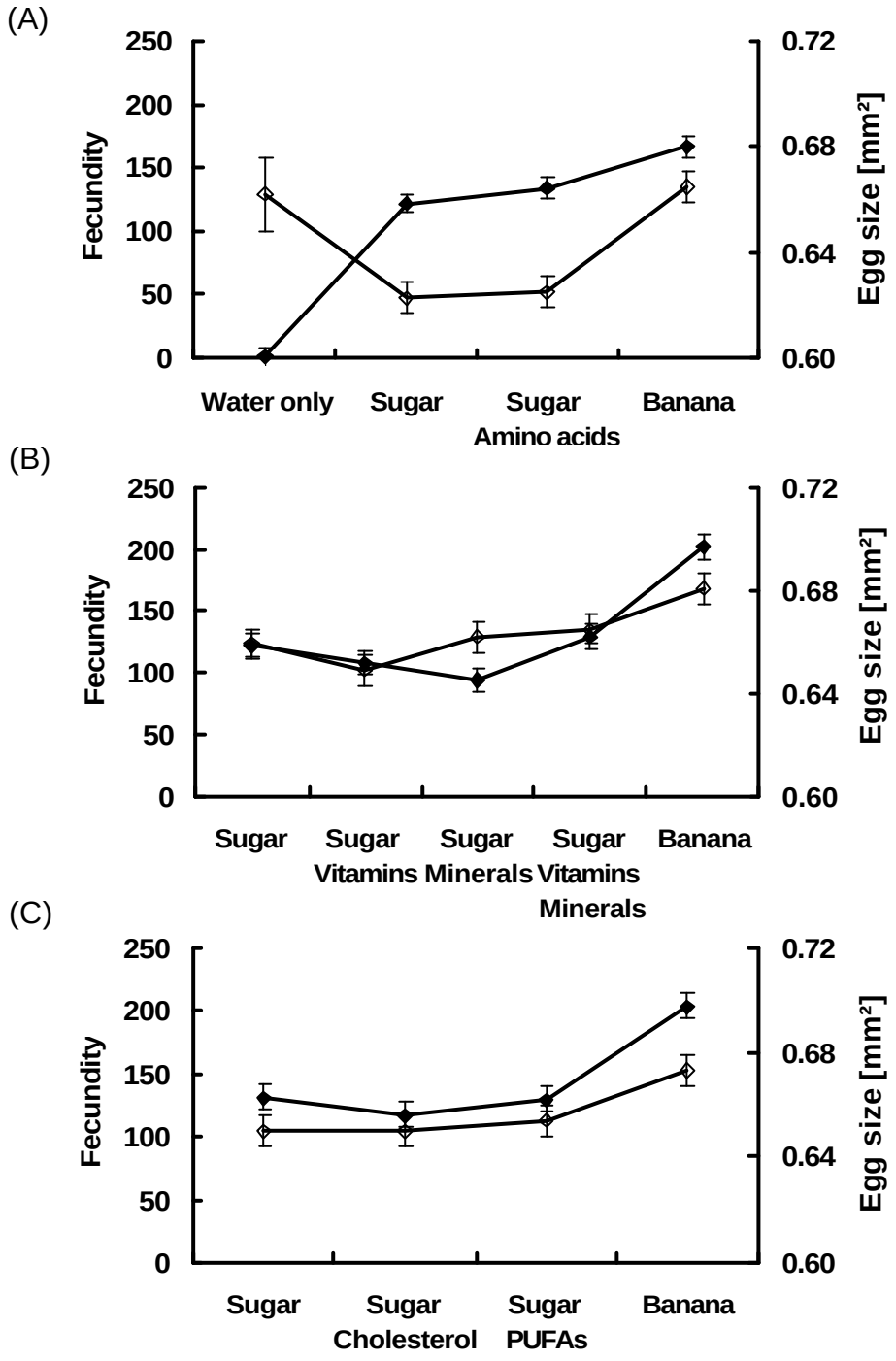

Fig. 4. Effects of different adult diets on lifetime fecundity (filled symbols) and mean egg size (open symbols; mean with SE in parenthesis) in *Bicyclus anynana*; without carbohydrates hardly any eggs are produced; none of the tested admixtures to a sucrose solution accounts for the increased reproductive output of banana fed females as compared to females fed on a pure sugar solution. Note that sugar concentrations vary: (A) 40V%, (B) 25V%, (C) 40V%; for further details compare chapters 6.2 and 6.3; PUFAs: polyunsaturated fatty acids.

However, two lines of evidence suggest that the increased reproductive output in banana-fed females is not associated with the decay of fruit (Fig. 5). (1) Performance was independent of the stage of fruit decay, as females feeding on fresh or decaying banana were statistically indistinguishable. (2) Neither supplementation of a sucrose solution with yeast (mimicking fermentative products of proven value to insect frugivores) nor ethanol (as a likely volatile attractant and as a energy source; Bokor and Pecsenye 2000; Heberlein et al. 2004) enhanced reproductive output as compared to a pure sugar solution.

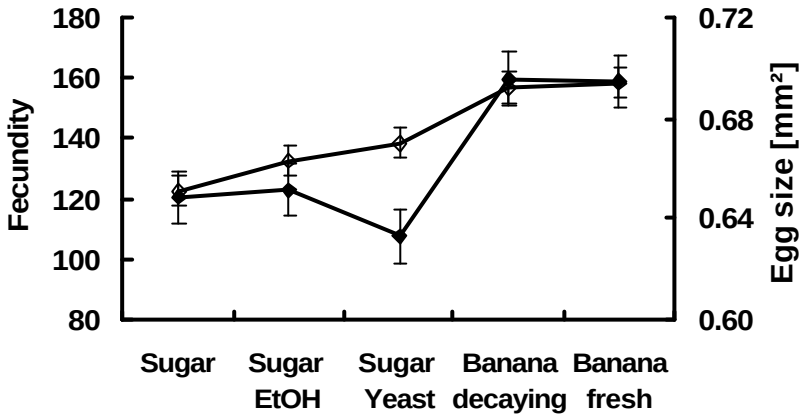

Fig. 5. Effects of different adult diets on lifetime fecundity (filled symbols) and mean egg size (open symbols; mean with SE in parenthesis) in *Bicyclus anynana*; neither ethanol (2V%) nor yeast added to a sucrose solution accounts for the increased reproductive output of banana fed females; performance of banana-fed females was independent of the stage of fruit decay. Sugar concentration was 20V%; for further details compare chapter 6.3.

The exceptionally high quality of banana (as compared to sucrose-based diets used) is further supported by the fact that the trade-off between egg size and number (Smith and Fretwell 1974) seems to be less pronounced in banana-fed females, suggesting that its expression depends on dietary quality (also compare chapter 6.2).

It seems highly unlikely that the lack of response to the provided admixtures is caused by differences in intake rates based on an intrinsic preference for bananas (due to e.g. odour, see e.g. Honda et al. 1998; Andersson 2003) as all butterflies were facing no-choice-situations. Likewise, considering the variety of substances tested, it is unlikely that other, not yet identified compounds may be responsible for the high advantage of feeding on banana. Therefore, reproduction presumably does not only depend on a small number of adult-derived nutrients (such as e.g. carbohydrates, where the crucial role is obvious), but on a larger number having

relatively small effects each. Thus, resource congruence (the use of nutrient types in a specified ratio; Bazzaz 1996; see also O'Brien et al. 2004; Fischer et al. 2004) rather than any specific component may be the key to answer the question.

In addition, the importance of adult dietary compounds, such as amino acids and lipids, may depend on the nutritional status of the butterfly. When given *ad libitum* access to larval food, protein and lipid resources accumulated during the larval stage seem to be already sufficient to realize the full reproductive potential in this butterfly. Thus, the need for adult-derived amino acids and lipids may become more apparent when access to larval food is restricted, which has recently been confirmed for adult-derived amino acids (see Jervis and Boggs 2005; Mevi-Schütz and Erhardt 2005). Still, this does not explain the greatly enhanced reproductive output in banana-fed females, the issue being addressed here.

Overall, the large advantage of feeding banana compared to sugar solutions seems extraordinary, as is the high dependence of reproduction on adult income in *B. anynana* (Fischer et al. 2004). To find out whether *B. anynana* comprises an exception with regard to reproductive resource allocation, whether such patterns are more wide-spread among insects or are maybe related to a fruit-feeding life style, remains a task for the future.

3

Summary (English and German)

3.1 Summary

Arthropod egg and thus progeny size is an evolutionary and ecologically significant trait, showing tremendous variation within and across species. The high variation found is caused by a complex set of interacting proximate and evolutionary factors, but despite increasing effort this network is still only partially resolved. Using butterflies as model organisms, this study focuses on two main factors that are assumed to strongly shape variation in offspring size and number: maternal size and maternal nutrition. Both are assumed to affect reproductive traits and thereby maternal and offspring fitness.

Phenotypic correlations between maternal size and egg size within various butterfly species (chapter 5.1) as well as a two-trait selection experiment on simultaneous changes in both traits in the butterfly *Bicyclus anynana* (chapter 5.2) clearly demonstrated that the importance of maternal size in shaping variation in egg size is limited, both in sense of a morphological as well as an evolutionary constraint. These results strongly contrast to the general assumption of a positive scaling relationship between both traits. This is one of the few studies addressing the issue of evolutionary constraints directly, employing artificial two-trait selection which has been proven to be a powerful tool to unravel genetic variances and covariances that underlie the evolution of traits.

While the importance of maternal size in shaping variation in egg size is limited, proximate factors including larval and adult crowding as well as the quantity and quality of available food during the larval and adult stage affect variation in reproductive traits to a high degree.

In the butterfly *B. anynana*, larval and adult densities (chapter 6.1) had surprisingly little effects on female reproduction, whereas dietary limitations yielded strong responses in female reproductive output. Larval food stress reduced fecundity and reproductive investment (mediated through a reduction in body size), but effects on egg size were overall marginal. Additional negative effects of adult food stress on fecundity were largely confined to females being fed as larvae *ad libitum*, while those being previously starved showed reduced performance regardless of adult income. When abundantly fed during the larval stage, a limitation of adult resources reduced reproductive output, proving the need for adult feeding in *B. anynana* for egg production. Thus, restricted food access in different developmental stages of *B.*

anynana (chapter 6.1) sets different limits to reproduction, either posed by shortage of larval-derived storage reserves (i.e. nitrogenous compounds) or adult income (i.e. carbohydrates). Consequently, restrictions in both, larval- and adult-derived resources, limit reproduction in *B. anynana*.

Further, this study deals with questions regarding effects of different adult dietary compounds for a fruit-feeding butterfly being novel in its reductionist approach and in the breadth of different nutrient classes considered (chapters 6.2 and 6.3). This study demonstrates that *B. anynana* relies to a large extent on adult feeding in order to realise full reproductive output. Female *B. anynana* require adult-derived carbohydrates for egg production and exhibit a tremendous gain in reproductive output when fed on fruit as compared to sucrose solutions. Contrary to initial expectations, I could not pinpoint a single pivotal substance (in addition to sucrose) that was able to elicit a comparably high reproductive performance as banana, although I tested the micronutrients being most abundantly available in banana (minerals: potassium and magnesium chloride; a mixture of vitamins and a combination of both) and all major substances known to be involved in insect egg production (amino acids, cholesterol, polyunsaturated fatty acids). Further, it is also excluded that the growth of microorganisms and fungi (associated with the production of fermentative products like organic acids and alcohols, thereby providing access to additional resources) explains the found results. In conclusion, reproduction does not only depend on a small number of adult-derived nutrients, but on a larger number having relatively small effects each. Thus, resource congruence (the use of nutrient types in a specified ratio) rather than any specific component may be the key to answer the question.

3.2 Zusammenfassung

In Arthropoden ist die Eigröße und damit die Größe der Nachkommen ein evolutionär und ökologisch wichtiges Merkmal, das enorme Variation zwischen und innerhalb von Arten zeigt. Diese hohe Variabilität wird durch ein komplexes Geflecht interagierender proximater und evolutionärer Faktoren bestimmt, das trotz zunehmender wissenschaftlicher Bemühungen noch immer nur teilweise verstanden ist. Die vorliegende Studie, in der Schmetterlinge als Modellorganismen verwendet werden, setzt den Fokus auf zwei Faktoren, die in einem starken Ausmaß Größe und Anzahl der Nachkommen bestimmen: die Körpergröße der Mutter und ihre Ernährung. Beide Faktoren beeinflussen Fortpflanzungsmerkmale und infolgedessen die Fitness der Mutter und ihrer Nachkommen.

Sowohl phänotypische Korrelationen zwischen der Größe der Mutter und der Größe ihrer Eier innerhalb verschiedener Tagfalter-Arten (Kapitel 5.1) als auch ein antagonistisches Zwei-Merkmal-Selektionsexperiment an der Art *Bicyclus anynana* (Kapitel 5.2) zeigten, dass der Einfluss der maternalen Größe auf die Variation der Eigröße nur von untergeordneter Bedeutung ist. Dies trifft sowohl auf ihre Bedeutung als morphologischer Zwang als auch auf ihren Einfluss als evolutionärer Zwang zu. Diese Ergebnisse stehen im Widerspruch zu der allgemein vertretenen Auffassung, dass die Körpergröße der Mutter und die Größe ihrer Eier, basierend auf einer allometrischen Beziehung und einer genetischen Kovarianz, positiv miteinander korrelieren.

Die vorliegende Studie ist eine der wenigen Untersuchungen, die das Thema evolutionärer Zwang in einem Selektionsexperiment experimentell angeht. Künstliche Selektion ist eines der wenigen geeigneten Mittel, um genetische Varianzen und Kovarianzen zu erfassen, die der Evolution von Merkmalen zugrunde liegen.

Während die maternale Körpergröße für die Variation der Eigröße nur von untergeordneter Bedeutung ist, beeinflussen Umweltfaktoren wie larvale und imaginale Individuendichten ebenso wie die Quantität und Qualität der verfügbaren Nahrung während beider Lebensabschnitte die Größe der Eier und andere reproduktive Merkmale in einem starken Ausmaß.

Beim Schmetterling *B. anynana* hatten erhöhte Individuendichten weder während der Larvalphase noch während der Imaginalphase einen erkennbaren Einfluss auf die weibliche Fortpflanzung, wohingegen Hungerphasen (in beiden Lebensabschnitten) sich sehr stark auf die Reproduktionsleistung der weiblichen

Falter auswirkten. Hungerphasen während der larvalen Entwicklung verminderten die Fekundität und Fortpflanzungsleistung der Weibchen (mittelbar durch eine Reduktion der Körpergröße), wohingegen nur geringe Effekte auf die Eigröße gefunden wurden. Zusätzliche negative Effekte von Hungerphasen während des Imaginalstadiums wurden nur bei Weibchen gefunden, die während der Larvalphase unlimitierten Zugang zu Nahrung hatten, während Weibchen, die bereits während der larvalen Entwicklung Hungerstress ausgesetzt waren, eine verminderte Fortpflanzungsleistung unabhängig von der Imaginalernährung zeigten. Hungerphasen während des Imaginalstadiums (ohne vorangegangene Hungerphasen während der larvalen Entwicklung) verminderten die Fortpflanzungsleistung, was die Bedeutung der Imaginalernährung für die Ei-Produktion in *B. anynana* deutlich hervorhebt. Somit steckt eine Limitierung der verfügbaren Nahrungsmenge in den verschiedenen Entwicklungsstadien (Kapitel 6.1) der Fortpflanzungsleistung qualitativ sehr verschiedenartige Grenzen, die entweder durch eine Limitierung der larvalen Ressourcen (d.h. stickstoffhaltiger Verbindungen) oder durch eine Limitierung der imaginalen Ressourcen (d.h. Kohlenhydrate) gesetzt werden.

Neben der Menge der verfügbaren Nahrung spielt auch deren Qualität eine bedeutende Rolle für alle Merkmale der Lebensgeschichte. Von besonderer Bedeutung sind hier der reduktionistische Ansatz der Experimente und die enorme Breite der unterschiedlichen Substanzklassen, die hier behandelt wurden (Kapitel 6.2 und 6.3). In Bezug auf die Ernährung der Imagines zeigt die vorliegende Studie, dass die Fortpflanzungsleistung von *B. anynana* zu einem sehr hohen Anteil von der Verfügbarkeit adäquater Nahrung während der Imaginalphase abhängt, um das volle reproduktive Potential ausschöpfen zu können. Weibliche *B. anynana* benötigen essentiell Kohlenhydrate, um Eier produzieren zu können. Zudem zeigen sie eine enorme Steigerung ihrer Reproduktionsleistung bei Fütterung mit Früchten (Banane) im Vergleich zu Zuckerlösungen. Es konnte jedoch keine Schlüsselsubstanz oder Substanzklasse identifiziert werden, die als Zusatz zu einer Zuckerlösung eine vergleichbar hohe Reproduktionsleistung wie Banane hervorruft. Dies widerspricht der Erwartung, da neben den Mikronährstoffen, die in der Banane relativ am meisten vorkommen (Mineralien: Kalium- und Magnesium; eine Vitaminmischung und eine Kombination aus beiden) auch alle Substanzen untersucht wurden, von denen bekannt ist, dass sie bei der Ei-Produktion von Insekten eine herausragende Rolle spielen (Aminosäuren, Cholesterol, mehrfach ungesättigte Fettsäuren). Zudem

konnte ausgeschlossen werden, dass das Wachstum von Mikroorganismen und Pilzen (während des Zerfallsprozesses der Früchte), das mit der Produktion von Gärungsprodukten wie organischen Säuren und Alkoholen verbunden ist, für die Befunde ausschlaggebend ist. Es wird gefolgert, dass die Fortpflanzungsleistung nicht nur von einigen wenigen wichtigen Nährstoffen abhängt, sondern vielmehr von einer größeren Anzahl, die jeweils relativ geringe Auswirkungen haben und erst in ihrer kumulativen Wirkung Bedeutung erlangen. Folglich sind die Verfügbarkeit einer Vielzahl an Nährstoffen und ihre Mengenverhältnisse zueinander der Schlüssel zu einem besseren Verständnis der Bedeutung der Imaginalernährung für die Fortpflanzung von Insekten.

4

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5

Maternal body size and butterfly reproduction

5.1 Maternal body size as a morphological constraint on egg size and fecundity in butterflies

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Abstract

It is a widespread notion that in arthropods female reproductive output is strongly affected by female size. In butterflies egg size scales positively with female size across species, suggesting a constraint imposed by maternal size. However, in intraspecific comparisons body size often explains only a minor part of the variation in progeny size. We here include representatives of various butterfly families to test the generality of this phenomenon across butterflies. Phenotypic correlations between egg and maternal body size were inconsistent across species: correlations were non-significant for *Pararge aegeria* and *Lycaena tityrus*, significantly positive for *Papilio machaon*, significantly negative for *Araschnia levana*, and contradictory for *Pieris napi*. Thus, there was no general pattern linking egg size to maternal size, e.g. caused by an allometric relationship. Consequently, there was at best limited evidence for maternal size acting as a morphological constraint on egg size within butterfly species. Realized fecundity depended on maternal size in *P. napi* and *A. levana*, but not in *P. aegeria*, suggesting that maternal size may affect egg number more strongly than egg size. Yet, variation in fecundity was primarily explained by variation in longevity as is expected for income breeders. Heritability estimates across species were rather similar for pupal mass (ranging between 0.14-0.19), but more variable for egg size (0.17-0.31).

Introduction

Arthropod egg and offspring size often varies substantially across species, populations within species, females within populations, and among the progeny produced by a single female (Fox & Czesak 2000; Fischer et al. 2002). Although egg size is an evolutionary and ecologically significant trait, there is still a rather poor understanding of the complex set of proximate and ultimate factors determining the high variation found (Azevedo et al. 1996; Bernardo 1996; Fox and Czesak 2000; Fischer et al. 2002). One factor that is thought to often exert strong effects on egg size is maternal size (e.g. Wickman and Karlsson 1989; Leather 1988; Bernardo 1996). In butterflies, for instance, there is evidence from a broad-based interspecific comparison for a presumably non-adaptive scaling relationship between egg size and female size resulting from an allometric scaling of organs, which is commonly found (Wiklund and Karlsson 1984; Wiklund et al. 1987; García-Barros 1994; 2002). This suggests that egg size may at least partially be subject to constraints imposed by

maternal size (Berrigan 1991; Fox and Czesak 2000; Fischer et al. 2002). If this correlation was causal in nature (e.g. arising from an allometric relationship due to internal constraints of some common developmental mechanism or a morphological constraint, e.g. oviduct size), a corresponding relationship is expected at the intra-specific level (Fischer et al. 2002).

Yet, patterns within species are much less clear than those across species. All sorts of correlations between egg and maternal size (positive, negative, and non-significant) have been described in the literature (e.g. Wiklund and Karlsson 1984; Boggs 1986; Svärd and Wiklund 1988; Bernardo 1996; Oberhauser 1997, Fox and Czesak 2000; Fischer et al. 2002).

The reasons for this broad variation, which is in apparent contradiction to the notion of a morphological constraint, are not understood. One possible factor involved in provoking such variation might be adaptive evolution, which may alter the expression of the presumed baseline allometric relationship between maternal size and egg size. Yet, the reported variation may also result from experimental deficiencies as many of the previous results are based on data derived from non-standardised conditions (e.g. using field-caught females) and/or small sample sizes. Consequently, weak or non-significant correlations may be due to various causes including limited statistical power, ageing of females, and differing environmental conditions (Fischer et al. 2002). Only few studies are based on an extensive data set obtained under highly standardised conditions (e.g. Fischer et al. 2002). To settle this issue there is a need for a more intensive exploration of egg to body size allometries in different species under identical settings. This will allow for more rigorous tests of the role of maternal size for variation in egg size, and whether the lack of pattern is wide-spread or caused by unaccounted environmental variation or just by chance.

We here follow this route by investigating representative species from various butterfly families in order to test whether the presumed positive scaling relationship between maternal size and egg size is indeed a general phenomenon across species, and whether this is likely to constrain adaptive evolution. Butterflies were reared under highly standardised conditions in high numbers to obtain reliable phenotypic correlations between maternal size and egg size. Closely connected to variation and covariation in maternal and egg size is fecundity, as (1) fecundity has been found to be strongly correlated to female size (e.g. Honek 1993; Tammaru et al. 1996; Tammaru et al. 2002) and (2) fecundity and egg size are assumed to be

competing for limited maternal resources resulting in a phenotypic trade-off between both traits (e.g. Smith and Fretwell 1974). Therefore, we also investigated egg numbers (and longevity) in some of the species studied. Finally, we will present some heritability estimates for egg and body size.

Material and methods

The following species were selected for this study based on accessibility and amenability to laboratory studies (e.g. Wiklund and Kaitala 1995; Van Dyck and Wiklund 2002): Pieridae: *Pieris napi* (33 females); Lycaenidae: *Lycaena tityrus* (16); Nymphalidae (Nymphalinae): *Araschnia levana* (14); Nymphalidae (Satyrinae): *Pararge aegeria* (10); Papilionidae: *Papilio machaon*. Females of the respective species, except for *P. machaon* (see below), were caught in the vicinity of Bayreuth (Germany, numbers are given in parentheses above) and afterwards transferred to climate chambers at the University of Bayreuth.

Butterflies were reared under controlled environmental conditions (27 °C, 70% humidity, L:D 18:6). Field-caught females were placed individually in translucent plastic pots (1 L) containing leaves of suitable oviposition plants (see Table 1), nectar plants (as found in the field), highly concentrated sucrose solution and water. Nectar plants and sugar solutions were replaced every other day, oviposition plants when necessary. Eggs were collected and transferred (separated by female) to elongated, sleeve-like gauze cages containing suitable larval host plants (Table 1). Larvae were reared in family groups in these cages until pupation, with host plants being replaced when necessary. Resulting pupae were collected daily and weighed two days after pupation (Sartorius microscale MC 210 P). Pupal mass is a reliable proxy for adult body size in butterflies (see e.g. Wiklund and Karlsson 1984; Bauerfeind and Fischer 2005a). Afterwards, pupae were placed individually in translucent plastic pots (125 ml) until adult eclosion.

Following eclosion, females were individually marked and set up for mating with random males. Mating cages were observed continuously, and sexes were separated at the end of each day to avoid unnoticed copulations. Mating couples were removed from the cages and allowed to end copulation in isolated translucent plastic pots (1 L). For females failing to mate on the eclosion day the procedure was repeated the next two days. Only virgin males and females from different families were allowed to mate to avoid sib matings.

For logistic reasons, *P. machaon* was purchased as diapausing pupae (offspring of field-caught females) from a commercial supplier. Pupae were weighed upon arrival (Sartorius microscale MC 210 P), then placed individually in translucent plastic pots (1 L) and transferred to a climate chamber (25 °C, 70% humidity, L:D 18:6) until eclosion. All females were individually marked and hand-paired with randomly selected males; some males had to be used twice to ensure fertilization of all females. Mated females were kept individually in cylindrical hanging cages for oviposition (diameter 30 cm, height 38 cm).

For oviposition, laboratory-raised F1 females of all species were treated as described above for the parental, field-caught females (Table 1). Eggs for size measurements were collected starting on the day after copulation. For all species collection continued until at least ten eggs had been sampled (some butterflies laid up to 60 eggs on their first day of oviposition). All correlations between maternal and egg size are based on the very first eggs deposited, thus effectively controlling for confounding effects of female age (e.g. Karlsson 1987 and references therein). Per female, the sizes of ten eggs were measured and their mean used for further analyses.

Table 1. Larval host and oviposition plants used in this study.

Species	Larval host	Oviposition
Araschnia levana	*Urtica dioica*	*Urtica dioica*
Lycaena tityrus	*Rumex acetosa*	*Rumex acetosa*
Papilio machaon	-	*Anethum graveolens,* *Daucus carota ssp. sativus,* *Petroselinum crispum*
Pararge aegeria	*Lolium perenne*	*Lolium perenne*
Pieris napi	*Brassica napus ssp. oleifera,* *Alliaria petiolata*	*Alliaria petiolata*

Egg size was measured as cross-sectional projections [mm^2] using a digital camera (Leica DC300) connected to a stereo microscope. The resulting images were

analyzed using Scion Image public software (Scion Corporation 2000). This method has been found to provide a highly reliable measurement of egg size in all species investigated, as confirmed by tight correlations between egg area (applying image analysis) and egg mass (determined to the nearest 0.001 mg using Supermicroscale Sartorius 4504 MP8; results not shown). All species used deposit eggs singly, except for *A. levana*, which deposits eggs in turret-shaped clutches. In this species we weighed egg turrets (Sartorius microscale MC 210 P) and divided their mass by the number of eggs, thus obtaining mean egg masses of individual turrets.

Additional data on longevity and lifetime fecundity were acquired for *P. aegeria*, *P. napi* and *A. levana*, whose eggs were collected every other day until the death of the females. For *P. napi* two consecutive generations were reared and the heritability for egg and pupal size was estimated using parent-offspring regression and full-sib analyses. For *P. aegeria* and *A. levana*, exclusively full-sib families were used.

Data analysis

For all species phenotypic correlations between maternal body size, egg size and lifetime fecundity were analysed using Pearson's product moment correlation. If available, data were controlled for effects of common ancestry of sisters within families by using family means, weighted by the number of individuals per family, as the level of replication. This approach avoids patterns found to be biased by peculiarities restricted to certain families. Otherwise, phenotypic correlations were assessed using data of individual females. The slopes of the regression lines (correlation of egg size and maternal size) of both generations of *P. napi* were compared using an analysis of (co-)variance (ANCOVA) including generation as random factor and egg size as covariate. Analyses of variance (ANOVAs) were used to analyse the effects of family (random factor) and sex on pupal size and egg size in *P. napi* (parent and offspring generation), *A. levana* and *P. aegeria*. Heritabilities of these traits were estimated by variance component analyses (REML, restricted error maximum likelihood). In *P. napi* additionally narrow-sense heritabilities of body and egg size were calculated by parent-offspring regressions (by regressing the mean body or egg size of full-sib sisters, weighted by the number of females in each full-sib family, on the body or egg size of the mother). Effects of pupal mass, longevity and egg size on lifetime fecundity were investigated using multiple regressions (stepwise forward addition of variables, Ridge regression) in *A. levana*, *P. aegeria* and *P. napi*

(parental generation. All statistical tests were performed using Statistica 6.1 (StatSoft 2003) or JMP version 4.02 (SAS Institute 2000). Throughout the text all means are given ± 1 SE.

Results

Phenotypic correlations between maternal size and egg size

Body and egg size varied substantially within all species investigated (Fig. 1). There was no general pattern linking egg size to maternal size within species: correlations were non-significant for *P. aegeria* (r = -0.07, n = 51, p = 0.637), *L. tityrus* (r = 0.05, n = 60, p = 0.694), and *P. napi* (in the offspring generation: r = -0.08, n = 139, p = 0.375), significantly positive for *P. napi* (in the parental generation: r = 0.30, n = 216, p < 0.001) and *P. machaon* (r = 0.47, n = 48, p < 0.001), and significantly negative for *A. levana* (r = -0.22, n = 143, p = 0.008; Fig. 1). These results were not seriously affected by common ancestry of sisters within families: correlations using family means gave qualitatively similar results (available for *P. aegeria*: r = -0.14, n_{fam} = 10, n_{ind} = 51, p = 0.339; *A. levana*: r = -0.32, n_{fam} = 9, n_{ind} = 143, p < 0.001; *P. napi*, parental generation: r = 0.25, n_{fam} = 24, n_{ind} = 216, p < 0.001). Only for *P. napi* (offspring generation) the previously non-significant correlation became significant using family means (r = -0.31, n_{fam} = 28, n_{ind} = 139, p < 0.001). A comparison of the regression lines of both generations of *P. napi* yielded a significant difference ($F_{1,352}$ = 11.4; p < 0.001 for interaction of generation and egg size).

Heritability of pupal and egg size

Pupal mass was significantly affected by family and sex in *P. napi* (parental and offspring generation), *P. aegeria* and *A. levana* (Table 2). Males were larger than females in *P. napi* (mean ± 1SE: parental generation: 142.0 ± 0.6 vs. 129.3 ± 0.6 mg; offspring: 131.7 ± 0.8 vs. 116.6 ± 0.8 mg), but smaller in *P. aegeria* (123.8 ± 1.0 vs. 142.2 ± 1.0 mg) and *A. levana* (104.3 ± 0.8 vs. 122.4 ± 0.8 mg). The factor sex accounted for 27-53% of the total variance in pupal mass, while family effects (= h^2) explained 16-18% (Table 2). The significant interaction between family and sex in the parental generation of *P. napi* (Table 2) was due to a high variability of the sexual size dimorphism across families (with the difference between male and female mass ranging from 0-30 mg). Egg size was significantly affected by the factor family in *P. napi* and *A. levana*, but not in *P. aegeria* (Table 2). Family effects accounted for 17-

31% (h^2) of the total variance found in egg size, showing more variability across species (and generations) than pupal mass estimates (Table 2). Narrow-sense heritability for *P. napi* as estimated by means of parent-offspring regression was 0.36 ± 0.16 (r = 0.40, n_{fam} = 31, n_{ind} = 453, p < 0.001) for pupal mass, while it was not significant (h^2 = 0.04 ± 0.30, r = 0.03, n_{fam} = 28, n_{ind} = 139, p = 0.803) for egg size.

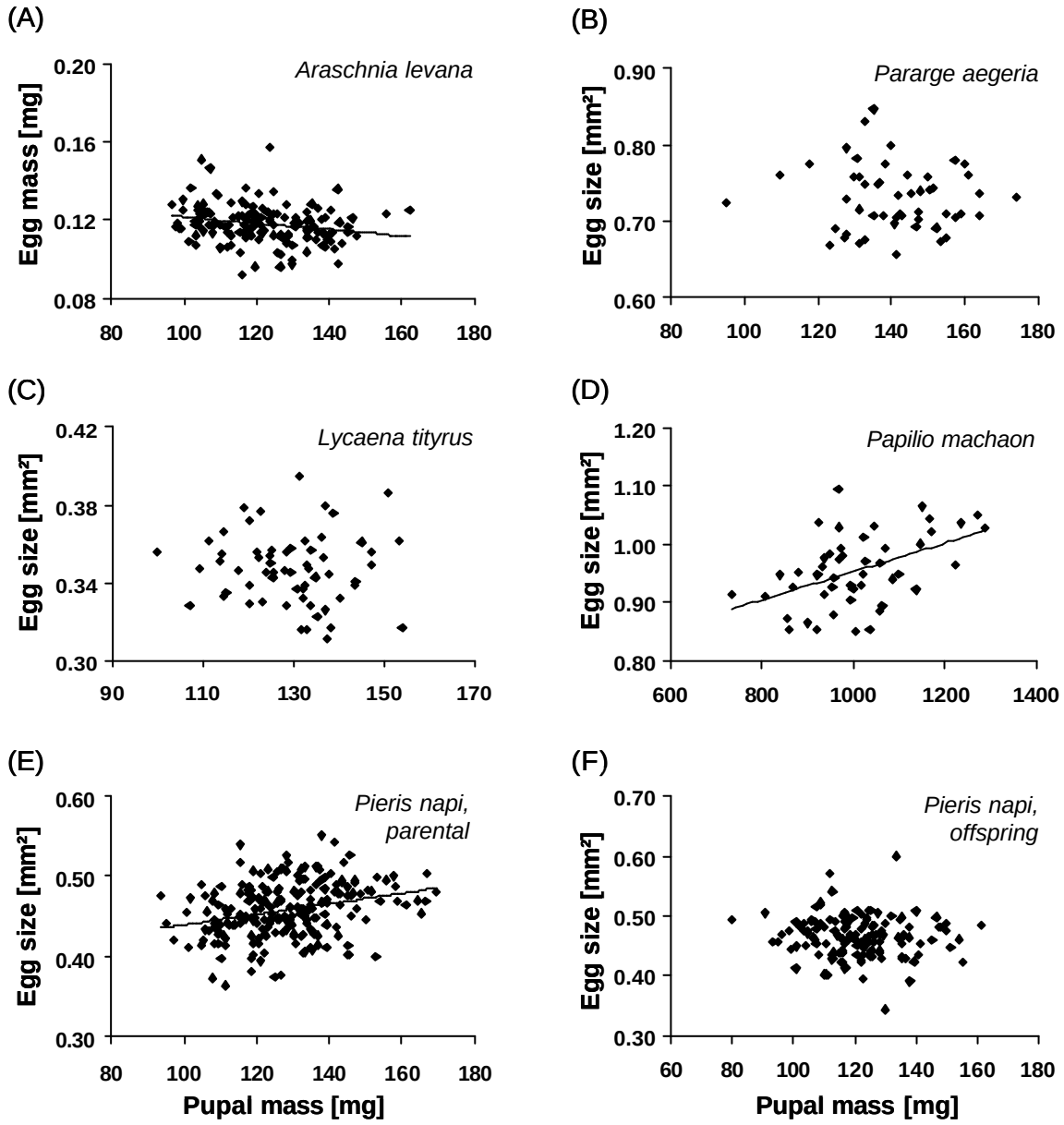

Fig. 1. Correlations between egg size and pupal mass for individual females of (A) *Araschnia levana* (coefficients of variation: CV_{pupal} = 11.3%; CV_{egg} = 8.3%), (B) *Pararge aegeria* (CV_{pupal} = 10.4%; CV_{egg} = 5.8%), (C) *Lycaena tityrus* (CV_{pupal} = 8.9%; CV_{egg} = 5.2%), (D) *Papilio machaon* (CV_{pupal} = 11.9%; CV_{egg} = 6.4%), (E) *Pieris napi* (parental generation; CV_{pupal} = 11.4%; CV_{egg} = 7.4%) and (F) *Pieris napi* (offspring generation; CV_{pupal} = 11.7%; CV_{egg} = 7.3%); regression lines are indicated if p < 0.05.

Table 2. Results of (A) two-way analyses of variance (ANOVAs) for the effects of "Family" (random factor) and "Sex" on pupal mass and (B) one-way analyses of variance (ANOVAs) for the effects of family (random factor) on egg size in *Pieris napi*, *Pararge aegeria* and *Araschnia levana* (given are whole model coefficients of determination r^2; $p < 0.05$ in bold). The proportion [%] of the total variance (pct of total) in both traits as explained by different sources is given, estimated by using the REML method of JMP (assuming random effects for all variables; defining "Sex" as fixed factor prevents JMP from calculating the necessary variance components).

(A)

Pupal mass	**Source**	***Mean Squares***	***df***	***F***	***p***	**pct of total**
Pieris napi, parental generation $n = 1362$; $r^2 = 0.35$	Family	3334.6	21	17.3	**<< 0.001**	16.8
	Sex	57932.6	1	300.3	**<< 0.001**	27.1
	Family x Sex	505.2	21	2.6	**<< 0.001**	
	Error	192.9	1318			
Pieris napi, offspring $n = 919$; $r^2 = 0.38$	Family	1641.0	34	7.3	**<< 0.001**	16.3
	Sex	26810.8	1	119.3	**<< 0.001**	26.6
	Family x Sex	263.8	34	1.2	0.230	
	Error	224.8	849			
Pararge aegeria $n = 305$; $r^2 = 0.54$	Family	1372.0	9	11.9	**<< 0.001**	13.9
	Sex	17628.3	1	152.7	**<< 0.001**	50.6
	Family x Sex	156.6	9	1.4	0.208	
	Error	115.4	285			
Araschnia levana $n = 439$; $r^2 = 0.60$	Family	2857.4	8	29.0	**<< 0.001**	18.6
	Sex	23490.1	1	238.5	**<< 0.001**	52.7
	Family x Sex	158.3	8	1.6	0.121	
	Error	98.5	421			

Table 2. *continued*.

(B)

Egg size	Source	*Mean Squares* *10^{-3}	*df*	*F*	*p*	pct of total
Pieris napi, parental generation	Family	2.20	16	2.4	**0.003**	16.5
n = 196; r^2 = 0.16	Error	0.92	179			
Pieris napi, offspring	Family	1.58	27	1.9	**0.014**	31.3
n = 139; r^2 = 0.27	Error	0.85	111			
Pararge aegeria	Family	1.96	9	1.4	0.232	23.6
n = 51; r^2 = 0.20	Error	1.43	41			
Araschnia levana	Family	0.35	8	4.5	**<< 0.001**	26.4
n = 143; r^2 = 0.20	Error	0.08	134			

Maternal body size and fecundity and the egg size – number trade-off

Maternal size and liftetime fecundity were significantly positively correlated (using family means) in *P. napi* (parental generation; $r = 0.39$, $n_{fam} = 21$, $n_{ind} = 77$, $p < 0.001$) and *A. levana* ($r = 0.90$, $n_{fam} = 7$, $n_{ind} = 55$, $p < 0.001$), though not in *P. aegeria* ($r = -0.28$, $n_{fam} = 10$, $n_{ind} = 39$, $p = 0.090$). Multiple regressions for the effects of longevity, pupal mass and egg size on lifetime fecundity revealed that the most important (and for *P. aegeria* the only significant) predictor of lifetime fecundity was female longevity, explaining 16-19% of the variation found (Table 3). In *A. levana* and *P. napi,* also female mass affected lifetime fecundity. Only in *P. napi* egg size contributed significantly to variation in egg number, showing a positive correlation.

The turret-like clutches deposited by *A. levana* contained 3-23 eggs each (mean ± 1SE: 7.7 ± 0.08). Female pupal mass and egg number per turret were positively correlated ($r = 0.37$, $n_{fam} = 9$, $n_{ind} = 142$, $p < 0.001$). Within egg turrets a trade-off between the number and size of eggs became apparent: egg sizes decreased with increasing numbers of eggs aligned in a turret ($r = -0.20$, $n_{fam} = 9$, $n_{ind} = 142$, $p = 0.019$).

Table 3. Results of multiple regressions (stepwise forward addition of variables; Ridge regression; lambda = 0.10; $F > 1.0$ for inclusion) for the effects of longevity, pupal mass and egg size on lifetime fecundity in *Araschnia levana* ($n = 55$), *Pararge aegeria* ($n = 38$) and *Pieris napi* (parental generation; $n = 76$). Given are standardised partial regression coefficients Beta (standard error in parentheses), multiple coefficients of determination r^2_{mult}, *F*-value and significance level. $p < 0.05$ in bold.

	Predictor	Beta	r^2_{mult}	*F*	*p*
Araschnia levana	Longevity	0.319 (0.123)	0.19	12.2	**<0.001**
	Pupal mass	0.258 (0.123)	0.26	5.1	**0.028**
	Egg size	-0.146 (0.114)	0.28	1.6	0.207
Pararge aegeria	Longevity	0.398 (0.142)	0.17	7.8	**0.008**
Pieris napi	Longevity	0.394 (0.091)	0.16	13.8	**<0.001**
	Egg size	0.266 (0.094)	0.28	12.7	**<0.001**
	Pupal mass	0.260 (0.094)	0.35	7.6	**0.007**

Discussion

Phenotypic correlations between maternal size and egg size

Maternal body size is generally believed to underlie variation in reproductive output in arthropods, influencing both egg numbers and sizes (Berrigan 1991). For instance, mainly based on interspecific comparisons, body size is considered to be an important morphological constraint on egg size in butterflies (García-Barros 1994; Bernardo 1996). However, intraspecific comparisons frequently revealed contradictory results (e.g. Wiklund and Karlsson 1984; Bernardo 1996). As such inconsistencies can result from assay conditions, we here used highly standardised methods to more rigorously test for causal relationships between maternal size and egg size across various butterfly species representing the major butterfly families. Our study shows that there is no general relation linking egg size to body size within the butterfly species investigated. A rather strong positive relationship between egg and body size was found only in *P. machaon*. Even when comparing two successive generations of the same species (*P. napi*) considerable variation in the relationship

between both traits was found (one positive, negative the other), indicating that environmental and/or maternal effects may play an important role even in highly standardised designs.

Thus, within species egg size does not seem to be generally subject to a morphological constraint imposed by female body size. This is in contrast to expectations arising from various findings including that scaling of body organs is common, that interspecific comparisons do suggest such a constraint and that positive genetic correlations between both traits were repeatedly found (Berrigan 1991; García-Barros 1994; Fox and Czesak 2000; Fischer et al. 2006).

We conclude therefore that the role of maternal body size as a constraint on egg size has been previously overemphasized and other factors are likely to be more important. These may include selective pressures imposed by the environment, feeding patterns, parent-offspring conflict and trade-offs with fecundity (Smith and Fretwell 1974; Parker and Begon 1986; Wiklund et al. 1987; Bernardo 1996; Klingenberg and Spence 1997). Such factors are likely to result in deviations from any underlying allometric relationship, and may well mask any potentially occurring scaling relationships (Wiklund et al. 1987). For instance, the mode of resource acquisition is likely to considerably affect correlations between maternal size and reproductive traits (Boggs 1986; Wickman and Karlsson 1989; Fox and Czesak 2000; Jervis and Ferns 2004; Calvo and Molina 2005). For capital breeders, relying mostly or entirely on resources acquired during larval development for egg production, a strong positive correlation between maternal size and reproductive traits (especially egg numbers) is expected, and has repeatedly been found (Marshall 1990; Tammaru et al. 1996; but see Tammaru et al. 2002 for contradictory results). This relationship should become less important with increasing dependence on adult feeding (income breeding, Boggs 1992; Bauerfeind and Fischer 2005b). The species used in this study are neither pure capital nor income breeders, but line up along the continuum between these extremes, with *P. machaon* (eclosing with the majority of eggs mature, cf. Wiklund and Karlsson 1984) being closest to a capital breeder. As could be expected, the strongest correlation between maternal size and egg size was found in this species.

Heritability of pupal and egg size

The heritability of pupal size is rather low in the species investigated (h^2 ca. 0.1-0.2; see also Mousseau and Roff 1987; Steigenga et al. 2005) as inferred from variance component analyses (as well as parent-offspring regression for *P. napi,* h^2 ca. 0.4). Heritabilities for pupal mass were rather similar across species in the present study, although heritabilities frequently vary among species, among populations within species and across environmental conditions (Falconer 1981).

Heritabilities for egg sizes varied to a higher degree across species, and were low to moderately high (not significant in *P. aegeria* and in *P. napi* when applying parent-offspring-regression), as could be expected for a life-history trait (Roff 1997, compare also Fischer et al. 2004; Steigenga et al. 2005). Interestingly, heritability estimates varied not only across, but also within species (in *P. napi*: $h^2_{parental}$ = 0.17 vs. $h^2_{offspring}$ = 0.31). Note that, in contrast to the full-sib analyses, parent-offspring-regression did not yield a significant heritability for egg size in two species (see above). Lower narrow-sense heritabilities (e.g. from parent-offspring regressions) are expected because full-sib analyses usually overestimate trait heritability, as variances between families can also be due to non-additive genetic or environmental effects (Roff 2002). Thus, the results stress the rather low heritability of egg size. The contradictory results found for pupal mass of *P. napi* cannot be explained, and most likely reflect limitations of the approach (compare e.g. Falconer 1981). While the broad-sense heritability of pupal mass was similar to the one of egg size in the parental generation, it was far lower in the offspring generation. This may suggest that body size is more closely related to fitness than is egg size, and/or that body size is more intensely subject to environmentally induced variation than is egg size (e.g. Teuschl et al. 2007 and references therein).

Maternal body size and fecundity and the egg size – number trade-off

In contrast to the patterns linking maternal size and egg size, the assumed positive correlation between body size and egg number was more apparent (cf. Honek 1993; Leather 1988). Only in *P. aegeria* no such correlation could be detected (but see Karlsson and Wickman 1990 for contradictory results). However, realized fecundity was found to be primarily dependent on female longevity, as is expected for income breeders (see also Jervis and Ferns 2004). In contrast, the frequently assumed trade-off between egg size and number (Smith and Fretwell 1974) was

supported – though not significantly – in *A. levana* only, but not in *P. aegeria* (no correlation) and *P. napi* (positive correlation between both traits). Thus, surprisingly, the only significant correlation between fecundity and egg size obtained was a positive one. Even the basic assumption of a trade-off between egg size and number in life-history theory may not be found due to various reasons including external factors such as dietary quality or male nuptial gifts, affecting its expression (Karlsson 1998; Sgrò and Hoffmann 2004; Bauerfeind and Fischer 2005b).

In *A. levana* weak trade-offs between egg size and number were not only found with regard to lifetime fecundity, but also within single egg turrets. In combination with the positive correlation between pupal mass and egg number per turret, which suggests a constraint on turret size due to maternal size, this results in a negative correlation between female size and egg size (Fig. 1). Note that in contrast to all other species used in this study *A. levana* spends its early larval period gregariously (Ruf 2002 and references therein). Depending on external conditions clutch sizes vary and can be adaptively adjusted by the female in order to maximize larval survival (see Ruf 2002; Bergström et al. 2006). Thus, in this species optimisation of clutch size seems to be at a selective premium, while interactions between group size and individual offspring size still need to be tested.

Conclusions

Intraspecific patterns linking maternal size and egg size varied strongly across species, indicating a subordinate role of maternal size in determining egg size variation within species. Rather, egg size variation seems to be primarily shaped by selective pressures, physiological features (such as feeding patterns) and environmental cues (Parker and Begon 1986; Bernardo 1996; Klingenberg and Spence 1997). Our data at least suggest that there is no general morphological constraint imposed by female size on egg size, and that the role of such potential constraints has been previously overestimated. Even though the lack of phenotypic correlations does largely rule out a morphological constraint within populations, this does not necessarily imply that maternal size does not bias or limit evolutionary changes in egg size due to genetic correlations (and thus developmental constraints).

Anyway, one should be extremely careful when trying to predict patterns within species based upon interrelations found on an interspecific level. Such intra- and interspecific allometric relationships do not need to share the same slope of correlation (Fischer et al. 2002). Within a population the major source of variability derives from the reproductive adaptations of individuals interacting with age structure and environmental variation, while between species it will largely result from accumulated genetic differences (Wickman and Karlsson 1989).

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5.2 Maternal body size as an evolutionary constraint on egg size in a butterfly

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Abstract

Genetic and developmental constraints have often been invoked to explain patterns of existing morphologies. Yet, empirical tests addressing this issue directly are still scarce. We here set out to investigate the importance of maternal body size as an evolutionary constraint on egg size in the tropical butterfly *Bicyclus anynana*, employing an artificial two-trait selection experiment on simultaneous changes in body and egg size (synergistic and antagonistic selection). Selection on maternal body size and egg size was successful in both the synergistic and the antagonistic selection direction. Yet, responses to selection and realized heritabilities varied across selection regimes: the most extreme values for pupal mass were found in the synergistic selection directions, while in the antagonistic selection direction realized heritabilities were low and non-significant in three out of four cases. In contrast, for egg size the highest values were obtained in the lines selected for low pupal mass. Thus, selection on body size yielded a stronger correlated response in egg size than vice versa, which is likely to bias (i.e. constrain), if weakly, evolutionary change in body size. However, it seems questionable whether this will prevent evolution towards novel phenotypes, given enough time and that natural selection is strong. Correlated responses to selection were overall weak. Egg and larval development times tended to be associated with changes in maternal size, while variation in pupal development times weakly tended to follow variation in egg size. Lifetime fecundity was similar across selection regimes, except for females simultaneously selected for large body mass and small egg size, exhibiting increased fecundity. Multiple regressions showed that lifetime fecundity and concomitantly reproductive investment were primarily determined by longevity, as expected for an income breeder, while egg size was primarily determined by pupal mass. Evidence for a phenotypic trade-off between egg size and number was weak.

Introduction

After years of emphasising the almost omnipotence of natural selection in shaping life histories, it is now generally acknowledged that phenotypes are not only shaped by selection, but also by constraints that limit or channel evolutionary change (Stearns 1992; Schlichting and Pigliucci 1998; Roff 2002; Beldade et al. 2002a; Brakefield 2006). Thus, life histories involve compromises between what selection can achieve (adaptation) and what selection is prevented from achieving (constraints;

Barnes and Partridge 2003). Evolutionary constraints may stem from genetic or developmental interrelations among a suite of traits, and have often been invoked to explain patterns of existing morphologies (Yang 2001; Beldade et al. 2002a, 2002b; Zijlstra et al. 2003; Frankino et al. 2005; Brakefield 2006; Griswold 2006). However, to date empirical data testing evolutionary constraints directly are still scarce (Beldade et al. 2002a; Frankino et al. 2005; Rose et al. 2005; Brakefield 2006).

Offspring and body size are traits of key importance within each individual's development, both being related to fitness (e.g. Stearns 1992; Fox and Czesak 2000; Roff 2002). Egg size in insects, for instance, can considerably affect the fitness of the progeny resulting from these eggs; larger offspring were frequently found to e.g. mature earlier, to have improved ability to withstand competition, or to survive better in stressful environments as compared to small offspring (Azevedo et al. 1997; Fox and Czesak 2000; Czesak and Fox 2003; Roff 2002; Fischer et al. 2003a, 2003b, 2006; but see Karlsson and Wiklund 1984). The considerable and wide-ranging ecological importance of a large body size is clearly demonstrated by its positive effects on various fitness-related traits (e.g. metabolic rate, dispersal, competitive abilities, capability to withstand starvation; Schmidt-Nielsen 1984; Blanckenhorn 2000). Of particular interest here is its role in shaping the expression and evolution of reproductive traits (see e.g. Berrigan 1991; Honek 1993).

In butterflies there is evidence from a broad-based interspecific comparison for a non-adaptive scaling relationship between egg size and female body size (García-Barros 1994, 2000), suggesting that egg size is at least partially subject to constraints imposed by female size and morphology (see also Berrigan 1991; Fox and Czesak 2000). However, there is growing evidence that the importance of maternal size as a morphological constraint on egg size has been previously overemphasized, as variation in egg size has been found to be at best weakly related to variation in maternal body size within various butterfly species and populations (Wiklund and Karlsson 1984; Fischer et al. 2002). This lack of phenotypic correlations does not necessarily imply that maternal size does not bias or limit evolutionary change in egg size, as life-history data can yield large differences between phenotypic and genetic correlations (Rose and Charlesworth 1981; Reznick 1985). Thus, while the importance of maternal size as a morphological constraint on variation in egg size may be limited, its role as an evolutionary constraint still needs to be assessed.

One of the most powerful tools to examine genetic variances and covariances that underlie the evolution of traits are artificial selection experiments (Brakefield 2003; Frankino et al. 2005; Fuller et al. 2005; Brakefield 2006). If, e.g., two traits are genetically correlated, selection for a change in one trait is likely to result in the evolution of a change in the second trait (Brakefield 2003). In the tropical butterfly *Bicyclus anynana* (Butler, 1879), selection experiments yielded clear evidence for a genetic coupling between maternal size and egg size (Fischer et al. 2002, 2006, 2007), which seems to be common in insects (Azevedo et al. 1997; Fox and Czesak 2000). However, while selection on body size yielded a strong correlated response in egg size, selection on egg size yielded a much weaker response in body size (Fischer et al. 2006, 2007). Based on the existence of a genetic correlation, it might be predicted that concerted changes of both traits will be more readily produced than those opposing the presumed genetic correlation (see Zijlstra et al. 2003; Brakefield 2003). Such genetic correlations have been widely documented for many morphological and life history traits, and are generally accepted to demonstrate developmental constraints that can bias or limit the evolutionary independence of coupled traits (Falconer and Mackay 1996; Roff 1997; Brakefield 2003).

To test for such an evolutionary bias (i.e. a constraint) we applied a two-trait selection experiment, targeted at simultaneous changes in maternal size and egg size. Selection was in the same (synergistic) and the opposite (antagonistic) direction as the presumed positive genetic correlation between both traits (Fischer et al. 2002, 2006, 2007). Although antagonistic selection experiments have been proven to be a powerful tool to unravel the constraining power of genetic covariances, they have been rarely used thus far as opposed to single-trait selection experiments (Zijlstra et al. 2003; Beldade et al. 2002a; Brakefield 2003; Fuller et al. 2005). Further, most existing examples focused on morphological traits, such that exploring more complex and integrated life history traits is still a largely unexplored task. Thus, this study considerably extends the scope of previous ones, by using antagonistic two-trait selection and focussing on life-history traits. The results of such experiments are likely to gain new insights into the genetic and developmental coupling respectively independency of life history traits in general, and thereby on the evolution of life histories. Maternal and egg size, for instance, are likely to be connected to other key life-history traits, such as fecundity (e.g. Smith and Fretwell 1974; Honek 1993) or growth trajectories (e.g. Blanckenhorn 2000; Teuschl et al. 2007). Therefore,

selection on maternal size and egg size is also likely to result in correlated responses in related traits, rendering the variance and covariance of these traits particularly important for gaining a better understanding of life history evolution.

Material and Methods

Study organism

Bicyclus anynana is a tropical, fruit-feeding butterfly ranging from Southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen et al. 2004). Reproduction is essentially confined to the warmer wet season when oviposition plants are abundantly available, and where 2-3 generations occur. During the colder dry season reproduction ceases and butterflies do not mate before the first rains at the beginning of the next wet season (Brakefield 1997; Windig 1994). Morphs are gradually replaced during seasonal transitions. Thus, both phenotypes may occur simultaneously (Brakefield and Reitsma 1991). Apart from the lack of conspicuous eyespots, dry season morphs show several differences from the wet season morphs including increased egg and body size, larger fat bodies and a reproductive diapause in order to survive the unfavourable dry season (Brakefield and Reitsma 1991). Thus, at least during the dry season butterflies are likely to be under selection for increased body and egg size. This, however, is not necessarily the case during the wet season (Fischer et al. 2003).

A laboratory stock population was established at Bayreuth University, Germany, in 2003 from several hundred eggs derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from 80 gravid females caught at a single locality in Malawi. In each generation several hundred individuals are reared maintaining high levels of heterozygosity at neutral loci (Van't Hof et al. 2005). For this study butterflies from the Bayreuth stock population were used.

Selection procedure

Throughout, butterflies were reared in a climate room at 27°C (exclusively inducing wet season phenotypes throughout), high relative humidity (70%) and a

photoperiod of L 12 : D 12 (24 h light cycle). Larvae were fed on maize plants in ample supply, adults on moist banana. Selection was targeted simultaneously at female pupal mass and egg size (no selection on males). All possible phenotypic combinations of both traits were included: (1) small body size / small egg size (hereafter 'be'); (2) large body size / large egg size ('BE'); (3) small body size / large egg size ('bE'); (4) large body size / small egg size ('Be'). 'be' and 'BE' are referred to as the synergistic direction of selection (both traits selected in the same direction, either larger or smaller), while 'bE' and 'Be' are referred to as the antagonistic direction of selection (both traits selected in opposing directions). Minor or capital letters indicate the direction of selection: b / B: small / large body size; e / E: small / large egg size. Pupal mass and egg size of a total of 628 female stock butterflies were measured at generation 0. Females were split randomly into two groups, from which two sets of independent replicate lines per selection regime as well as two unselected controls ('C') were derived. Control lines were established by selecting 40 randomly chosen females each, while for selection lines the 40 females with the most extreme phenotypes (see below) were chosen.

To initiate the first generation, stock population pupae were collected on a daily basis. All female pupae were weighed to the nearest 0.01 mg two days after pupation (Sartorius microscale MC 210 P). Pupal mass was used as a proxy for adult body size because of tight correlations between both traits ($r^2 = 0.80$, $P < 0.0001$, cf. Bauerfeind and Fischer 2005a; see also Wiklund and Karlsson 1984; Fischer et al. 2002). Weighed female pupae were placed individually in translucent plastic pots (125 ml) until adult eclosion. Following eclosion, all females were marked by individual numbers written on the left hindwing (using a felt-tip pen, Staedtler Lumocolor permanent), and given a premating time of two days before an equal number of randomly chosen virgin males (from their own replicate line for later generations) was added to the females' cages for mating. After a mating period of two days, females were placed individually in translucent plastic pots (1 L, covered with gauze) containing a fresh cutting of maize for egg-laying. Thus, oviposition started for all females on day five of adult life. Throughout, all butterflies had access to moist banana for adult feeding. Eggs were collected within the next two days for egg measurements; per female, the sizes of ten eggs were measured and their mean was used for further analyses. Thus, in this experiment only the first eggs deposited were used, thereby effectively controlling for effects of female age. Egg size was

measured as cross-sectional projections [mm²] using a digital camera (Leica DC300) connected to a stereo microscope. The resulting images were analyzed using Scion Image public software (Scion Corporation 2000). This method provides a highly reliable measurement of egg size for the nearly perfectly spherical eggs of *B. anynana* (Fischer et al. 2002). All egg size measurements [mm²] were converted into egg mass [mg] using a conversion factor of 0.63, derived from samples of control line eggs.

Selection was based on the ranks of the phenotypic values for each female's pupal mass and egg size. Depending on the respective selection regime, ranks were either added (synergistic direction of selection) or subtracted (antagonistic direction of selection) from one another (cf. Beldade et al. 2002a). Females with minimum and maximum values derived from an addition of ranks represented the females selected for the 'be'- and 'BE'-lines, respectively; females with minimum and maximum values derived from a subtraction of ranks represented the females selected for the 'bE'- and 'Be'-lines. In subsequent generations, pupal mass and egg size of 100 to 160 females were measured per replicate line, from which the 40 with the most extreme trait combinations (as detailed above) were selected as parents (for control lines 40 females were chosen at random; there were no significant differences in the proportion of individuals contributing to the next generation across selection lines; Kruskal-Wallis-ANOVA, $P = 0.264$). Selection was carried out for 11 generations.

Correlated responses to selection

Correlated responses to selection on female body size and egg size were assessed in all 10 replicate lines ('BE', 'be', 'bE', 'Be', 'C'; two replicates each). The eggs for this experiment were collected in generation 11. The resulting hatchlings were divided among ten sleeve-like gauze cages per replicate line, containing 30 larvae each (i.e. larvae were reared in groups). All cages were arranged in a randomized block design within a single climate chamber (27°C; L 12 : D 12; 70% relative humidity). For all individuals we measured pupal development time and pupal mass (as above). Subsamples of ca. 50 females per replicate line were scored for longevity, egg sizes and numbers. Females were kept individually in plastic pots as outlined above, with eggs being collected, counted and weighed to the nearest 0.01 mg (Sartorius microscale MC 210 P) every other day until the death of the female. To obtain average egg masses, this value was divided by the respective number of eggs

per sample. Egg water content was assessed as the difference between egg fresh and dry mass (after drying the eggs at 70°C for 24 h), and is given as percent of egg fresh mass throughout. Reproductive investment was calculated as the sum of the egg dry masses produced during the females' lifetimes.

In an additional experiment we recorded larval development times (experimental setup as above, but here eggs within cages were collected within a 12 h light period to allow for the calculation of larval times) as well as egg development times and hatching success. For the latter two, eggs collected within 12 h light periods were placed into Petri dishes lined with moist filter paper, and containing a small cutting of maize for hatching caterpillars (ten replicate dishes per replicate line, each containing 50 randomly chosen eggs from the respective population). Dishes were checked daily for hatchlings until no more hatchlings were found on three consecutive days.

Data analysis

Realized heritabilities (h^2) were calculated by fitting least-square regressions to pupal and egg mass (relative to unselected controls) on cumulated selection differentials, with heritabilities being estimated as twice the slope of the regression line (as selection was on females only). Analyses of (co-)variance (ANCOVAs) were used to compare the slopes of the regression lines, using selection regime as fixed factor, replicate line as random factor, and cumulated selection differential as covariate. Pearson's product moment correlations were used to assess phenotypic correlations within selection regimes.

General linear models were applied to test for differences in target and non-target traits across selection regimes in F12 butterflies (followed by Tukey's HSD). To account for the fact that individuals within lines are statistically not independent, replicate line was nested within selection regime (and, if applicable, cage was nested within replicate line and selection regime). Unless otherwise stated, replicate line and cage were treated as random effects. Because in analyses treating replicate line as a random effect statistical power is exceedingly low (because of the low number of replicate lines; e.g. Underwood 1997), analyses were repeated treating replicate line as fixed factor in case of non-significant results. A recurrence of a negative result indicates that this is not purely due to limited statistical power, while a positive one may at least indicate trends (though non-independence of individuals within lines is

not controlled for anymore; see also Fischer et al. 2006). Pupal mass was added as covariate when appropriate.

To test for effects of female age on egg sizes and numbers, repeated measurement ANOVAs (until day 12 of the oviposition period) were carried out (note that repeated measurement ANOVAs do not support random factors; thus all factors were treated as fixed effects). Hatching success across selection regimes and replicate lines was analysed using nominal logistic regressions on binary data. Further, multiple regressions (stepwise forward addition of variables; Ridge regression, to control for interrelations between factors) were carried out to investigate the effects of life history traits (pupal mass, longevity and reproductive traits) on lifetime fecundity, egg fresh mass and reproductive investment within selection regimes. All statistical tests were performed using Statistica 6.1 (StatSoft 2003) or JMP version 4.02 (SAS Institute 2000). Throughout the text all means are given ± 1 SE.

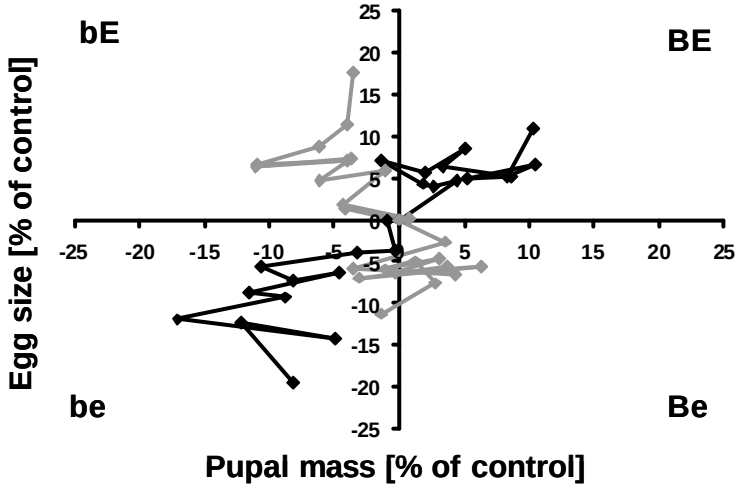
Results

Artificial selection on female body and egg size

After 11 generations of selection, a significant response to selection was observed in egg size (‘bE’ > ‘BE’ > ‘C’ > ‘Be’ > ‘be’; based on the size of the first eggs deposited; Tukey HSD after ANOVA) and a statistical trend in female pupal mass (Figs. 1, 2; Table 1). Treating replicate line as fixed factor indicates that the latter result might be caused by insufficient statistical power ($F_{4,2217}$ = 99.1, p < 0.001; ‘BE’ > ‘Be’ > ‘C’ > ‘bE’ > ‘be’; Tukey HSD). For both target traits, there was significant variation across replicate lines and cages (Table 1; Fig. 2). Relative to controls, egg size changed by +8.7% (‘BE’), +13.1% (‘bE’), -10.2% (‘Be’), and -19.8% (‘be’), and pupal mass by +5.9% (‘BE’), +3.1% (‘Be’), -7.5% (‘bE’) and -10.5% (‘be’) over the course of selection (averaged across replicates; cf. Fig. 1). Thus, the highest responses for both target traits were found in the lines selected for small body size, and egg size generally responded more strongly to selection than pupal mass. Pupal mass additionally varied across sexes, with females (209.7 ± 1.6 mg, n = 1261) being larger than males (164.0 ± 1.3 mg, n = 1061). A significant sex by selection regime interaction (Table 1) indicates some minor variation in the sexual size dimorphism across selection regimes, with females being 23.0% (‘BE’), 22.4% (‘Be’), 19.6% (‘bE’), 21.7% (‘be’) and 22.2% (‘C’) larger than males.

Although selection was on the size of the first eggs deposited, results were qualitatively identical for mean egg fresh (selection regime: $F_{4,5}$ = 14.5, p = 0.006) and dry mass ($F_{4,5}$ = 12.2, p = 0.008) averaged over the first 18 days of the oviposition period (Table 1). There was significant variation across replicate lines for both traits (egg fresh mass: $F_{5,490}$ = 12.5, p < 0.001; egg dry mass: $F_{5,490}$ = 6.9, p < 0.001).

a)



b)

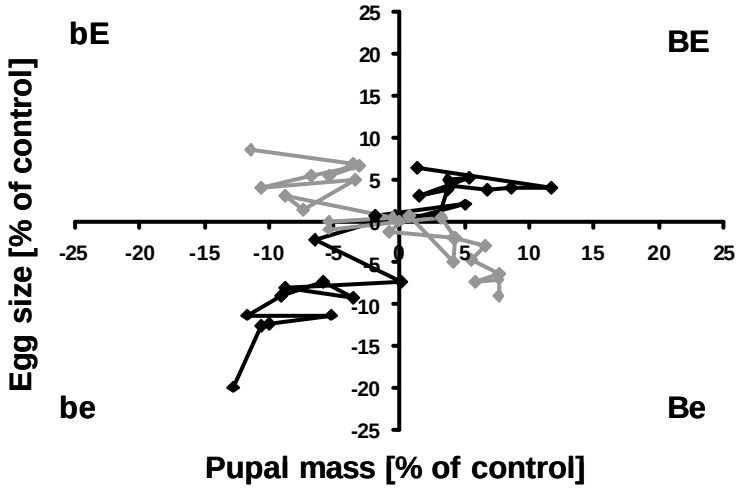
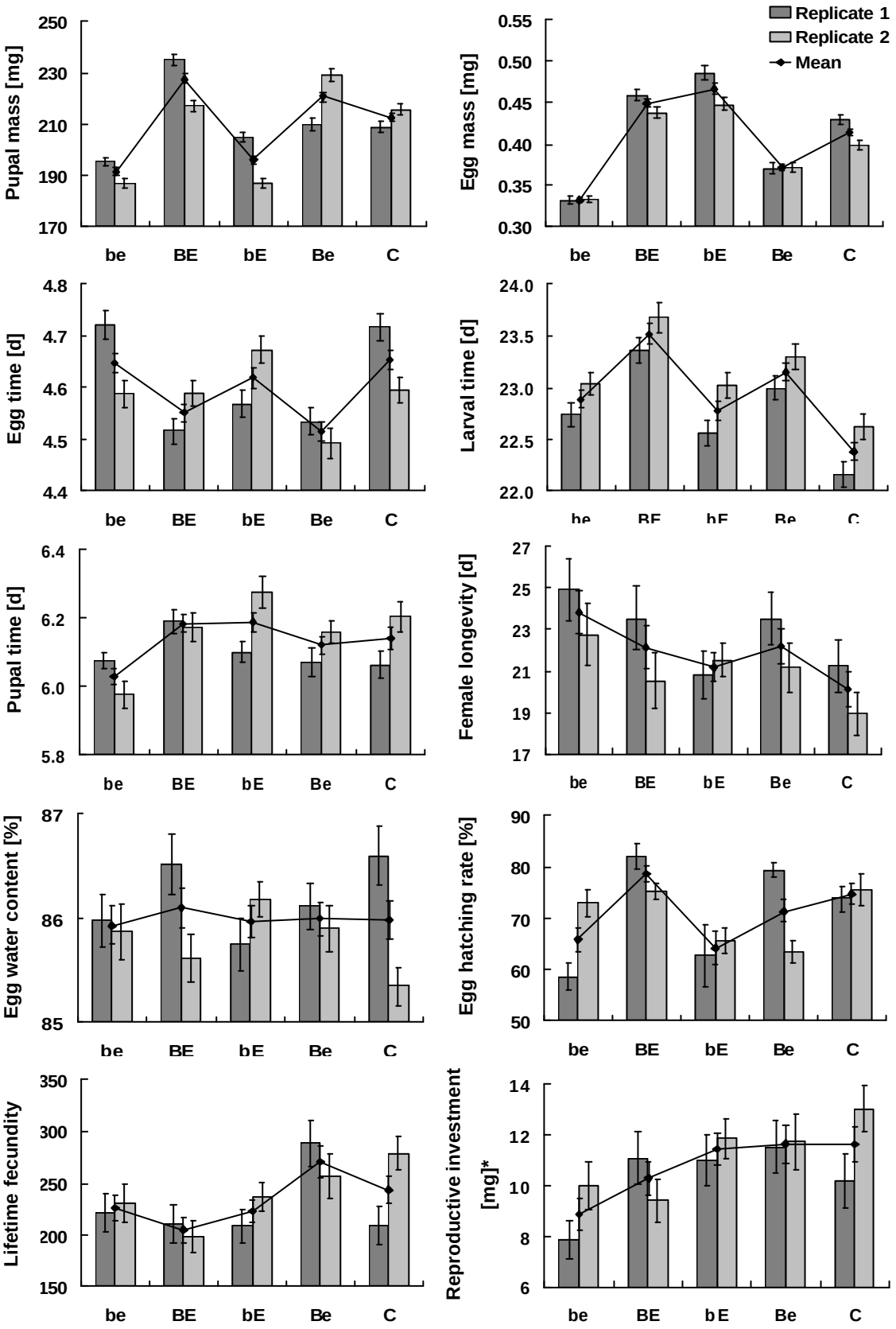


Fig. 1. Response to selection on pupal mass and egg size (for first eggs laid) relative to controls over 12 generations of artificial synergistic and antagonistic selection in *Bicyclus anynana* in replicate lines 1 (a) and 2 (b); minor or capital letters indicate direction of selection: b / B: small / large pupal size; e / E: small / large egg size.



* calculated as sum of egg dry masses [mg] per female over the oviposition period

Fig. 2. *see next page*

Fig. 2. Mean values (± 1 SE) of female pupal mass, egg fresh mass of the first eggs deposited, egg development time, female larval and pupal development time, female longevity, egg water content (averaged across the first 18 days of the oviposition period), egg hatching rate, lifetime fecundity and reproductive investment in selection lines of *Bicyclus anynana* in generation 12. Lines connect mean values of selection regimes (minor or capital letters indicate direction of selection: b / B: small / large body size; e / E: small / large egg size; C: control).

Table 1. Nested analyses of (co-)variance for the effects of selection regime (fixed) and replicate line (random) on various traits of *Bicyclus anynana*. Cage (random), sex (fixed), and pupal mass (covariate) were added as appropriate. Throughout, replicate was nested within selection regime, and cage within replicate and regime; $p < 0.05$ in bold.

Trait	Factor	*MS*	*df*	*F*	*p*
Pupal mass [mg]	Regime	71551	4,5	4.1	0.076
n = 98-145	Replicate [regime]	17387	5,92	24.0	**< 0.001**
	Cage [regime x line]	729	90,2217	1.5	**0.002**
	Sex	1096559	1,2217	2260.7	**< 0.001**
	Regime x Sex	3565	4,2217	7.4	**< 0.001**
Egg mass [mg]	Regime	0.28	4,5	25.6	**0.001**
n = 44-58	Replicate [regime]	0.01	5,479	6.7	**< 0.001**
	Pupal mass	< 0.01	1,479	2.7	0.100
Egg development time	Regime	2.7	4,5	1.6	0.311
n = 294-407	Replicate [regime]	1.7	5,91	2.0	0.080
	Dish [regime x line]	0.9	90,3476	3.7	**< 0.001**
Larval development time	Regime	69	4,5	3.3	0.114
n = 130-161	Replicate [regime]	21	5,91	3.0	**0.016**
	Cage [regime x line]	7	90,2701	3.4	**< 0.001**
	Sex	1513	1,2701	703.5	**< 0.001**
	Regime x Sex	6	4,2701	2.6	**0.037**
Pupal development time	Regime	2.7	4,5	1.0	0.488
n = 98-145	Replicate [regime]	2.7	5,94	10.9	**< 0.001**
	Cage [regime x line]	0.3	90,2216	1.2	0.128
	Sex	76.3	1,2216	358.0	**< 0.001**
	Regime x Sex	0.6	4,2216	2.7	0.300

Table 1. *continued*.

Trait	Factor	*MS*	*df*	*F*	*p*
Female longevity	Regime	179.9	4,5	1.6	0.288
n = 41-55	Replicate [regime]	112.8	5,447	1.5	0.189
	Pupal mass	15.7	1,447	0.2	0.648
Egg water content	Regime	0.5	4,5	0.05	0.994
n = 44-58	Replicate [regime]	10.6	5,490	3.8	**0.002**
	Pupal mass	0.8	1,490	0.3	0.598
Lifetime fecundity	Regime	63932.8	4,5	1.9	0.236
n = 44-58	Replicate [regime]	34467.4	5,493	2.0	0.072
	Pupal mass	10035.6	1,493	0.6	0.441
Reproductive investment	Regime	107.7	4,5	1.5	0.311
n = 44-58	Replicate [regime]	72.0	5,480	1.6	0.159
	Pupal mass	54.9	1,480	1.2	0.271

Repeated measures ANOVAs revealed significant effects of female age on egg fresh ($F_{5,1260}$ = 4.8, p < 0.001) and dry mass ($F_{5,1250}$ = 34.9, p < 0.001). While egg fresh mass weakly declined with increasing female age, egg dry mass reached maximum values on days 4 and 6 of the oviposition period (Fig. 3). In neither case, female age interacted significantly with selection regime (all p-values > 0.2). Thus, selection on the first eggs deposited yielded continuous differences in egg fresh and dry mass throughout the whole oviposition period.

On average, realized heritabilities (h^2) were higher for egg size (0.39 ± 0.17) than for female pupal mass (0.14 ± 0.07; paired t-test: t = -4.7, p = 0.002), with the 'be' lines showing the highest values (Table 2). Realized heritabilities for pupal mass, but not for egg size tended to be higher in the synergistic selection direction than in the antagonistic one. For female pupal mass, three out of four lines even yielded non-significant realized heritabilities in the antagonistic direction of selection (Table 2). The slopes of regressions fitted to pupal mass on cumulated selection differentials did not differ significantly among selection regimes as assessed by ANCOVA ($F_{3,44}$ = 1.9, p = 0.152 for interaction of selection regime with cumulated selection differential). Among replicate lines within selection regimes, P-values were > 0.4 for interactions of replicate lines with cumulated selection differential, except for 'Be' lines ($F_{1,22}$ = 6.7, p = 0.017). Realized heritabilities for egg size were overall higher in lines selected for low compared to high pupal mass (Table 2). The slopes of

regressions fitted to egg size on cumulated selection differentials differed among selection regimes (ANCOVA $F_{3,44}$ = 3.9, p = 0.015 for interaction of selection regime with cumulated selection differential), but not among replicate lines (ANCOVAs; all p-values for interactions ≥ 0.08). Phenotypic correlations between both target traits were positive in four out of six cases, but overall weak (significant in three out of six cases only; Table 3).

Table 2. Realised heritabilities (h^2) of pupal mass and egg size for replicated selection lines of *Bicyclus anynana*. Least-square regressions were fitted to trait values (relative to unselected controls) on cumulated selection differentials, with heritabilities being estimated as twice the absolute values of the slope of the regression lines (as selection was on females only). Minor or capital letters indicate direction of selection: b / B: small / large pupal size; e / E: small / large egg size; $p < 0.05$ in bold; numbers indicate replicate line.

	Selection direction	r^2	t	p	h^2
Pupal size	**be1**	0.47	-3.1	**0.009**	0.23
	be2	0.65	-4.5	**< 0.001**	0.22
	BE1	0.67	-4.7	**< 0.001**	0.16
	BE2	0.35	-2.4	**0.035**	0.13
	bE1	0.26	-2.0	0.073	0.12
	bE2	0.17	-1.48	0.167	0.11
	Be1	0.003	0.2	0.855	0.01
	Be2	0.47	-3.1	**0.010**	0.12
Egg size	**be1**	0.91	-10.8	**< 0.001**	0.62
	be2	0.87	-8.6	**< 0.001**	0.56
	BE1	0.45	-3.0	**0.012**	0.18
	BE2	0.70	-5.1	**< 0.001**	0.16
	bE1	0.86	-8.4	**< 0.001**	0.54
	bE2	0.90	-9.9	**< 0.001**	0.40
	Be1	0.50	-3.3	**0.007**	0.28
	Be2	0.70	-5.1	**< 0.001**	0.36

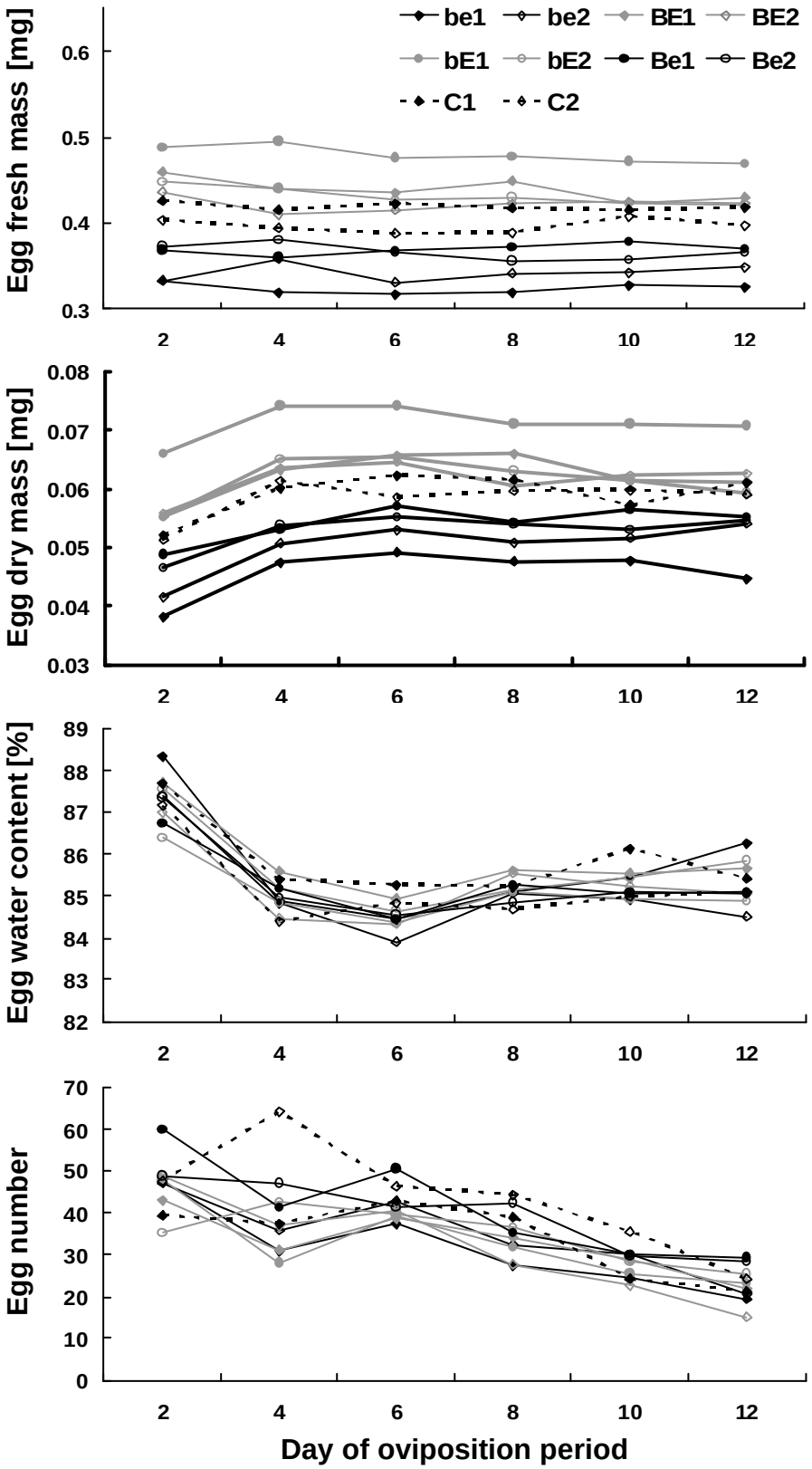


Fig. 3. Egg fresh and dry mass, egg water content and egg number over time in selection lines (*n* = 26 - 59; F12) of *Bicyclus anynana*; minor or capital letters indicate direction of selection: b / B: small / large pupal size; e / E: small / large egg size; C: control; numbers indicate replicate line. SE are omitted for enhanced visibility.

Table 3. Phenotypic correlations between pupal mass and egg size before (stock population, G0) and after selection (G12; averaged across selection lines); minor or capital letters indicate direction of selection: b / B: small / large pupal size; e / E: small / large egg size; $p < 0.05$ in bold.

Selection direction	*r*	*t*	*p*	*n*
Stock (G0)	0.18	4.6	**< 0.001**	628
be	0.06	0.6	0.556	112
BE	0.30	3.1	**0.003**	95
bE	0.29	3.1	**0.003**	105
Be	-0.04	-0.4	0.681	108
Control	-0.10	-1.1	0.295	104

Correlated responses to selection

Nested ANOVAs with replicate line as random factor failed to reveal any significant effects of selection regime on egg, larval or pupal development time, female longevity, lifetime fecundity, reproductive investment or egg water content (Table 1; Fig. 2). There was significant variation across replicate lines for larval and pupal development time and egg water content, but not for egg development time, female longevity, lifetime fecundity or reproductive investment. Additionally, egg and larval times, but not pupal times varied significantly across dishes / rearing cages. Treating replicate line as fixed factor, however, yielded significant results for egg ($F_{4,3476} = 3.2$, $p = 0.016$), larval ($F_{4,2701} = 9.6$, $p < 0.001$) and pupal development time ($F_{4,2216} = 10.8$, $p < 0.001$), fecundity ($F_{4,493} = 3.8$, $p = 0.005$), and threshold significances for reproductive investment ($F_{4,480} = 2.4$, $p = 0.050$) and female longevity ($F_{4,447} = 2.4$, $p = 0.050$), while there was no significant effect on egg water content ($F_{4,490} = 0.2$, $p = 0.952$). Those results indicate some tendencies to be analysed in more detail below.

Egg development times appeared to be reduced in lines selected for high female pupal mass as compared to those selected for low pupal mass and controls. In contrast, there was no indication for a link between egg size and egg development time (Fig. 2). Selection for large female size tended to prolong larval development

times as compared to lines selected for small female size, while the 'C' lines exhibited the shortest development times (Fig. 2). Further, larval times differed between sexes with males (21.5 ± 0.1 d) having a quicker development than females (22.9 ± 0.1 d; Table 1). A significant selection regime by sex interaction indicates variation in the sex differences in development times, with females showing by 6.8% ('BE'), 7.4% ('Be'), 6.7% ('bE'), 5.7% ('be') and 5.6% ('C') longer development times than males (averaged across selection regimes). Pupal development times tended to be shorter in 'be' lines, but were otherwise very similar across selection regimes (Fig. 2). Consistently across selection regimes, males (6.5 ± 0.03 d) had longer pupal development times than females (6.1 ± 0.03 d; Table 1).

Fecundity tended to be higher in 'C' and 'Be' lines than in other selection regimes (Fig. 2), but was very similar in lines selected for small female size regardless of variation in egg size. Thus, there is very little support for a trade-off between egg size and number in these lines.

Repeated measures ANOVAs on egg numbers yielded a significant effect of female age ($F_{5,1865}$ = 80.7, p < 0.001): egg numbers declined during the course of the oviposition period (Fig. 3). A significant interaction between female age and selection regime ($F_{20,1865}$ = 3.3, p < 0.001) indicates that the decrease in egg numbers with female age varied across selection regimes (Fig. 3). Throughout, female pupal mass (if added as covariate) did not affect reproductive traits (Table 1).

While selection regime did not affect egg water content (see above), repeated measures ANOVAs revealed a significant female age effect ($F_{5,1250}$ = 52.0, p < 0.001; Fig. 3). Egg water content was higher in first compared to later eggs, associated with the opposite pattern in dry mass, while egg fresh mass remained very similar throughout (Fig. 3).

Finally, egg hatching success was significantly affected by selection regime (Wald c^2 = 69.0, p < 0.001) and replicate line (Wald c^2 = 61.6, p < 0.001). Interestingly, egg hatching success did not seem to be related to egg size, but rather to female pupal mass: lowest hatching rates were found in 'be' and 'bE' lines (Fig. 2).

Predictors of lifetime fecundity and egg size

Multiple regression analyses showed that longevity, showing a positive correlation, was the most important predictor for lifetime fecundity, except for the 'C' lines (Table 4a). Its importance varies across selection regimes: while it explains a

mere 5-7% (r^2) of the variation in fecundity in the 'C' lines and the synergistic selection regimes, it explains 23% of the variation in the antagonistic selection regimes. Effects of egg fresh mass, in contrast, were weak throughout (and significant in three out of five cases only), explaining 2-8% of the variation in lifetime fecundity. Female pupal mass had only minor effects on fecundity, explaining = 2% of its variation.

Table 4. Multiple regressions (stepwise forward addition of variables, Ridge regression, lambda = 0.10, $F > 1.0$ for inclusion) for the effects of life-history traits on (a) lifetime fecundity and (b) egg fresh mass across selection regimes (n = 86 - 98). Given are standardized partial regression coefficients Beta, multiple coefficients of determination r^2_{mult}, F-value ad significance level; minor or capital letters indicate direction of selection: b / B: small / large pupal size; e / E: small / large egg size; $p < 0.05$ in bold.

a)

Lifetime fecundity	Predictor	Beta (SE)	r^2_{mult}	F	p
be	Longevity	0.25 (0.09)	0.06	6.6	**0.012**
	Egg fresh mass	-0.14 (0.09)	0.09	2.2	0.145
	Pupal mass	Not included in the model			
BE	Longevity	0.22 (0.10)	0.05	4.0	**0.048**
	Egg fresh mass	-0.12 (0.10)	0.07	2.4	0.126
	Pupal mass	-0.12 (0.10)	0.10	1.3	0.258
bE	Longevity	0.46 (0.09)	0.23	26.6	**< 0.001**
	Egg fresh mass	-0.29 (0.09)	0.31	9.3	**0.003**
	Pupal mass	0.10 (0.09)	0.32	1.3	0.266
Be	Longevity	0.51 (0.09)	0.23	28.4	**< 0.001**
	Egg fresh mass	-0.19 (0.09)	0.27	5.0	**0.029**
	Pupal mass	Not included in the model			
C	Egg fresh mass	-0.27 (0.10)	0.08	7.7	**0.007**
	Longevity	0.28 (0.10)	0.15	6.9	**0.010**
	Pupal mass	0.14 (0.10)	0.17	1.9	0.167

Table 4. *continued.*

b)

Egg fresh mass	Predictor	Beta (SE)	r^2_{mult}	*F*	*p*
be	Pupal mass	0.20 (0.09)	0.05	4.5	**0.036**
	Fecundity	-0.13 (0.09)	0.06	1.9	0.176
	Longevity	Not included in the model			
BE	Pupal mass	0.27 (0.10)	0.09	8.5	**0.005**
	Fecundity	-0.13 (0.10)	0.10	1.1	0.305
	Longevity	Not included in the model			
bE	Pupal mass	0.34 (0.09)	0.12	12.3	**< 0.001**
	Fecundity	-0.33 (0.10)	0.20	8.0	**0.006**
	Longevity	0.16 (0.10)	0.22	2.3	0.129
Be	Longevity	0.36 (0.11)	0.07	6.6	**0.011**
	Fecundity	-0.23 (0.10)	0.11	4.8	**0.030**
	Pupal mass	Not included in the model			
C	Fecundity	-0.28 (0.10)	0.08	7.7	**0.007**
	Pupal mass	-0.13 (0.10)	0.11	2.4	0.126
	Longevity	0.11 (0.10)	0.12	1.0	0.313

For egg fresh mass, multiple regression analyses showed that female pupal mass was overall of highest importance, explaining 3-12% (r^2) of the variation in egg fresh mass (except in the ‘Be’ lines; Table 4b). The importance of female pupal mass tended to be higher in the lines selected for large egg size. Across all regimes, lifetime fecundity was negatively correlated with egg fresh mass, reaching significance in the ‘C’ lines and the antagonistic selection regimes. In those, longevity had minor effects on egg fresh mass, except for the ‘Be’ lines where it was the most important predictor for egg fresh mass, explaining 7% of the variation found.

Discussion

It is generally assumed that constraints stemming from genetic and developmental interrelations between traits may at least partly explain patterns of existing morphologies (Beldade et al. 2002b, 2002b; Frankino et al. 2005; Griswold 2006). However, empirical tests addressing this issue directly are still scarce (Brakefield 2006). For the butterfly *B. anynana* as well as many other insects there is evidence for a positive genetic correlation between egg size and maternal body size, which may bias or limit the independent evolution of both traits (Parsons 1964; Fischer et al. 2002, 2006, 2007; Fox and Czesak 2000; Czesak and Fox 2003). This study aimed at investigating the role of maternal body size as an evolutionary constraint on egg size, employing two-trait artificial selection on simultaneous changes in maternal size and egg size to draw upon the general role of genetic correlations in constraining life history evolution.

Response to selection

Both target traits did respond to artificial selection (though pupal mass only weakly; Fig. 1; Table 1). All target phenotypes (i.e. those in the synergistic and in the antagonistic direction) could – albeit to varying extents – be achieved even within a short evolutionary time frame. Thus, there was no evidence for a strong genetic constraint. Yet, responses to selection and realized heritabilities varied across selection regimes. The most extreme phenotypes for female body size, accompanied by higher realized heritabilities, were found in the synergistic selection directions, while in the antagonistic direction trait values were less extreme and realized heritabilities were non-significant in three out of four cases (Table 2). This indicates a bias in evolutionary change with regard to the independent evolution of female size and egg size, and thus a 'slight' constraint. Yet, it seems questionable whether this will prevent evolution towards novel phenotypes, given enough time and that natural selection is strong (Fuller et al. 2005; Brakefield et al. 2006). For egg size the pattern is more complex. Here, the 'be' lines, but not their 'BE' counterparts showed the most extreme phenotypes (Fig. 2). Further, there was no consistent difference in realized heritabilities between the synergistic and antagonistic selection direction (Table 2). Rather, heritabilities for egg size were higher in females selected for low pupal mass. Overall, h^2-values of egg size exceeded those of female size. This may suggest that body size is more closely related to fitness than is egg size, and / or that egg size is

less affected by environmentally induced variation (i.e. is more canalized) than is body size (e.g. Teuschl et al. 2007 and references therein).

This asymmetric pattern is in agreement with earlier results from single-trait selection experiments using *B. anynana*, showing that selection on body size yields a large correlated response in egg size, while selection on egg size affects body size to a much smaller extent (Fischer et al. 2002, 2006, 2007). These results support the notion that genetic responses to divergent selection may not follow the simple paradigm of diverging frequencies of alleles at the same set of genes; rather, different loci may contribute to the responses in different selection regimes (Fuller et al. 2005). In addition, pleiotropy, linkage disequilibrium or epistatic genetic effects may be involved in mediating these patterns (Gromko 1995; Fuller et al. 2005).

We found significantly positive phenotypic correlations between maternal size and egg size in 'BE' and 'bE' lines only (Table 4). Overall, however, these correlations between pupal mass and egg size were weak, suggesting that any potential morphological constraint imposed by female size on egg size (e.g. due to oviduct size) has to be small in this species (Fischer et al. 2002).

Though selection was on the first eggs laid only, relative differences in egg size across selection regimes remained constant as females aged (Fig. 3). The slight decrease in egg size with female age probably reflects resource depletion (Begon and Parker 1986; Karlsson 1987; Giron and Casas 2003). Overall, this decline in egg size was very small in this study, likely reflecting the beneficial feeding conditions and the strong dependence of *B. anynana* on adult-derived nutrients (income breeding; Tammaru and Haukioja 1996; Fischer et al. 2004; Bauerfeind and Fischer 2005b; Karl et al. 2007). Fischer et al. (2006) found a steeper decrease in large compared to small single-trait egg-selected lines, which might be the result of a much higher variation in egg size.

Note that neither direct responses nor correlated responses (below) are likely to be strongly affected by maternal effects, which in principle may influence selection (Fox 1998; Fox et al. 1999). This is because all lines were subject to identical rearing and feeding conditions, rendering maternal environmental effects highly unlikely. Potential maternal genetic effects, in contrast, would form part of the response to selection.

Correlated responses in development times and adult female longevity

Generally, a positive genetic correlation between larval development time and body size is expected as body size is a function of the overall duration of the growth period and the mass accumulated therein (e.g. Roff 2002; Tammaru et al. 2004; Davidowitz et al. 2005). Accordingly, selection for increased body size tended to prolong the larval period here (Fig. 2; Table 1; see also Nunney 1996; Teuschl et al. 2007; but compare Fischer et al. 2007 for contradictory results). In agreement with Fischer et al. (2007), there was no support for the notion that larger egg and thus initial larval size reduces larval development times in this species (as e.g. proposed by Azevedo et al. 1996, 1997; Bernardo 1996; Yampolski and Scheiner 1996).

Overall, pupal development times remained rather invariant across selection regimes (Fig. 2; Table 1). Only the 'be' lines tended to have reduced and the lines selected for large egg size to have marginally prolonged pupal times compared to other lines (also Fischer et al. 2006 for the latter). This might be related to changes within the hormonal system affecting both development and reproduction.

Female longevity did not vary across selection regimes (Table 1; see also Pijpe et al. 2006). Therefore, being large neither translates into enhanced reproductive output (see below) nor into prolonged lifespan. This contrasts with the prediction that larger individuals should do better in terms of reproduction and survival as their accumulated energy should last longer (see Reim et al. 2006 and references therein). We believe this to be related to the strong dependence of *B. anynana* on adult income which mitigates the importance of a large body size in this species, and to the benign feeding conditions used (Bauerfeind and Fischer 2005b; Fischer et al. 2004).

Correlated responses in female reproductive traits

Lifetime fecundity and reproductive investment

The effect of female body size on fecundity and reproductive investment has proven to be of subordinate importance in this study (Tables 1 and 4; Fischer et al. 2006). There was little evidence for positive genetic correlations between these traits (e.g. Honek 1993; Tammaru et al. 1996; 2002; Leather 1988), except that lifetime fecundity tended to increase in the 'Be' lines (Fig. 2; Table 1). This indicates that large females invest relatively less into reproduction, and therefore allocate relatively more resources to survival and general maintenance than small females (cf. Reznick

1985; Van Noordwijk and De Jong 1986; Stearns 1992; Ellers 1996; Hoffmann and Merilä 1999; Reznick et al. 2000; Zera and Harshmann 2001). Reproductive investment did also not vary with egg size, a matter that attracted some controversy in recent years (Winkler and Wallin 1987; Schwarzkopf et al. 1999; Caley et al. 2001; Czesak and Fox 2003; Fischer et al. 2006; Karl et al. 2007). While the influence of female size was negligible, female longevity proved to be of prime importance in determining lifetime fecundity, as is expected for an income breeder (Table 4; Tammaru and Haukioja 1996; Blanckenhorn and Heyland 2004; Bauerfeind and Fischer 2005). Further, we found weak negative correlations between egg size and number within selection lines, suggesting a tendency towards a phenotypic trade-off between both traits (Smith and Fretwell 1974; see also Schwarzkopf et al. 1999).

Egg water content and hatching success

Relative egg water content remained invariant across selection regimes (Table 1), contrasting with previous results showing that larger eggs contain relatively more water (Fischer et al. 2006; Karl et al. 2007). Again, this difference is likely to be attributable to the much larger variation in egg size in the earlier studies. Highest egg water contents were found at the beginning of the oviposition period (coinciding with the lowest egg dry masses; Fig. 3), indicating that the first eggs deposited are not necessarily those of highest quality. This finding challenges common wisdom proposing an increased maternal investment into first eggs, as these are likely to be the most important ones for female reproductive success in the wild (e.g. Wiklund and Karlsson 1984; Begon and Parker 1986). However, whether such variation in egg composition affects egg viability remains to be tested.

Egg hatching success across selection regimes was unrelated to egg size (see also Karlsson and Wiklund 1984), but rather followed variation in maternal size (Fig. 2). Lowest hatching rates occurred in the lines selected for small female size. Similarly, those lines exhibited the longest egg development times. These results indicate that large egg size does not necessarily indicate egg quality (e.g. Jaeckle 1995; Royle et al. 1999; Karl et al. 2007; but see e.g. Seko and Nakasuji 2004 for contradictory results). However, these data need to be interpreted with caution, as inbreeding might be a problem here, despite following an identical experimental protocol (e.g. Saccheri et al. 1996; Fischer 2006; Fischer et al. 2006). Yet, we did not

find elevated numbers of crippled adults or unviable pupae (results not shown), both of which being indicative of increased levels of inbreeding (Saccheri et al. 1996).

Although correlated responses to selection were overall weak, it should be noted that some of the patterns above are probably 'real'. In evaluating the significance of these results two caveats should be mentioned: First, the low power of the statistical approach makes it very difficult to prove the existence of weak effects. Second, selection was done for a rather short period of time only. Given more time, stronger correlated responses would be expected.

Conclusions

This study provides evidence for an albeit slight evolutionary constraint on body size, i.e. a bias in evolutionary change, as responses to artificial selection in female size were more readily achieved in the synergistic compared to the antagonistic direction of selection (Beldade et al. 2002a; Brakefield 2006). At the same time, body size seems to constrain variation in egg size as the strongest response in egg size was found in combination with small female size. Nevertheless, selection resulted in responses in both the synergistic and antagonistic directions. Therefore, evolution towards novel phenotypes will most likely not be prevented, given enough time and that natural selection is strong (cf. Brakefield et al. 2006). However, such genetic constraints will slow down adaptive evolution and thus impact on the success of any given phenotype, especially in rapidly changing environments (Beldade et al. 2002b and references therein). Our results suggest that the importance of genetic correlations in shaping evolutionary trajectories may have been overemphasized in the past. In particular the importance of maternal size in shaping variation in egg size seems to be very limited, at least in our study organism, both in the sense of an evolutionary and a morphological constraint (this study; Fischer et al. 2002).

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6

Maternal nutrition and butterfly reproduction

6.1 Effects of food stress and density in different life stages on reproduction in a butterfly

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Abstract

Availability of adequate nutrition and (rearing) density are among the most important factors affecting growth, development and reproduction in animals. In holometabolous insects diets and energetic needs change between life stages, with storing of larval resources, adult feeding and reproduction being linked strategies. Nevertheless, studies investigating nutritional (and density) effects across metamorphic boundaries are largely lacking. We aim at disentangling the functional basis of reproductive patterns by independently manipulating larval and adult (1) density and (2) access to food, respectively, in the tropical butterfly, *Bicyclus anynana*. (1) A high larval rearing density had, contrary to common wisdom, very little impact on body size, but reduced larval development time through increased growth rates. The latter is thought to be an adaptation to high densities, driven by the risk of larval food resources becoming exhausted before reaching metamorphosis. Larval density and male company during oviposition (i.e. adult density) had no detectable effects on female reproduction. (2) Larval food stress prolonged larval development time and reduced larval growth rate, body size, fecundity and reproductive investment. Detrimental effects on female reproduction were mediated through a reduction in body size. Additional negative effects of adult food stress on fecundity were largely confined to females being fed as larvae *ad libitum*, while those being previously starved showed reduced performance regardless of adult income. Effects on egg size were inconsistent and, overall, marginal. Our results show that restricted food access in different developmental stages may set different limits to reproduction, either posed by shortage of larval-derived storage reserves (i.e. nitrogenous compounds) or adult income (i.e. carbohydrates). Thus, one should be cautious when stating that one or the other type of nutrients is ultimately limiting to reproduction. Rather, our findings highlight the importance of resource congruence and of considering both, larval- *and* adult-derived resources for reproduction.

Introduction

The pattern of resource allocation has critical consequences for individual fitness and is fundamental to numerous fields of research in behavioral, evolutionary and population ecology. In particular the availability of adequate nutrition comprises one of the most important factors affecting growth, development and reproduction in

animals. Food shortage during juvenile development was repeatedly found to increase development time, and to decrease growth rates, body size and reproductive output later in live (e.g. Briegel 1990; Berrigan and Charnov 1994; Blanckenhorn 1998; Fischer and Fiedler 2001a). Likewise, limited food access in the adult stage was found to diminish performance in various ways (e.g. Boggs and Ross 1993; Braby and Jones 1995; Fischer and Fiedler 2001b). Among the most obvious effects are those on reproductive output, as reproduction is a nutrient-limited process, triggered only if sufficient nourishment is available (Braby and Jones 1995; Wheeler 1996; Boggs 1997a). Studying the allocation of limited resources to reproduction is particularly interesting in holometabolous insects because diets and energetic needs change between life stages (e.g. between the herbivorous larval and the usually nectarivorous adult stage in butterflies).

Resources allocated to reproduction may be derived to varying degrees from stored reserves or current feeding, with pure capital or income breeders being exceedingly rare (e.g. Sibly and Calow 1984; Wheeler 1996; Boggs 1997a; O'Brien et al. 2004). In insects different resource types (e.g. carbohydrates, lipids and amino acids) may also be differentially drawn from reserves and current feeding (e.g. Boggs and Ross 1993; Boggs 1997b). Given the need for resource congruence (the use of nutrient types in a specified ratio; Bazzaz 1996), storage, foraging and reproduction are linked strategies in these animals. Understanding the relative importance of stored (usually larval-derived) and current (usually adult-derived) resources for reproduction is thus a critical element to understanding the functional basis of reproductive patterns.

Despite the need for considering both, the role of larval- *and* adult-derived resources for reproduction, only few studies have investigated nutritional effects across metamorphic boundaries (e.g. Boggs 1997b; O'Brien et al. 2002; O'Brien et al. 2003; O'Brien et al. 2004; Fischer et al. 2004). These studies basically investigated the importance of storage and current feeding for egg manufacture by using radio-labeled or stable isotopes. However, apparently no attempts have been made thus far to distinguish between the effects of food limitation during the larval and adult stage, though we do have a fairly rich knowledge on the effects of food shortage on life-history and reproduction in general (see above). Depending on the respective life-history strategy (i.e. the position within in the continuum between income versus capital breeder; Tammaru and Haukioja 1996), stresses applied

during different stages may have strikingly different implications (e.g. Boggs 1997b; Boggs et al. 2003).

Against this background we here adopt a full-factorial experimental design, enabling us to separately test for the effects of food limitation during the larval and adult stage as well as for interactions between the two in the tropical, fruit-feeding butterfly *Bicyclus anynana* (Butler, 1879) (Lepidoptera: Nymphalidae). In the majority of butterfly species the larval diet is the primary supply of protein, which is stored for use during metamorphosis and oogenesis (e.g. O'Brien et al. 2004). As adults, butterflies typically feed on diets that are rich in carbohydrates and poor in amino acids such as nectar or rotting fruit (Romeis and Wäckers 2000; O'Brien et al. 2004), though some species supplement their diet with substrates ranging from pollen to mud, dung or carrion or by preferring amino acid-containing nectars (e.g. Gilbert 1972; Boggs 1987; DeVries et al. 1997; Beck et al. 1999; Rusterholz and Erhardt 2000; Mevi-Schütz et al. 2003). Informal speculations that fruit could be a richer source of nitrogenous compounds than is nectar, could not be confirmed by recent data; both resources appear to be rather similar in nutrient composition (Bosque and Pacheco 2000; Omura and Honda 2003; Fischer et al. 2004).

However, adult carbohydrate income, which has been found to largely affect reproductive output at least in some butterfly species (e.g. Hill 1989; Boggs and Ross 1993; Fischer and Fiedler 2001b), can be used to synthesize non-essential amino acids using endogenous sources of nitrogen (O'Brien et al. 2002). In contrast, essential amino acids stored from larval feeding cannot be replaced and are therefore likely ultimately limiting to reproduction (O'Brien et al. 2002). The extent to which adult diet is important for reproduction in butterflies depends on the timing of egg provisioning, oviposition and the nutritional physiology (nutrient synthesis and turnover; O'Brien et al. 2004). While some butterfly species eclose with the majority of their eggs mature, others eclose with few or no mature eggs and require adult feeding to realize their potential reproductive output (Ramaswamy et al. 1997; O'Brien et al. 2004).

B. anynana females eclose with no mature eggs and essentially require adult income for maturing eggs, without which no eggs will be laid (Fischer et al. 2004). Accordingly, stable isotope analyses revealed that adult diet is an important source of egg nutrients, with approximately half of the egg carbon originating from adult feeding. The carbon that the adult diet provides is incorporated into developing eggs

quite rapidly, as the first eggs laid already show a nearly even ratio of larval- and adult-derived carbon (Fischer et al. 2004). Egg nitrogen isotopes, in contrast, resembled larval diet more closely than adult diet, indicating that the adult diet is primarily a source of carbon rather than nitrogen (Fischer et al. 2004). Based on these findings we predict that food limitation during larval development *and* in the adult stage will affect reproductive traits, with reduced storage reserves potentially interacting with food restrictions in the adult stage.

Additionally we investigate effects of larval and adult density (i.e. female harassment, see below) on life-history traits. Effects of larval density on adult body size and egg size have been reported for several insect species, with higher densities generally resulting in reduced size (at least in nongregarious larvae as is the case here), albeit theoretical models usually predict an increase to enhance competitive ability (e.g. Hawley 1985; Credland et al. 1986; Partridge et al. 1987; Fox and Czesak 2000; Agnew et al. 2002). However, whether such effects are caused by density itself (i.e. chemical or physical cues; Burns 1995), by food shortage affecting size parameters directly or by food shortage affecting some correlated traits remains hitherto largely unclear. Apart from larval rearing density, harassment by males may also affect female reproduction. For instance, Partridge et al. (1987) found reduced female longevity and increased early fecundity in *Drosophila melanogaster* females facing male company during oviposition. This effect was traced back to male accessory gland products by Chapman et al. (1995). To disentangle the effects of food shortage versus density *per se* we manipulate density independently of food availability.

Material and Methods

Study organism

B. anynana is a tropical, fruit-feeding butterfly ranging from Southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen et al. 2004). A laboratory stock population was established at Bayreuth University, Germany, in 2003 from several hundred individuals derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from 80 gravid females

caught at a single locality in Malawi. In each generation several hundred individuals are reared maintaining high levels of heterozygosity (Saccheri and Bruford 1993). For this study butterflies from the Bayreuth stock population were used.

Experimental design

All butterflies were reared and maintained in an environmental cabinet at a constant temperature of 27°C, high humidity (70 %) and a photoperiod of L 12 : D 12 (24h light cycle). These conditions are similar to those at which the butterflies develop and reproduce during the favorable wet season in the field (Brakefield 1997). Larvae were fed on young maize plants, adults on moist banana. Throughout all experiments, banana was replaced every other day. Two separate experiments were performed to investigate effects of food stress and density on life-history traits.

Experiment 1: Effects of larval food stress and larval density on life-history traits

For this experiment eggs collected from several hundred females were kept in plastic pots (20 x 15 x 6 cm) containing moistened filter paper and fresh cuttings of maize. Three days after hatching larvae were assigned to one of four treatment groups and transferred to elongated, sleeve-like gauze cages containing potted maize plants. The following treatments were used: (1) high density (= 50 individuals per cage) with or without food stress and (2) low density (= 20 individuals per cage) with or without food stress. Larvae from the food stress treatments were starved for 24 ± 2h on day 15 after hatching (i.e. in the last larval instar), whereas the control groups were provided food in ample supply throughout. A rather late time for the starvation period was chosen because daily weight gain is highest in the last instar, and concomitantly effects of food shortage should be most pronounced. For the high density treatments three replicates each, for the low density treatments eight replicates each were used (i.e. 22 replicates in total). Sleeve cages (= replicates) were checked on a daily basis and plants were replaced if necessary. To even out minor temperature differences within the environmental cabinet, the cages were rotated every other day.

Pupae were collected daily and weighed to the nearest 0.01 mg the day following pupation (Sartorius microscale MC 210 P). Afterwards, they were placed individually in translucent plastic pots (125 ml) until adult eclosion. Following eclosion, all males were pooled (thus randomizing male larval feeding regime), while females were

individually marked and kept separated by eclosion day. All females were given a premating time of two days separated from males, after which an equal number of random virgin males was added to the females' cages for two days. After the mating period females were placed individually in translucent plastic pots (1 L, covered with gauze) containing a fresh cutting of maize for egg-laying. Thus, oviposition started for all females on day five of adult life. Eggs were collected, counted and measured daily during the first seven days of the oviposition period (see below).

Experiment 2: Effects of larval and adult food stress and male company on life-history traits

In the second experiment, larvae were reared in big population cages (50 x 50 x 50 cm) containing potted maize plants in ample supply, which were replaced if necessary. There were two food stress levels: either no food stress or two-times 24 ± 2h of starvation with one feeding day in-between during the last larval instar. A more intense stress regime was used here to exaggerate starvation effects. Per treatment, two replicates containing about 300 individuals each were used. Pupae and butterflies were treated in the same manner as described above until the onset of oviposition. During oviposition, both larval treatment groups were randomly assigned to one of four treatments: (1) female alone in egg-laying pot, access to moist banana throughout; (2) female plus one male in egg-laying pot, access to moist banana throughout; (3) female alone, access to moist banana every other day only; (4) female plus one male, access to moist banana every other day only. All individuals had access to water throughout. Male butterflies were added in order to test whether female harassment affects the females' reproductive output. Females are unlikely to re-mate under those restricted conditions (1 L pots, no re-matings were observed during the course of the experiment), and moreover only old, non-virgin males were used. Males that died during the course of the experiment were replaced daily. Thus, there were eight treatment groups in total (including different levels of larval food stress). Again, eggs were collected, counted and measured daily during the first seven days of the oviposition period (see below).

Data analysis

Pupal mass was measured for all animals (males and females); early fecundity (number of eggs laid within the first seven days of the oviposition period; early

fecundity correlates closely (r^2 = 0.67) with lifetime fecundity in *B. anynana,* Brakefield et al. 2001), egg size and reproductive investment (calculated as the product of mean egg size (averaged over oviposition period) and fecundity) were recorded for all females. In *experiment 1* we additionally measured adult fresh mass, larval and pupal development time and larval growth rate. Tight correlations between pupal and adult mass (males: r^2 = 0.74, p < 0.0001, n = 187; females: r^2 = 0.80, p < 0.0001, n = 186) suggest that pupal mass is a reliable proxy for adult body size. Hence, adult mass was not measured in *experiment 2*. Growth rate was calculated as (ln pupal weight / larval development time) * 100. As the eggs of *B. anynana* are nearly perfectly spherical, egg size was measured as cross-sectional area [mm²] using a digital camera (Leica DC300) connected to a binocular microscope. The resulting images were analyzed using Scion Image public software (Scion Corporation 2000). Tight correlations between egg area (applying image analysis) and egg mass as well as hatchling size confirm that this method provides a highly reliable measurement of egg size in *B. anynana* (Fischer et al. 2002). To calculate egg size for individual females, the mean across all measurement days was used as between-day variation in egg size was negligible.

All data were analysed using nested analyses of variance (ANOVAs), with treatment and sex as fixed factors and replicates nested within treatments (to control for environmental differences across replicates). Pupal mass was added as a covariate as appropriate. All statistical tests were performed using JMP version 4.02 (SAS Institute 2000). Throughout the text all means are given ± 1 SE.

Results

Experiment 1: Effects of larval food stress and larval density

Larval density significantly affected larval development time, larval growth rate and pupal mass (Table 1). High larval densities reduced larval development time and pupal mass, but increased larval growth rate (Fig. 1). Larval food stress significantly reduced larval growth rate, pupal and adult mass, whereas larval development time was significantly prolonged (Table 1, Fig. 1). Significant interactions between larval density and food stress for pupal and adult mass (Table 1) suggest that larvae reared at a high density suffered more from food limitation than those reared at a low density (Fig. 1).

Table 1. Nested analyses of (co-)variance (AN(C)OVA) for the effects of larval density, larval food stress, replicates and sex on life-history traits in *Bicyclus anynana*. Only significant interaction terms are given. Significant *p*-values are printed in bold.

Trait	Source	*df*	*Mean squares*	*F*	*p*
Larval time [d]	Larval density	1	36.2	10.4	**0.001**
r^2 = 0.30	Larval food stress	1	40.7	11.6	**0.001**
	Replicates [Density, Stress]	17	15.4	4.4	**<0.0001**
	Sex	1	308.3	88.1	**<0.0001**
	Error	453	3.5		
Larval growth rate [%]	Larval density	1	43.0	5.7	**0.018**
r^2 = 0.22	Larval food stress	1	162.8	21.6	**<0.0001**
	Replicates [Density, Stress]	17	36.1	4.8	**<0.0001**
	Sex	1	133.7	17.7	**<0.0001**
	Error	453	7.6		
Pupal time [d]	Larval density	1	0.26	1.62	0.203
r^2 = 0.16	Larval food stress	1	0.39	2.38	0.123
	Replicates [Density, Stress]	17	0.27	1.66	**0.048**
	Sex	1	11.84	72.90	**<0.0001**
	Density x Sex	1	0.72	4.40	**0.036**
	Density x Stress x Sex	1	0.81	5.02	**0.026**
	Error	478	< 0.01		
Pupal mass [mg]	Larval density	1	5193.2	10.0	**0.002**
r^2 = 0.52	Larval food stress	1	14198.8	27.3	**<0.0001**
	Replicates [Density, Stress]	17	1244.9	2.4	**0.001**
	Sex	1	199102.2	383.4	**<0.0001**
	Density x Food stress	1	2425.0	4.7	**0.031**
	Error	462	519.3		
Adult mass [mg]	Larval density	1	16.0	0.2	0.682
r^2 = 0.73	Larval food stress	1	3517.4	37.0	**<0.0001**
	Replicates [Density, Stress]	17	74.9	0.8	0.707
	Sex	1	74760.0	786.6	**<0.0001**
	Density x Food stress	1	1028.7	10.8	**0.001**
	Error	350	352.5		
Mean egg size [mm²]	Larval density	1	<0.01	0.1	0.721
r^2 = 0.14	Larval food stress	1	<0.01	1.9	0.166
	Replicates [Density, Stress]	17	<0.01	1.3	0.225
	Pupal mass	1	0.01	6.8	**0.010**
	Density x Food stress	1	0.01	3.8	0.051
	Error	212	<0.01		
Early fecundity	Larval density	1	2704.9	0.7	0.402
r^2 = 0.15	Larval food stress	1	4631.1	1.2	0.273
	Replicates [Density, Stress]	17	4937.8	1.3	0.202
	Pupal mass	1	23097.1	6.0	**0.015**
	Error	211	3831.2		
Reproductive investment [mg]	Larval density	1	939.4	0.6	0.435
r^2 = 0.15	Larval food stress	1	2456.5	1.6	0.208
	Replicates [Density, Stress]	17	1760.0	1.1	0.313
	Pupal mass	1	12055.4	7.8	**0.006**
	Error	211	1537.4		

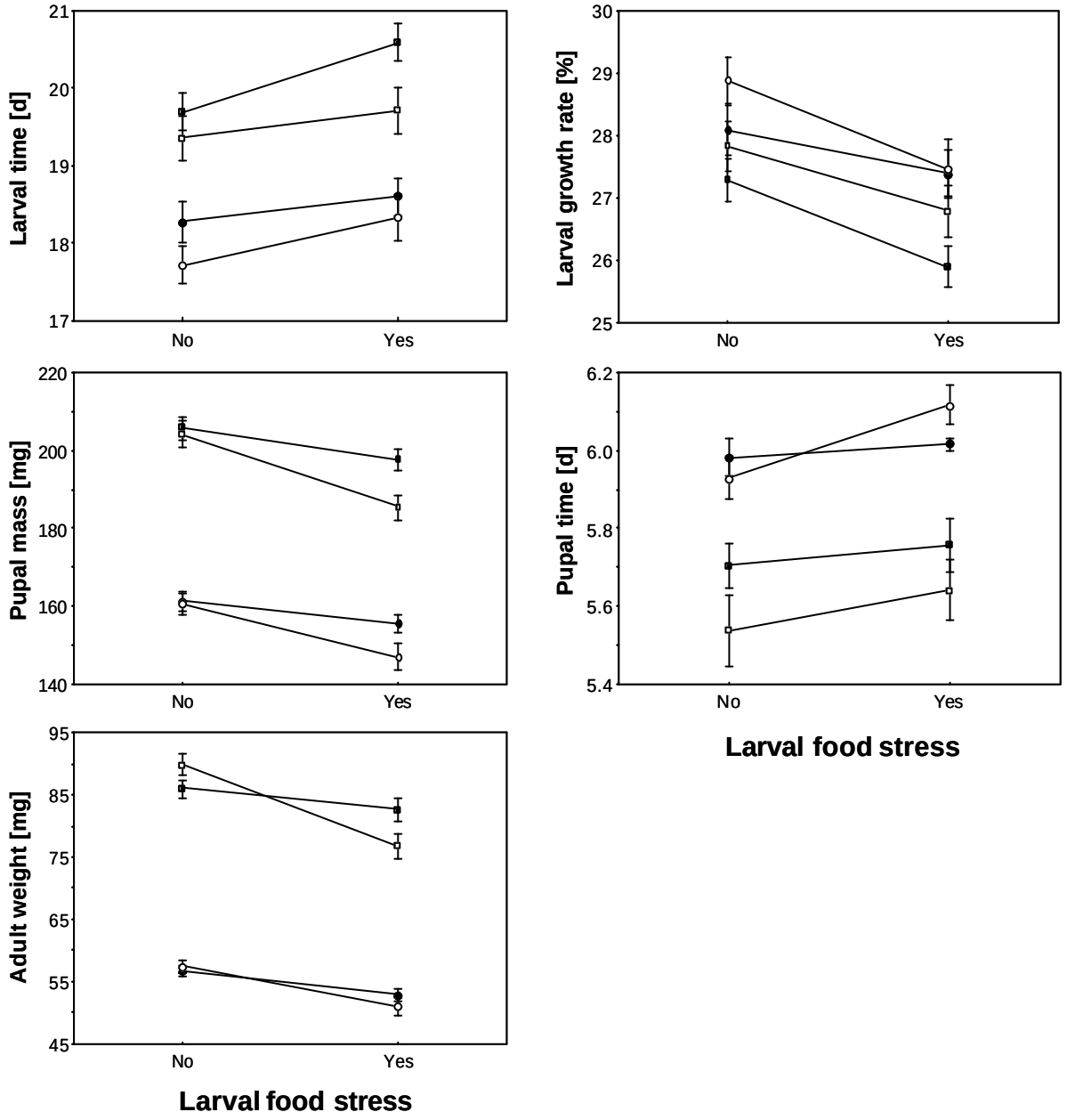

Fig. 1. Effects of larval density and larval food stress on life-history traits (means ± 1 SE) in *Bicyclus anynana*. All data were pooled for replicates; group sample sizes range between 43 - 74 (total *n* = 478). Squares: females; circles: males; filled and open symbols: low respectively high larval rearing densities.

Neither larval food stress nor density affected egg size, early fecundity or reproductive investment directly (Table 1), though pupal mass significantly influenced all traits. Removing the covariate pupal mass from the analyses presented in Table 1 shows that larval food stress does significantly affect these traits (egg size: $F_{1,234}$ =

1.9, p = 0.029; early fecundity: $F_{1,233}$ = 1.2, p = 0.036; reproductive investment: $F_{1,233}$ = 4.5, p = 0.034) (see Fig. 2).

Thus, the effect of larval food limitation on egg size, fecundity and reproductive investment is at least largely mediated through its effect on pupal mass. Larval density, in contrast, had no effect on either of the reproductive traits (all p-values > 0.4).

However, a significant interaction between larval density and food stress concerning egg size emerged when removing the covariate pupal mass ($F_{1,234}$ = 3.8, p = 0.029). Females reared at a low density showed no difference in egg size irrespective of the level of food stress, whereas those reared at a high density laid smaller eggs when access to food was limited. When pupal mass was controlled for, this interaction was also nearly significant (Table 1).

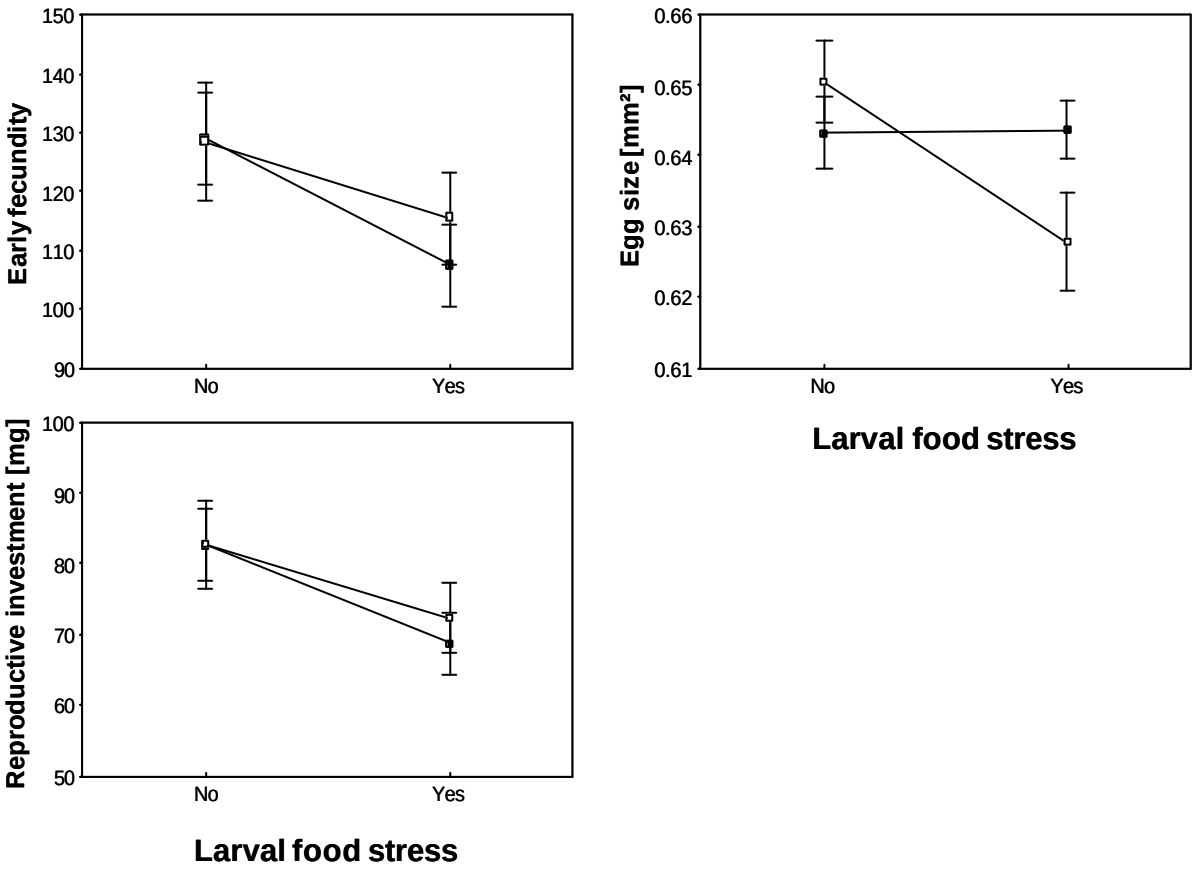

Fig. 2. Effects of larval density and larval food stress on female reproductive traits (means ± 1 SE) in *Bicyclus anynana*. All data were pooled for replicates; group sample sizes range between 44 - 66 (total n = 233). Filled and open symbols: low and high larval rearing densities.

Females and males of *B. anynana* differed strikingly in larval growth rate, larval and pupal development time, pupal mass and adult mass (Table 1), with females having on average lower larval growth rates (27.0 ± 0.2 % versus 28.0 ± 0.2 %), longer larval times (19.8 ± 0.1 days versus 18.2 ± 0.1 days), slightly shorter pupal times (5.7 ± 0.3 days versus 6.0 ± 0.3 days), higher pupal masses (199.1 ± 1.4 mg versus 156.3 ± 1.6 mg) and higher adult masses (84.5 ± 0.7 mg versus 54.7 ± 0.7 mg) than males.

The significant interactions between sex and larval density respectively food stress found for pupal development times reflect some marginal variation in this trait (Table 1, Fig. 1). Replicates differed significantly in larval growth rate, larval and pupal development time and pupal mass, though the magnitude of effects was generally small.

Experiment 2: Effects of larval food stress, adult food stress and male company

In this experiment larval food stress significantly reduced pupal mass (females 154.4 ± 1.5 mg versus 143.0 ± 1.6 mg; males 128.5 ± 1.5 mg versus 117.3 ± 1.6 mg), tended to increase egg size and tended to reduce fecundity (Table 2, Fig. 3). If the covariate pupal mass (affecting all reproductive traits) is removed from the analyses presented in Table 2, the significant effect of larval food stress on egg size disappears ($F_{1,350}$ = 5.5, p = 0.105), whereas significant effects of larval food limitation on early fecundity ($F_{1,350}$ = 3.1, p < 0.001) and reproductive investment ($F_{1,350}$ = 1.9, p = 0.001) emerge. The comparison of the ANCOVA and ANOVA results once again suggests that detrimental effects of larval food limitation on fecundity and reproductive investment are largely mediated through effects on pupal mass. The non-significant result for egg size suggests that, overall, effects of larval food limitation on egg size were negligible (Fig. 3), with the significant effect in the ANCOVA analysis being caused by the smaller females from the starvation treatment laying eggs similar in size to those of the larger control females. Adult food stress caused a significant reduction in egg number and reproductive investment, but had no detectable impact on egg size (Table 2). The presence of a male butterfly during oviposition had no effect on egg size, early fecundity or reproductive investment (Table 2).

Table 2. Nested analyses of (co-)variance (AN(C)OVA) for the effects of larval food stress, adult food stress, adult density (male company), replicates and sex (pupal mass only) on life-history traits in *Bicyclus anynana*. Only significant interaction terms are given. Significant *p*-values are printed in bold.

Trait	Source	*df*	*Mean squares*	*F*	*p*
Pupal mass [mg]	Larval food stress	1	29713.2	56.5	**<0.0001**
r^2 = 0.28	Replicates [Larval stress]	2	3489.1	6.6	**0.001**
	Sex	1	156143.0	297.0	**<0.0001**
	Error	913	525.7		
Mean egg size [mm²]	Larval food stress	1	0.010	5.5	**0.020**
r^2 = 0.05	Adult food stress	1	<0.001	0.2	0.657
	Adult density	1	0.002	1.1	0.295
	Pupal mass	1	0.017	9.8	**0.002**
	Replicates [Larval stress]	2	0.001	0.6	0.531
	Error	339	0.002		
Early fecundity	Larval food stress	1	8051.2	3.1	0.080
r^2 = 0.24	Adult food stress	1	12171.8	4.6	**0.032**
	Adult density	1	2659.0	1.0	0.314
	Pupal mass	1	200948.9	76.8	**<0.0001**
	Replicates [Larval stress]	2	462.6	0.2	0.838
	Error	339	2617.8		
Reproductive Investment [mg]	Larval food stress	1	2002.9	1.9	0.171
r^2 = 0.27	Adult food stress	1	5624.4	5.3	**0.022**
	Adult density	1	1462.2	1.4	0.241
	Pupal mass	1	94101.8	88.6	**<0.0001**
	Replicates [Larval stress]	2	236.9	0.2	0.800
	Error	339	1061.6		

While the ANCOVA analyses failed to reveal any significant interactions (all *p*-values > 0.1), all interactions between larval and adult food stress approached significance when analyzed with ANOVAs (egg size: $F_{1,350}$ = 2.2, p = 0.047; early fecundity: $F_{1,350}$ = 1.4, p = 0.019; reproductive investment: $F_{1,350}$ = 1.8, p = 0.010). While either larval or adult food stress had themselves very little impact on egg size (see above), adult food stress tended to increase egg size in animals having experienced food limitation during larval development, but tended to decrease egg size in control animals. Regarding early fecundity and reproductive investment, adult food stress diminished performance for those animals having experienced no larval food stress, while those being starved during larval development remained completely unaffected and exhibited a lower performance throughout (Fig. 3). The similarity of patterns shows that variation in reproductive investment is basically driven by variation in egg number.

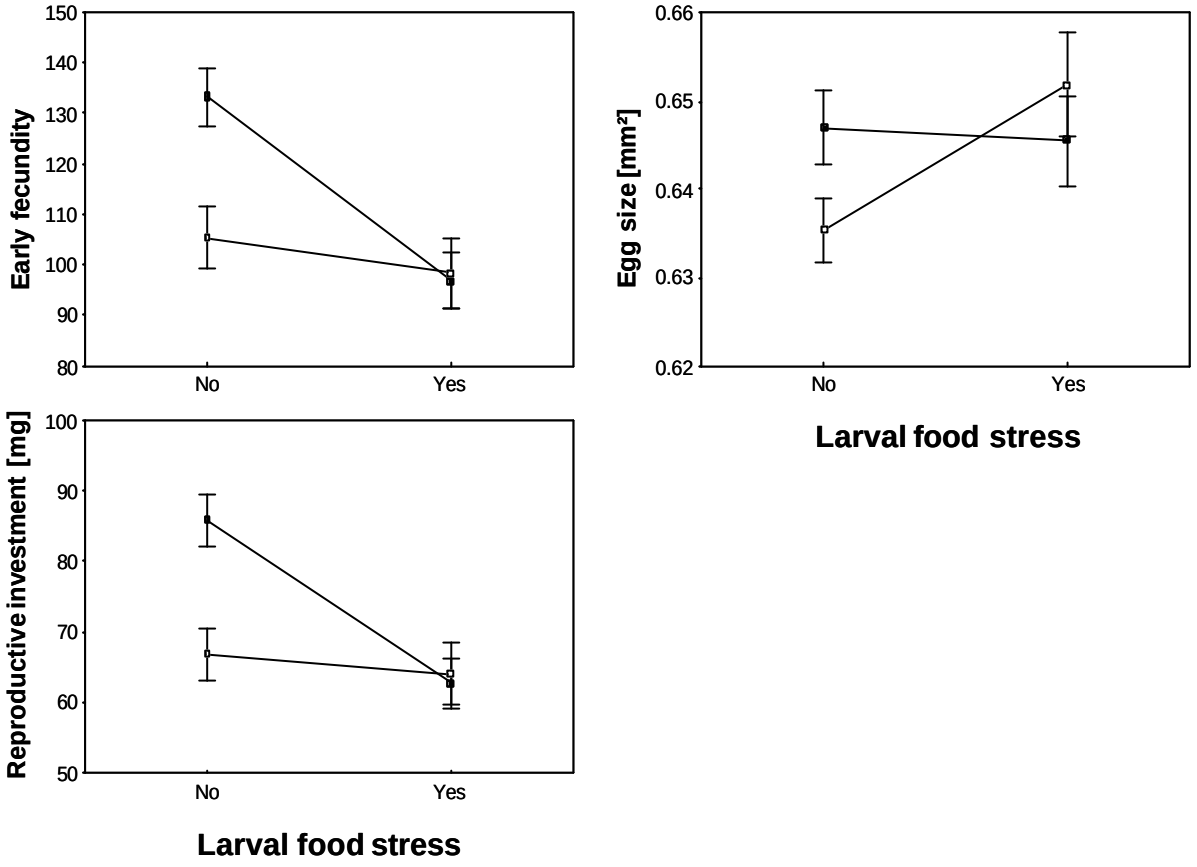

Fig. 3. Effects of adult and larval food stress on female reproductive traits (means ± 1 SE) in *Bicyclus anynana*. All data were pooled for replicates and adult densities; group sample sizes range between 68 - 104 (total n = 350). Filled symbols: no adult food stress; open symbols: adult food stress.

As in *experiment 1*, sexes differed in pupal mass with females being significantly larger than males (149.2 ± 1.1 mg versus 123.4 ± 1.1 mg; Table 2). Also, replicates differed to a lesser extent in pupal mass, but not in any of the reproductive traits.

Discussion

Effects of larval food stress and density on life-history traits

As expected, food stress during the larval stage significantly influenced life-history traits by reducing body size and larval growth rate, a result believed to be universal and generally predicted by life-history models (Berrigan and Charnov 1994; Gotthard and Nylin 1995; Arendt 1997; Blanckenhorn 1999). Larval development time was significantly prolonged, suggesting that larvae facing food stress

compensate for temporarily reduced nutrient intake by extending the larval period and thus the time available for feeding.

Nevertheless those larvae could not fully compensate for a 24h-period of starvation, as they remained smaller than control individuals (Fig. 1). Comparable results were found in other insects (e.g. Collins 1980; Blanckenhorn 1999; Fischer and Fiedler 2001b) suggesting that development time *and* body size are generally important fitness parameters (e.g. Peters 1983; Yampolski and Scheiner 1996). If only one of these traits were of prime importance, either a full compensation in body size (by further increasing larval time) or no change in larval time at all (at the expense of an even smaller body size) would be expected.

A high larval rearing density in the nongregarious larval stage resulted in reduced larval development time (and pupal mass), but increased larval growth rate (Fig. 1). Thus, the pattern differs strikingly from the effects of food limitation, causing a prolonged larval time and decreased growth rate (see above). This difference has several important implications. First, growth rates are not maximized under 'normal' conditions, as has been traditionally postulated, but may vary according to environmental cues (Abrams et al. 1996; Arendt 1997). Second, accelerated growth (as found here and e.g. by Leonard 1968 for the gypsy moth, *Lymantria dispar*) is likely to be an adaptation to high density, presumably driven by the risk of larval food resources becoming exhausted before metamorphosis (Blanckenhorn 1999). Obviously, reaching the adult stage at all, even at the expense of being smaller, is much more important than achieving a large body size and concomitantly enhancing competitive ability. Also, the increase in growth rate proves that densities were successfully manipulated independently of food stress in our experiment (i.e. food was not limiting even at high densities). Third, a reduction in pupal mass with increasing larval density, as was repeatedly found in several insects (e.g. Leonard 1968; Credland et al. 1986; Borash and Ho 2001; Agnew et al. 2002), may be a consequence of density *per se* (accompanying accelerated growth) or of food limitation.

It should be noted, however, that, under conditions where food is not limiting, there is very little evidence for effects of larval density on body size in our experiment (no evidence at all for adult mass, Table 1). A decrease in body size was found only if high density coincides with restricted food conditions (Fig. 1). The significant interaction between larval density and food stress, with larvae reared at a high

density suffering stronger from food limitation, suggests that the accelerated growth at high density impairs the ability to achieve a large body size. Anyway, as the same pattern of reduced body size may arise through different pathways, there is need for thorough experimental approaches effectively controlling for confounding effects of food shortage.

Sex-specific differences in life-history traits were found, as expected, in larval growth rate, larval and pupal development time, pupal and adult mass. A faster growth of males, also found in the majority of other butterflies and insects, is generally attributed to selection on protandry in males (to maximize the number of matings) and females (to minimize pre-reproductive period; e.g. Fagerström and Wiklund 1982; Zonneveld and Metz 1991), while larger female body size can be explained by fecundity selection (Roff 1992; Honek 1993). The shorter pupal development time in females (not affecting protandry) is special to *B. anynana* with no explanation being available (see also Fischer et al. 2002).

Effects of larval and adult food stress on female reproductive traits

Larval food limitation consistently had negative effects on fecundity and reproductive investment in both experiments, which were basically mediated through a reduction in body size. Obviously shortage of stored resources derived from larval feeding is responsible for these findings. Adult food limitation further reduced reproductive output, as was expected, because *B. anynana* cannot mature eggs without adult income (Fischer et al. 2004). Note that stressed females had access to banana every other day and thus, on principle, had the opportunity for compensatory feeding. However, the significant effects of adult feeding regime on fecundity and reproductive investment suggest that females were not able to fully compensate for the food limitation during starvation days. Thus, both, larval and adult resources are important to reproduction in this species. If resources are limited, allocation to maintenance or storage may take precedence over allocation to reproduction, resulting in decreased reproductive output (Zera and Harshman 2001). Accordingly Boggs and Ross (1993) found that decreased fecundity under adult food limitation was accompanied by increased oocyte resorption, with resources being reallocated to somatic maintenance (see also Brough and Dixon 1990). Further, food stress may trigger responses of the neuroendocrine system, resulting in additional effects on the onset and extent of egg production (e.g. Slansky 1980).

Most interestingly, however, we found clear interactions between larval and adult food stress regarding fecundity and reproductive investment. While adult food stress diminished performance for those animals having experienced no larval food stress, those being starved during larval development remained completely unaffected and exhibited a lower performance throughout. Thus, butterflies with reduced storage reserves could not take advantage of having access to adult income *ad libitum*, suggesting that the upper limit for the use of adult diet is set by nutrients accumulated during the larval stage. On the other hand, egg manufacture of individuals facing no larval food shortage (associated with larger storage reserves) is considerably impaired if adult diet is restricted, proving the importance of a sufficient carbohydrate intake. Thus, storage reserves *and* adult income are required for reproduction in *B. anynana*, with different nutrients becoming limiting under different circumstances (i.e. food limitation in the larval or adult stage; see also O'Brien et al. 2004; Fischer et al. 2004). Therefore, it is not obvious upon first sight what is ultimately limiting to reproduction, suggesting that either stressing the importance of larval- or adult-derived resources would inadequately reflect the truth. Rather, our findings stress the need for resource congruence.

While it is clear that carbohydrates from adult income are extensively used for egg manufacture in *B. anynana* (approximately 50 % of egg carbon derives from adult intake; Fischer et al. 2004), we can at this point only speculate what the limiting factor regarding larval-derived resources is, though it is very likely nitrogenous compounds that are essentially absent in the adult diet (Fischer et al. 2004). Recently, O'Brien et al. (2002) showed that essential amino acids are likely to be ultimately limiting to reproduction, as these cannot, in contrast to nonessential amino acids, be synthesized using carbohydrates from adult diet and endogenous nitrogen sources. Thus, the availability of either essential and/or nonessential amino acids could be limiting to reproduction, as even the synthesis of the latter requires endogenous nitrogen sources from the larval diet.

In contrast to effects on fecundity and reproductive investment, effects of larval or adult food stress on egg size were generally small or even absent, and were also in part inconsistent among experiments. Thus, in line with earlier studies (e.g. Collins 1980) food stress had, overall, very little impact on egg size, though some studies report changes in egg composition under resource limitation (Briegel 1990; Tessier and Consolatti 1991; Boggs and Ross 1993).

In spite of the above mentioned effects on life-history traits, larval rearing density did not affect either of the reproductive traits. Likewise, the presence of a male during the oviposition had no effect. Thus, neither did male company substantially limit the time available for oviposition or feeding (i.e. females were probably not harassed by males under the given experimental arrangement), nor could the females benefit from potentially occurring second matings (which were unlikely; see Methods).

In summary, our study highlights the importance of considering the role of larval-*and* adult-derived resources for reproduction in holometabolous insects (see also Boggs and Ross 1993). Restricted food access in different developmental stages may set strikingly different limits (either posed by shortage of nitrogenous compounds or carbohydrate) to reproduction. For our study organism it is not possible to state that either larval or adult resources are ultimately limiting to reproduction, as they are both: adult income is essential for maturing eggs (Fischer et al. 2004), but adult intake cannot compensate for reduced storage reserves. Similar situations are likely to apply to many arthropods, with, if anything, resource congruence being ultimately limiting. To fully understand the functional basis of reproductive patterns more studies elucidating the relative importance of stored and current resources are needed.

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6.2 Effects of adult-derived carbohydrates, amino acids and micronutrients on female reproduction in a fruit-feeding butterfly

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Abstract

It is generally believed that butterflies (and other holometabolous insects) rely primarily on reserves accumulated during the larval stage for reproduction, whereas the carbohydrate-rich adult diet is thought to mainly cover energy requirements. In at least some species though, realization of the full reproductive potential is extensively affected by post-eclosion nutrition. While the importance of carbohydrates is fairly well understood, the role of adult-derived amino acids and micronutrients is controversial and largely unknown, respectively. We here focus on the effects of different adult diets on female reproduction in the tropical, fruit-feeding butterfly *Bicyclus anynana* (Nymphalidae). Carbohydrates were the most important adult-derived nutrients affecting reproduction. Adding amino acids, vitamins or minerals to sucrose-based solutions did not yield a reproductive output equivalent to that of fruit-fed females, which showed the highest performance throughout. This suggests that either not yet identified compounds of fruit substantially contribute to reproduction, or that resource congruence (the use of nutrient types in a specified ratio) rather than any specific nutrient component is of key importance. Apart from adult income, realised fecundity depended on egg size and longevity, with the former dominating when dietary quality was low, but the latter when quality was high. Thus, the egg size-number trade-off seems to be affected by female nutrition.

Introduction

It is generally believed that Lepidoptera, as is the case in many holometabolous insects, rely primarily on nutrients accumulated during the larval stage for somatic maintenance and reproductive output (Leather 1995; Telang et al. 2001; Mevi-Schütz and Erhardt 2003a). In contrast to the protein-rich larval diet, adult diet (such as nectar or rotting fruit) is typically rich in carbohydrates and poor in amino acids (Watt et al. 1974; Baker and Baker 1975; 1983; Romeis and Wäckers 2000; O'Brien et al. 2004). However, some species supplement their adult diet with substrates ranging from pollen to mud, dung or carrion or by preferring amino acid-containing nectars (e.g. Gilbert 1972; Boggs and Jackson 1991; Braby and Jones 1995; Smedley and Eisner 1996; Beck et al. 1999; Rusterholz and Erhardt 2000). Adult feeding mainly covers energy requirements (general maintenance including flight expenditures, e.g. Willers et al. 1987; O'Brien 1999), but has also been found to affect reproductive

output in at least some butterfly and moth species (e.g. Murphy et al. 1983; Leather 1984; Hill 1989; Boggs and Ross 1993; O'Brien et al. 2000; Fischer and Fiedler 2001; Fischer et al. 2004).

As insect eggs consist primarily of protein (Engelmann 1999) female Lepidoptera are in high demand for amino acids. However, amino acids are generally scarce in their sugar-rich adult diet (O'Brien et al. 2002). Nonessential amino acids have been shown to be extensively synthesised by females using adult-derived carbon and endogenous nitrogen-sources (O'Brien et al. 2002), whereas essential amino acids are entirely larval in origin and therefore likely ultimately limiting to reproduction. Hence, the role of amino acids seems to be particularly relevant for the understanding of nutritional constraints on insect reproduction (O'Brien et al. 2002).

However, the role of amino acids in the diet of adult butterflies is controversial (e.g. Beck et al. 1999, Mevi-Schütz and Erhardt 2003b). Some species preferentially feed on nectars with high amino acid contents (e.g. *Colias*, Watt et al. 1974), whereas others do not (e.g. *Battus philenor*, Erhardt 1991; *Ornithoptera priamus*, Erhardt 1992). Selective feeding on nitrogenous diets may reflect the nutritional status of the butterflies (Rusterholz and Erhardt 2000; Mevi-Schütz and Erhardt 2003c), and seems to be restricted to females because of their higher demand for protein (e.g. Alm et al. 1990; Erhardt and Rusterholz 1998; Rusterholz and Erhardt 2000; Mevi-Schütz and Erhardt 2003c). Nevertheless, no or only weak effects of amino acids on longevity and reproductive success of female butterflies were found in various species (Hill 1989; Hill and Pierce 1989; O'Brien et al. 2000; Romeis and Wäckers 2002; Mevi-Schütz and Erhardt 2003c), whereas fitness improved substantially in others (e.g. pollen-feeding *Heliconius* butterflies, Gilbert 1972; Dunlap-Pianka et al. 1977; *Euphydryas editha,* Murphy et al. 1983; but see Moore and Singer 1987 for contradictory results on this species).

In contrast to the majority of nectar-feeding butterflies in temperate zones, many tropical species feed on rotting fruits (Braby and Jones 1995). In spite of further progress in understanding the role of nectar for reproductive output in butterflies (Boggs 1997; O'Brien et al. 2002; O'Brien et al. 2004), studies on the impact of fruit-feeding on butterfly life histories are scarce (e.g. Fischer et al. 2004). Informal speculations that fruit could be a richer source of nitrogenous compounds than is nectar could not be confirmed by recent data; both resources appear to be rather similar in nutrient composition (Bosque and Pacheco 2000; Omura and Honda 2003;

Fischer et al. 2004). However, rotting fruit may provide yeast to fruit-feeding Lepidoptera, which is an excellent source of protein to insect frugivores (Good and Tatar 2001) rendering rotting fruit a potentially important source for amino acids.

Feeding on rotting fruits, however, does not only provide carbohydrates and some amino acids, but also detectable amounts of micronutrients such as minerals (e.g. potassium, magnesium and phosphorous) and a variety of vitamins (cf. University of Hohenheim 1996), which could contribute to reproductive output (either indirectly via beneficial effects on overall performance or by a direct contribution to egg provisioning). Despite numerous studies focussing on larval nutrition (e.g. Vanderzant et al. 1962; Rodriguez 1972; Murugan and George 1992; Barbehenn et al. 1994; Stamp 1994; Barbehenn et al. 2001; Woods et al. 2002; Perkins et al. 2004), studies on the importance of adult-derived minerals and vitamins for somatic maintenance and reproductive output are still scarce (e.g. Smedley and Eisner 1996; Engelmann 1999). Nevertheless, such adult-derived micronutrients appear to affect egg production in at least some insect species (e.g. Pappas and Fraenkel 1977; Engelmann 1999). Currently, the best studied example for the importance of adult-derived micronutrients for butterfly reproduction is the uptake of sodium by mud-puddling males (e.g. Boggs and Jackson 1991; Smedley and Eisner 1995; Beck et al. 1999).

Here we investigate the relative importance of different nutritional components on female reproduction in the tropical, fruit-feeding butterfly *Bicyclus anynana* (Butler, 1879) (Lepidoptera: Nymphalidae). We performed two separate experiments specifically focussing on the fitness effects (rather than preferences that may not necessarily indicate a fitness advantage) of adult-derived nutrients. While in *experiment 1* the effects of carbohydrates and amino acids were investigated, *experiment 2* focussed on the role of micronutrients (minerals, vitamins) for female reproductive output.

Material and Methods

Study organism

B. anynana is a tropical, fruit-feeding butterfly ranging from Southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and

predation (Brakefield 1997; Lyytinen et al. 2004). A laboratory stock population was established at Bayreuth University, Germany, in 2003 from several hundred individuals derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from 80 gravid females caught at a single locality in Malawi. In each generation several hundred individuals are reared maintaining high levels of heterozygosity at neutral loci (Saccheri and Bruford 1993). For this study butterflies from the Bayreuth stock population were used.

Experimental design

All butterflies were reared and maintained in an environmental cabinet at a constant temperature of 27°C, high relative humidity (70 %) and a photoperiod of L12:D12 (24h light cycle). These conditions are similar to those at which the butterflies develop and reproduce during the favourable wet season in the field (Brakefield 1997). Larvae were reared in big population cages (50 x 50 x 50 cm) containing potted maize plants in ample supply, which were replaced if necessary. Following adult eclosion, all males were pooled and given access to moist banana, while females were individually marked and randomly assigned to different dietary treatments as described below. Females were given a premating time of one day separated from males, after which an equal number of random virgin males was added to the females' cages for two days. After the mating period females were placed individually in translucent plastic pots (1 L, covered with gauze) containing a fresh cutting of maize for egg-laying. Thus, oviposition started for all females on day four of adult life. Eggs were collected, counted and measured every other day until the death of the butterflies.

Experiment 1

In this experiment females were divided among the following five treatment groups: access to 1) water only, 2) a saturated solution of essential and non-essential amino acids (ca. 40 V% Miragel®-Gelatine, see Table 1a), 3) a highly concentrated sucrose solution (ca. 40 V% to provide the butterflies with an excess supply of carbohydrates; hereafter sugar), 4) a highly concentrated sucrose solution (ca. 40 V%) enriched with amino acids (Miragel®-Gelatine) or 5) moist banana.

Females were fed on the respective diets throughout their lives, i.e. from the eclosion day onwards.

As the above experiment indicated that a few females were able to mature a substantial number of eggs when having access to amino acids but not to any carbohydrate source (see Results), we tested by means of artificial selection whether this ability is heritable. Therefore, the number of eggs laid within the first four days of the oviposition period was counted for 200 female stock population butterflies, having access to an amino-acid solution only. Females laying more than 20 eggs were selected as parents for the next generation. In subsequent generations between 100 and 200 females were measured and treated the same way. Throughout, the same rearing, mating and oviposition schedule as described above was used. After four generations of selection the experiment was terminated and the resulting line was compared to an unselected control line.

Experiment 2

As *experiment 1* indicated that neither feeding carbohydrates, amino acids nor a combination of both yielded a reproductive output equivalent to that of banana-fed females, a second experiment was designed to assess the importance of other potentially fitness-relevant constituents of banana (namely minerals and vitamins). Thus, females were assigned to one of the following five treatment groups: access to 1) sucrose solution (25 V%; hereafter sugar), 2) sucrose solution (25 V%) enriched with a mixture of vitamins, 3) sucrose solution (25 V%) enriched with minerals, 4) sucrose solution (25 V%) enriched with a combination of vitamins and minerals or 5) moist banana (see Table 1b for concentrations of vitamins and minerals). We included in our diets the minerals and vitamins being most abundant in banana (cf. University of Hohenheim 1996), however, amplified concentrations ten- and twentyfold, respectively, to increase the probability of detecting potentially weak effects on reproductive output.

Table 1. Composition of diets used in *experiments 1* (a) and *2* (b). a) Amounts of amino acids per 100 g protein (Miragel®–Gelatine; intraGEL G.F. Menrath GmbH+Co. KG; 100 g Miragel® contain: 84 g protein, 14 g water, 2 g salts, 0 g carbohydrates); division in essential and nonessential amino acids (specifically for insects) follows Ito and Inokuchi (1972) and Barbehenn et al. (1994); b) amounts of minerals and vitamins per 1 L 25 V% sucrose solution.

a)

Essential amino acids			
Arginine	9.1 g	Methionine	0.9 g
Histidine	0.8 g	Phenylalanine	2.2 g
Isoleucine	1.3 g	Threonine	1.6 g
Leucine	3.1 g	Tryptophan	0.0 g
Lysine	4.2 g	Valine	2.4 g

Nonessential amino acids			
Alanine	8.9 g	Hydroxiproline	12.7 g
Asparagine	5.9 g	Proline	13.4 g
Glutamine	11.4 g	Serine	3.2 g
Glycine	17.5 g	Tyrosine	0.2 g
Hydroxilysine	1.0 g		

b)

Minerals			
Potassiumchloride	3900 mg	Magnesiumchloride	360 mg

Vitamins			
Retinolequivalent (Vitamin A)	4.6 mg	Pantothenic acid (Vitamin B5)	4.6 mg
Thiamine (Vitamin B1)	0.9 mg	Pyridoxine (Vitamin B6)	7.4 mg
Riboflavin (Vitamin B2)	1.1 mg	Ascorbic acid (Vitamin C)	240 mg
Niacinequivalent (Vitamin B3)	19.0 mg		

Data analysis

As the eggs of *B. anynana* are nearly perfectly spherical, egg size was measured as cross-sectional area [mm²] using a digital camera (Leica DC300) connected to a binocular microscope. The resulting images were analysed using Scion Image public software (Scion Corporation 2000). Tight correlations between egg area (applying image analysis) and egg mass as well as hatchling size confirm that this method

provides a highly reliable measurement of egg size in *B. anynana* (Fischer et al. 2002). To calculate egg size for individual females, the mean across all measurement days was used as between-day variation in egg size was negligible. Reproductive investment was calculated as the product of mean egg size (averaged over oviposition period) and lifetime fecundity.

Data were analysed using one-way ANOVAs; significant differences between treatment groups were identified using Tukey's HSD. As the prerequisites for the use of ANOVAs were not met in all cases, data were re-analysed using the non-parametric Kruskal-Wallis-test, which did not reveal any qualitative differences from the ANOVA results (results not shown). Effects of egg size and longevity on fecundity were additionally investigated using multiple regressions (stepwise forward addition of variables, Ridge regression). Survival times were scored using Mantel's procedure (Mantel 1967) followed by a c^2-test (based on sum of scores) to test for significance. All statistical tests were performed using Statistica 6.1 (StatSoft 2003). Throughout the text all means are given ± 1 SE.

Results

Experiment 1

Adult diet significantly affected survival probability (c^2_4 = 109.3, $p < 0.001$) and longevity ($F_{4,284}$ = 35.5, $p < 0.001$) of *B. anynana* females, which were lowest when no carbohydrates were provided (Table 2a, Fig. 1a). Access to carbohydrates (from sugar or banana) increased lifespan significantly, with females being additionally fed amino acids having intermediate life spans because of increased mortality rates after day 12 of the oviposition period compared to females fed on sugar only or banana (Fig. 1a).

Fecundity, mean egg size and reproductive investment were all significantly affected by dietary treatment (lifetime fecundity: $F_{4,285}$ = 95.4, $p < 0.001$; mean egg size: $F_{4,188}$ = 8.0, $p < 0.001$; reproductive investment: $F_{4,285}$ = 93.1, $p < 0.001$). Fecundity and reproductive investment depended strongly on carbohydrate intake, without which hardly any (water only) or only very few (amino acids) eggs were laid (Table 2a). Females fed on moist banana achieved the highest fecundity and reproductive investment, while amino acids added to sugar did not enhance either reproductive trait significantly (as compared to sugar only; Table 2a). However, the group of females being fed on sugar with amino acids deposited significantly more

eggs than females fed on banana during the first four days of the oviposition period (Tukey HSD after ANOVAs on daily fecundity), but showed a steep decline in egg numbers afterwards (Fig. 2a). Throughout, egg numbers peaked on oviposition days 3 - 4, with the subsequent decline being less accentuated in the banana-fed females than in the other groups (Fig. 2a). Egg size was largest in the banana-group, with all other treatments being statistically indistinguishable (note that females with access to water laid eggs comparable in size, however, only single eggs were deposited; Table 2a).

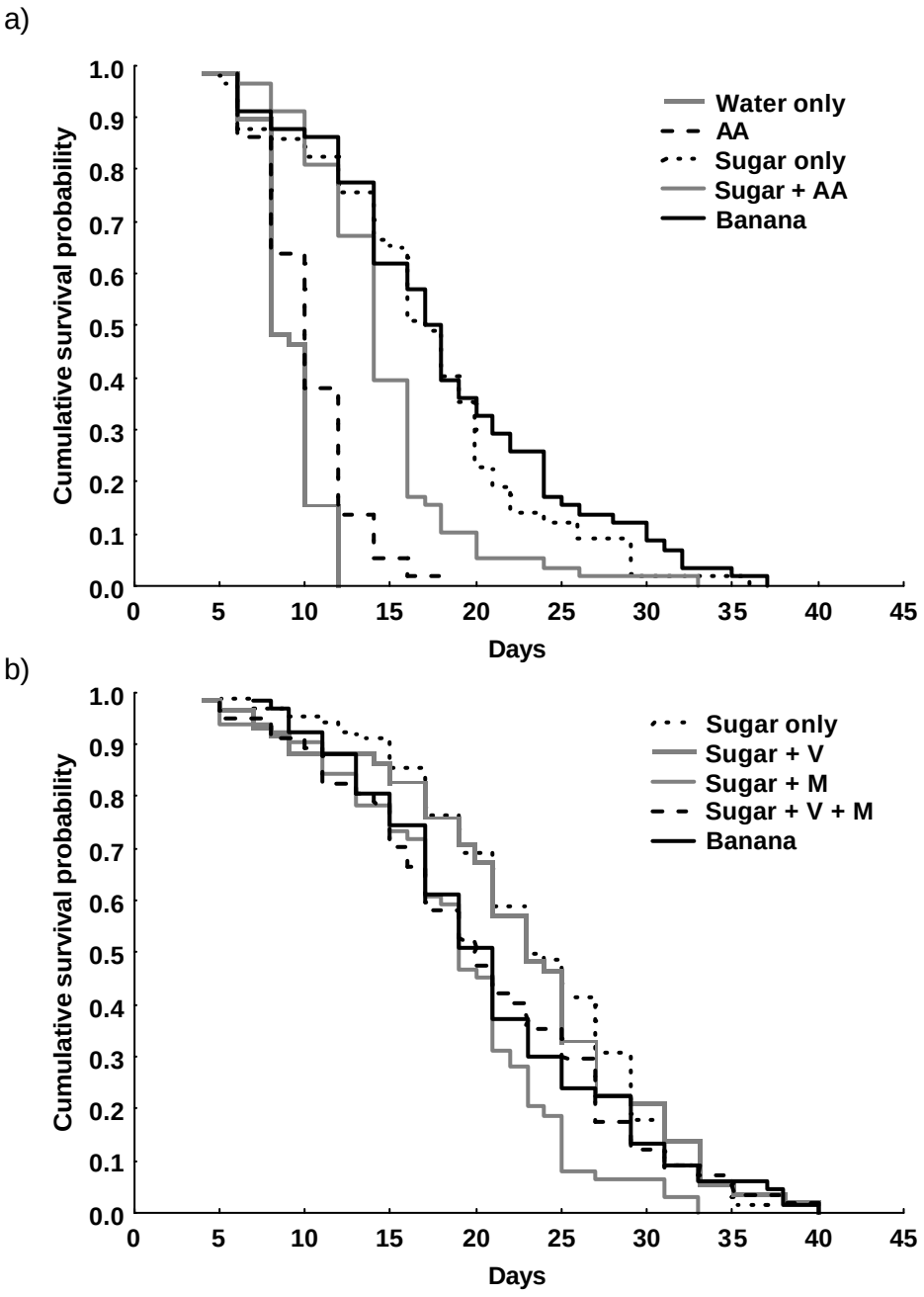
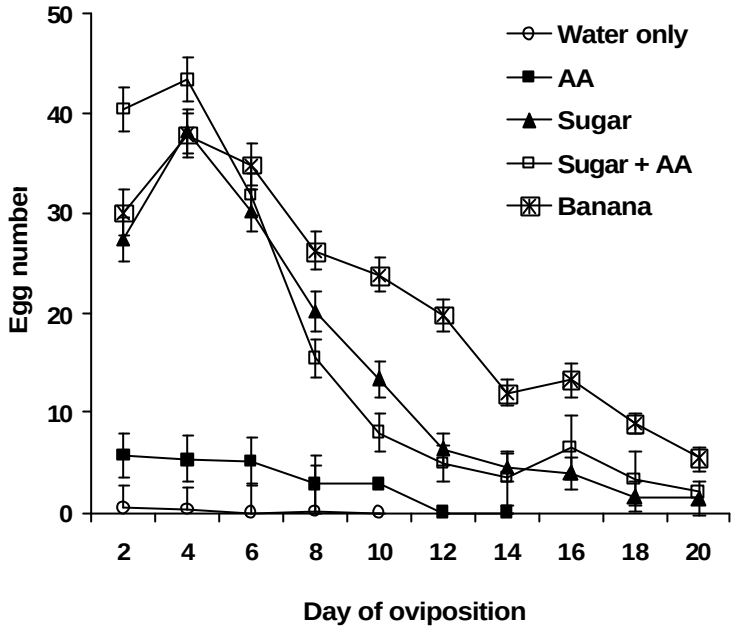


Fig. 1. Cumulative survival probabilities of female *Bicyclus anynana* fed on different diets for *experiments 1* (a) and *2* (b). AA: Amino acids; V: Vitamins; M: Minerals.

a)



b)

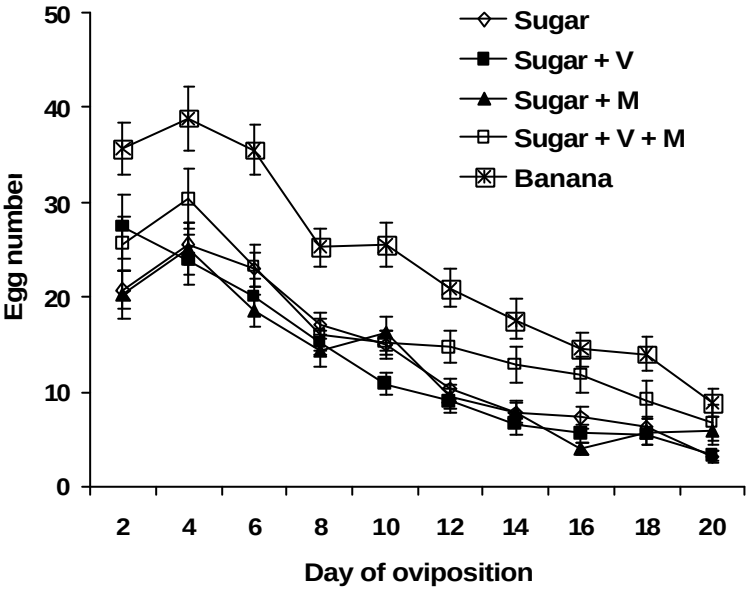


Fig. 2. Egg number over time (group means ± 1 SE) for female *Bicyclus anynana* fed on different diets for *experiments 1* (a) and *2* (b). AA: Amino acids, V: Vitamins, M: Minerals.

Table 2. Effects of adult diet on female longevity and reproductive traits (mean with SE in parenthesis) in *Bicyclus anynana* for *experiments 1* (a) and *2* (b). Different superscript characters within rows indicate significant differences between treatment groups (Tukey HSD after ANOVA). AA: Amino acids; V: Vitamins; M: Minerals (for diet composition see Methods).

a)

Trait	Water only	AA	Sugar	Sugar + AA	Banana
Longevity [d]	9.0 (0.7)^{a} *n* = 58	10.2 (0.7)^{a} *n* = 58	17.0 (0.7)^{b} *n* = 57	14.4 (0.7)^{c} *n* = 58	18.2 (0.7)^{b} *n* = 58
Lifetime fecundity	0.8 (7.7)^{a} *n* = 58	15.0 (7.7)^{a} *n* = 58	122.0 (7.7)^{b} *n* = 57	133.8 (7.7)^{b} *n* = 58	166.9 (7.7)^{c} *n* = 58
Egg size [mm²]	0.662 (0.014)^{a,b} *n* = 10	0.630 (0.010)^{a} *n* = 19	0.623 (0.006)^{a} *n* = 51	0.625 (0.006)^{a} *n* = 58	0.665 (0.006)^{b} *n* = 54
Reproductive investment [mg]	0.5 (4.9)^{a} *n* = 58	9.8 (4.9)^{a} *n* = 58	76.3 (4.9)^{b} *n* = 57	84.6 (4.9)^{b} *n* = 58	105.4 (4.9)^{c} *n* = 58

b)

Trait	Sugar	Sugar + V	Sugar + M	Sugar + V + M	Banana
Longevity [d]	23.4 (0.9)^{a} n = 68	22.7 (1.0)^{a} *n* = 58	18.8 (0.9)^{b} *n* = 64	20.5 (1.0)^{a,b} *n* = 57	20.9 (0.9)^{a,b} *n* = 67
Lifetime fecundity	122.0 (9.6)^{a} *n* = 68	108.1 (10.4)^{a} *n* = 58	94.5 (9.9)^{a} *n* = 64	129.7 (10.5)^{a} *n* = 57	202.8 (9.7)^{b} *n* = 67
Egg size [mm²]	0.659 (0.006)^{a} *n* = 66	0.649 (0.006)^{a} *n* = 54	0.662 (0.006)^{a,b} *n* = 56	0.665 (0.006)^{a,b} *n* = 54	0.681 (0.006)^{b} *n* = 66
Reproductive investment [mg]	80.5 (6.4)^{a} *n* = 68	70.1 (7.0)^{a} *n* = 58	63.2 (6.6)^{a} *n* = 64	86.3 (7.0)^{a} *n* = 57	137.7 (6.5)^{b} *n* = 67

Despite having no access to carbohydrates a few females being fed on an amino acid solution laid many more eggs than expected (10 out of 58 females laid more than 10 eggs; maximum: 207 eggs). In order to assess the significance of this finding a selection experiment was carried out (see Methods). The resulting line, however, showed no significant divergence from an unselected control after four generations of selection (selection line: 1.7 ± 0.4 eggs, n = 111 females; unselected control: 2.8 ± 0.5, n = 93; t-test: t = -1.8; p = 0.066).

Multiple regressions revealed positive correlations between lifetime fecundity and longevity (r^2_{mult}: 4 - 26 %), and negative correlations between lifetime fecundity and egg size (r^2_{mult}: 14 - 29 %, Table 3a), with the latter indicating a trade-off between egg size and number. In the two sugar treatments egg size (explaining ca. 25 % of the variance) is the most important predictor of lifetime fecundity followed by longevity (explaining ca. 5 %). The relative importance of these variables is reversed in banana-fed females, with egg size and longevity explaining 14 % and 26 % of the variance, respectively.

Experiment 2

As in *experiment 1* all traits under investigation were significantly affected by feeding treatment (longevity: $F_{4,309}$ = 3.7, p = 0.005; survival probability: c^2_4 = 16.5, p = 0.002; lifetime fecundity: $F_{4,309}$ = 18.5, p < 0.001; mean egg size: $F_{4,291}$ = 4.0, p = 0.003; reproductive investment: $F_{4,309}$ = 20.1, p < 0.001; Table 2b). Females fed on sugar or sugar enriched with vitamins lived longest, while females provided sugar enriched with minerals lived shortest (Table 2b, Fig. 1b). Overall, however, differences in longevity among groups were relatively small.

Lifetime fecundity and concomitantly reproductive investment was by far highest in the females feeding on moist banana (Table 2b), with the advantage being accumulated throughout the whole oviposition period (Fig. 2b). Egg numbers and reproductive investment of females feeding on sugar were not significantly influenced by any admixture (Table 2b). However, females fed on sugar enriched with both, vitamins and minerals, were able to maintain their egg production on a higher level late in life than females fed on sugar enriched with either vitamins or minerals, resulting in significant differences between treatments at days 13 - 16 of the oviposition period (Tukey HSD after ANOVA, Fig. 2b). Similar to *experiment 1*, egg size was largest in banana-fed females (Table 2b).

Table 3. Results of multiple regressions (stepwise forward addition of variables; Ridge regression; lambda = 0.10; $F > 1.0$ for inclusion) for the effects of life-history traits on fecundity for *experiments 1* (a) and *2* (b). Given are standardized partial regression coefficients Beta (standard error in parentheses), multiple coefficients of determination r^2_{mult}, F-value and significance level. $p < 0.05$ in bold. AA: Amino acids; V: Vitamins; M: Minerals.

a)

Treatment	Predictor	Beta	r^2_{mult}	*F*	*n*	*p*
Sugar only	Egg size	-0.541 (0.112)	0.291	20.14	50	**< 0.001**
	Longevity	0.232 (0.112)	0.349	4.30		**0.044**
Sugar + AA	Egg size	-0.420 (0.112)	0.227	6.42	57	**< 0.001**
	Longevity	0.192 (0.112)	0.266	2.95		0.091
Banana	Longevity	0.430 (0.105)	0.263	18.57	53	**< 0.001**
	Egg size	-0.361 (0.105)	0.402	11.88		**0.001**

b)

Treatment	Predictor	Beta	r^2_{mult}	*F*	*n*	*p*
Sugar	Egg size	-0.432 (0.108)	0.205	16.00	64	**< 0.001**
Sugar + V	Egg size	-0.568 (0.104)	0.367	29.62	52	**< 0.001**
	Longevity	0.181 (0.104)	0.403	3.01		0.089
Sugar + M	Egg size	-0.166 (0.129)	0.030	1.66	55	0.203
Sugar + V + M	Egg size	-0.371 (0.124)	0.151	8.93	52	**0.004**
Banana	Longevity	0.365 (0.114)	0.126	9.20	65	**0.003**
	Egg size	-0.128 (0.114)	0.143	1.27		0.263

The results of multiple regressions largely reflect those obtained from *experiment 1*. Again, there were negative correlations between lifetime fecundity and mean egg size throughout all treatments (r^2_{mult}: 2 - 37 %, Table 3b). Longevity, however, was only in the group provided banana a significant predictor of lifetime fecundity. As before, the relative importance of both variables changed in the banana-fed females.

Discussion

Effects of adult diet on reproduction and longevity

Females fed on diets lacking carbohydrates showed substantially reduced longevity and were not able to deposit significant numbers of eggs. Thus, carbohydrates play a decisive role for reproduction and somatic maintenance in *B. anynana*, which is already known for a number of butterflies (e.g. Murphy et al. 1983; Leather 1984; Gunn and Gatehouse 1985; Karlsson and Wickman 1990; Boggs 1997; Wei et al. 1998; O'Brien et al. 2000; Fischer et al. 2004; O'Brien et al. 2004). Stable isotope analyses in *B. anynana* revealed that the carbon provided by the adult diet is incorporated into developing eggs quite rapidly, and that the very first eggs laid show already a nearly even ratio of larval- and adult-derived carbon (Fischer et al. 2004). In total, approximately half of the egg carbon originated from adult feeding.

Feeding on a solution of essential and nonessential amino acids did not improve performance in any trait as compared to having access to water only (Table 2a). The fact that a few of these females laid unexpectedly many eggs is presumably not due to genetic factors (even though the selection experiment lasted for four generations only). Thus, for now we cannot explain this phenomenon. One possibility might be that it is the result of an accidental contamination with carbohydrate-rich fluids during the course of the experiment. Excluding the respective females from further analyses though did not change any result qualitatively (results not shown).

Despite their high need for amino acids and protein for egg provisioning (Engelmann 1999), female *B. anynana* were not able to benefit substantially (but see below) from having access to amino acids in addition to carbohydrates in their adult diet (see also Gunn and Gatehouse 1985; Moore and Singer 1987; Hill 1989; Hill and Pierce 1989; O'Brien et al. 2000; Romeis and Wäckers 2002; Mevi-Schütz and Erhardt 2003c). Thus, it seems that the larval-derived storage proteins are already sufficient to realize the full reproductive potential. This is in line with some other findings on nitrogen turn-over in Lepidoptera. Gunn and Gatehouse (1986), for instance, reported for female *Spodoptera exempta* that protein and lipid reserves, which are exhausted in the course of the egg-laying period, can be replenished by synthetic pathways utilizing adult-derived carbohydrates and the nitrogen pool in the haemolymph rather than adult-derived nitrogen. Similarly, O'Brien et al. (2002) found that nonessential amino acids can be extensively synthesised using adult carbon and

endogenous nitrogen in *Amphion floridensis*, rendering at least some Lepidoptera rather independent from adult nitrogen sources.

However, during the first four days of the oviposition period females fed on carbohydrates in combination with amino acids deposited a significantly higher number of eggs than females fed on sugar only or banana. This could potentially confer a substantial fitness advantage, as the eggs laid first tend to be the most important ones due to predation and other random sources of mortality (e.g. Begon and Parker 1986). The fact that these females do not have higher lifetime fecundities than females fed sugar only is due to a more rapid decline in egg numbers after day four, which might be caused by a sooner depletion of resources.

Interestingly, however, neither carbohydrates nor carbohydrates in combination with amino acids could fully account for the high reproductive output (in terms of lifetime fecundity *and* egg size) found in banana-fed females in *experiment 1*. This suggested that other dietary components such as micronutrients (e.g. minerals and vitamins) may additionally contribute to egg production in *B. anynana* – as has been reported for a few other insect groups (e.g. Pappas and Fraenkel 1977; Engelmann 1999). Though some studies suggest that vitamins are essential for normal growth, development and molting of butterfly larvae and other phytophagous insects (e.g. Dadd 1961; Vanderzant et al. 1962; Claret and Volkoff 1992; Barbehenn et al. 2001), surprisingly little is known about the role of vitamins in the adult diet. One exception we are aware of reports that a diet deficient of ascorbic acid results in reduced fecundity and egg hatching success, and that the resulting offspring is not able to reach maturity unless provided with ascorbic acid (Vanderzant et al. 1962). In *B. anynana* though, neither longevity nor reproduction were significantly affected by an admixture of vitamins to sugar.

Similarly, providing two minerals (potassium and magnesium) in addition to carbohydrates did not affect reproductive output, but decreased longevity. The latter suggests that the chosen mineral concentrations had adverse effects on overall performance. Since it was not possible to rise cation levels in the diet without increasing anion levels, the negative effect of potassium- and magnesium-chloride could be due to chloride anions (see also Stamp 1994). Nutritional imbalances can cause increased catabolism and excretion (Wulfsun and Stamp 1991), and are therefore likely to affect overall performance and lifespan of butterflies. Again, a much larger body of literature is available for the effects of dietary minerals in

caterpillars than in adult butterflies (e.g. Wulfsun and Stamp 1991; Stamp 1994; Perkins et al. 2004). However, it has been found that male *Gluphisia septentrionis* excrete excess potassium and magnesium, and that no significant quantities of these ions are passed to the female at mating (Smedley and Eisner 1996). This suggests that butterflies are in general not in shortage of either mineral (potassium is generally abundantly available in plants; Smedley and Eisner 1995).

Although lifetime fecundity and reproductive investment of sugar-fed females were not significantly influenced by either admixture, females fed on sugar enriched with vitamins *and* minerals were able to maintain their egg production late in life on a higher level than females fed on sugar enriched with either vitamins or minerals. Though the effect is weak and transient, it may suggest that micronutrients may become limiting late in life, and that micronutrients do not operate independently from each other but interact in complex ways (see also Barbehenn et al. 1994; Stamp 1994). In summary, none of the sugar-based diets tested in *experiments 1* or *2* yielded a reproductive output equivalent to that of banana-fed females. Whether different feeding regimes yielded qualitative changes in egg composition is unknown. This interesting issue remains a task for the future.

Factors affecting lifetime fecundity

Throughout all dietary treatments, lifetime fecundity was negatively correlated to egg size (though not always significantly), indicating a trade-off between egg size and number (resources available to reproduction are allocated to either few large or many small eggs; e.g. Smith and Fretwell 1974; Roff 1992). Further, longevity was positively related to fecundity in all cases where longevity was included in the models (significantly in three). In all sugar treatments – independent of varying admixtures – egg size was the most important predictor of lifetime fecundity, whereas in the banana-fed females longevity was of highest importance. Thus, when fed on a high-quality diet (as indicated by high fecundity and large egg size), lifespan and therefore the time available for oviposition is the most important factor influencing fecundity in *B. anynana* (see also Leather 1995). At the same time the fundamental trade-off between egg size and number becomes less important, indicating that animals fed on high-quality diets are (to a certain extent) able to increase offspring quality without having to sacrifice offspring number. This suggests that the egg size-number trade-

off, as is already known for some others, depends on environmental conditions (e.g. Messina and Fry 2003; Sgrò and Hoffmann 2004).

Conclusions

In summary we show that carbohydrates are the most important nutrients affecting reproduction and longevity in *B. anynana*. However, banana-fed females showed a significantly higher reproductive output than females fed solutions containing sucrose, amino acids, vitamins, minerals or some sucrose-based combinations of the latter. Although our results indicate that the importance of adult-derived micronutrients for reproduction has been underestimated, we were not able to identify the critical substances. This is somewhat surprising as we tested the micronutrients being most abundantly available in banana in our feeding experiments. There are three possible explanations for our results. First, the observed difference could be the result of increased intake rates based on an intrinsic preference for bananas (due to e.g. odour, see e.g. Honda et al. 1998; Andersson 2003 for effects of floral odours on adult butterfly feeding response). However, all females were facing no-choice situations with only one food source being provided, which was easily and abundantly available. Thus, it is not obvious why females should feed less than needed even if they would prefer a different diet when given a choice. Second, other, not yet identified compounds of banana (e.g. lipids or fatty acids) increase reproductive output. Third and perhaps most likely, reproduction does not only depend on a small number of adult-derived nutrients (such as e.g., carbohydrates, where the crucial role is obvious), but on a larger number having relatively small effects each. Thus, resource congruence (the use of nutrient types in a specified ratio; Bazzaz 1996; see also O'Brien et al. 2004; Fischer et al. 2004) rather than any specific component may be the key to answer the question. The exceptionally high quality of banana (compared to the other diets used) is further supported by the fact that the trade-off between egg size and number seems to be less pronounced in banana-fed females.

While a crucial role of carbohydrates is now known for a number of butterflies (e.g. Murphy et al. 1983; Leather 1984; Wei et al. 1998; O'Brien et al. 2000; Fischer et al. 2004; O'Brien et al. 2004), the large advantage of feeding banana compared to sugar solutions seems extraordinary, as is the high dependence of reproduction on adult income in this species (Fischer et al. 2004). To find out whether *B. anynana*

comprises an exception with regard to reproductive resource allocation, whether such patterns are more wide-spread among insects or are maybe related to a fruit-feeding life style, remains a task for the future.

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6.3 Effects of adult nutrition on female reproduction in a fruit-feeding butterfly: the role of fruit decay and dietary lipids

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Abstract

It was generally believed that butterflies and other holometabolous insects rely primarily on reserves accumulated during the larval stage for reproduction. Recent studies, however, highlight the often fundamental importance of adult nutrition to realize the full reproductive potential. While the importance of carbohydrates is fairly well understood, the role of most other adult-derived substances is only partially resolved. We here focus on the effects of dietary lipids (cholesterol, polyunsaturated fatty acids) and fruit decay (dietary yeast, ethanol) on female reproduction in the tropical, fruit-feeding butterfly *Bicyclus anynana* (Nymphalidae). We found that banana-fed control females outperformed all other groups fed on sucrose-based diets. Lipids, yeast or ethanol added to a sugar solution did not yield a similarly high reproductive output compared to fruit-fed females. Groups fed fresh or decaying banana showed no differences in reproductive performance. As we could not identify a single pivotal substance, we conclude that resource congruence (the use of nutrient types in a specified ratio) rather than any specific nutrient component is of key importance for maximum reproductive output. Further, dietary quality may affect egg hatching success in spite of no obvious effects on egg size and number. Thus, any implications about potential fitness effects of different diets need to consider egg (and hatchling) viability in addition to fecundity.

Introduction

Many holometabolous insects, as e.g. Lepidoptera, were generally believed to rely primarily on nutrients accumulated during the larval stage for reproductive output (Leather 1995; Telang et al. 2001; Mevi-Schütz and Erhardt 2003) neglecting the indispensable function of adult feeding for reproductive success in at least some insect species (e.g. Gilbert 1972; Braby and Jones 1995; Rusterholz and Erhardt 2000; Bauerfeind and Fischer 2005a). While the often crucial importance of adult-derived carbohydrates for female butterfly reproduction is fairly well understood (e.g. Leather 1984; Wei et al. 1998; Fischer et al. 2004; O'Brien et al. 2004; Bauerfeind and Fischer 2005b), the role of most other adult-derived substances is still only partially resolved (e.g. Dunlap-Pianka et al. 1977; Murphy et al. 1983; Moore and Singer 1987; Hill and Pierce 1989; Boggs and Jackson 1991; Smedley and Eisner 1996).

Lipids, for instance, are likely to be of key importance for insect reproduction, as they are major constituents of the oocyte dry mass and serve various functions including their role as the main energy source for the developing embryo (Stanley-Samuelson and Loher 1983; Stanley-Samuelson et al. 1988; Kawooya and Law 1988; Svoboda 1999; Ziegler and Van Antwerpen 2006). As most insects are neither able to synthesise long-chain polyunsaturated fatty acids (but Blomquist et al. 1982; Beenakkers et al. 1985) nor the tetracyclic steroid nucleus required for the synthesis of sterols (Behmer and Nes 2003) *de novo*, they depend on exogenous sources for successful development and reproduction (Al-Izzi and Hopkins 1982; Beenakkers et al. 1985; Turunen 1990; Behmer and Grebenok 1998; Svoboda 1999; Mondy and Corio-Costet 2000). In butterflies, lipid requirements are thought to be mainly covered by larval-derived storage depots in the fat body (Behmer and Grebenok 1998; Arrese et al. 2001). Fruit-feeding butterflies, though, may have access to noticeable amounts of adult-derived lipids, whose importance, in contrast to larval-derived ones, is currently unknown.

Apart from being in themselves a source of various substances including lipids (cf. University of Hohenheim 1996), fruits also support the growth of microorganisms and fungi (associated with the production of fermentative products like organic acids and alcohols; Morais et al. 1995; Molleman et al. 2005a). Among these yeast is of special concern, providing protein and other substances to insect frugivores (Rattray et al. 1975; Morais et al. 1995; Brown et al. 1996; Good and Tatar 2001). As a dietary supplement, yeast has been shown to dramatically increase egg production but to reduce longevity in *Drosophila melanogaster* and *Chrysoperla carnea* (Chippindale et al. 1993; McEwen and Kidd 1995; Simmons and Bradley 1997; Good and Tatar 2001).

The fermenting activity of yeasts results in the production of noticeable concentrations of e.g. ethanol (Leavey 2004). While low levels of ethanol are present in fruits of all developmental stages (ranging from trace amounts up to 0.5 %; Dominy 2004), its concentration rises more than tenfold during fermentation (Bokor and Pecsenye 2000; Dudley 2004; Milton 2004). Ethanol at low concentrations may serve as an energy source (apart from being an olfactory cue; e.g. Omura and Honda 2003), while high concentrations (> 5.0 - 7.5 %) are toxic (Bokor and Pecsenye 2000; Heberlein et al. 2004). Adaptation to environmental alcohol has been extensively studied in *Drosophila* only (e.g. McKenzie and McKechnie 1979; Bokor and

Pecsenye 2000; Fry 2001), while comparable studies in other insect species are largely lacking (but see Abramson et al. 2005).

In summary, our understanding of the effects of adult diet on insect life-history traits (survival and reproduction) is still far from being complete. We here focus on the relative importance of different compounds of the adult diet for reproduction in the tropical, fruit-feeding butterfly *Bicyclus anynana* (Butler, 1879) (Lepidoptera: Nymphalidae). In this species adult-derived carbohydrates are essential for egg production, without which no eggs will be produced (Fischer et al. 2004; Bauerfeind and Fischer 2005b). When feeding on fruit (banana), female *B. anynana* deposited more and larger eggs than females feeding on a pure sucrose solution (Bauerfeind and Fischer 2005b). As neither amino acids nor various micronutrients (vitamins, minerals) account for this discrepancy (Bauerfeind and Fischer 2005b), it is currently unclear whether the better performance of fruit-fed butterflies is caused by one critical, yet unidentified class of substances (e.g. lipids), a whole variety of substances contributing small effects each, or some volatile feeding cues increasing intake rates.

Against this background we performed two separate experiments focussing on fitness effects (rather than preferences that may not necessarily indicate a fitness advantage) of adult-derived nutrients of female *B. anynana*. While in *experiment 1* we focus on the effects of dietary compounds acquired during the process of decay (yeast, ethanol), *experiment 2* investigates the effects of adult-derived cholesterol and polyunsaturated fatty acids on survival and reproductive output of female butterflies. Additionally, the effects of diet composition on egg hatching success are investigated.

Methods

Study organism

Bicyclus anynana is a tropical, fruit-feeding butterfly ranging from Southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternate wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen et al. 2004). A laboratory stock population was established at Bayreuth University, Germany, in 2003 from several hundred individuals derived from a well-established stock population at Leiden University, The

Netherlands. The Leiden population was founded in 1988 from 80 gravid females caught at a single locality in Malawi. In each generation several hundred individuals are reared maintaining high levels of heterozygosity at neutral loci (Saccheri and Bruford 1993; Van't Hof et al. 2005). For this study butterflies from the Bayreuth stock population were used.

Experimental design

All butterflies were reared and maintained in an environmental cabinet at a constant temperature of 27°C, high relative humidity (70 %) and a photoperiod of L12:D12 (24h light:dark cycle). These conditions are similar to those at which the butterflies develop and reproduce during the favourable wet season in the field (Brakefield 1997). Larvae were reared in big population cages (50 x 50 x 50 cm) containing potted maize plants in ample supply, which were replaced if necessary. Pupae were removed daily from the rearing cages, transferred to a separate cage and checked daily for eclosed butterflies. Males and females were separated on the day of adult eclosion (note that male *B. anynana* butterflies do not mate on their first day). All males were pooled and given access to moist banana, while females were randomly assigned to different dietary treatments as described below. All males were provided the same diet to exclusively investigate effects of female nutrition on reproduction. Note, that *B. anynana* males are not providing females with nuptial gifts, and that there is no evidence for any effects of male treatment on female reproductive output (Fischer et al. 2003; Ferkau and Fischer 2006). Females were given a premating time of one and two days (*experiment 1* and *2*, respectively) separated from males, after which an equal number of random virgin males was added to the females' cages for two days. After the mating period females were placed individually in translucent plastic pots (1 L, covered with gauze) containing a fresh cutting of maize for egg-laying. Thus, oviposition started for all females on day four or five (*experiment 1* and *2*, respectively) of adult life. Eggs were collected, counted and measured every other day until the death of the butterflies or until day 12 of the oviposition period (*experiment 1* and *2*, respectively).

Experiment 1

Upon eclosion females were divided among the following five treatment groups (n = 55 - 57 each): access to 1) a pure sucrose solution, 2) a sucrose solution

supplemented with baker's yeast (*Saccharomyces cervisiae*), 3) a sucrose solution supplemented with ethanol (2 Vol%), 4) fresh moist banana mash or 5) decaying moist banana mash that was allowed to decompose for three days at 27°C and a relative humidity of 70 % prior to the experiment. Throughout, the applied sucrose concentration was 20 Vol%. Note that fresh banana contains approximately 20 % total carbohydrates (at 75 % water content, cf. University of Hohenheim). Thus, all feeding treatments provide the butterflies with comparable amounts of carbohydrates. Feeding solutions were replaced every other day. Additionally, all females had access to water throughout. Females were fed on the respective diets throughout their lives, i.e. from the eclosion day onwards.

Experiment 2

As the above and an earlier experiment demonstrated a significantly higher reproductive output in banana-fed females compared to all sugar-based solutions (cf. Bauerfeind and Fischer 2005b), we doubled the sucrose concentration in all sugar-based treatment groups to 40 Vol% in order to rule out that the effect was due to an energetic constraint. Females were assigned to the following seven treatment groups (n = 39 - 51 each): access to 1) a pure sucrose solution, 2) a sucrose solution enriched with liposomes (10 µl liposome solution in 2 ml sucrose solution; ca. 1 x 10^6 liposomes / ml suspension; see below) (hereafter liposomes), 3) a sucrose solution enriched with liposomes supplemented with cholesterol (hereafter cholesterol), 4) a sucrose solution enriched with liposomes supplemented with a mixture of polyunsaturated fatty acids (hereafter PUFAs; a balanced mixture of α-linolenic acid 18:3(n-3), linoleic acid 18:2(n-6), arachidonic acid 20:4(n-6) and eicosapentaenoic acid 20:5(n-3), 5) fresh moist banana, 6) a sucrose solution enriched with ethanol (5 Vol%) (hereafter EtOH) or 7) a sucrose solution enriched with baker's yeast (*Saccharomyces cervisiae*) (hereafter yeast). The latter two were once again included to investigate effects of dietary quality on egg hatching success (see below). Feeding solutions were daily supplemented with liposomes and ethanol and replaced every other day. Again, all females had access to water throughout.

Liposome stock suspensions were prepared from 3 mg 1-palmitoyl-2-oleoyl-phosphatidylglycerol and 7 mg 1-palmitoyl-2-oleoyl-phosphatidylcholin (Lipoid, Germany) dissolved in an aliquot of chloroform. Cholesterol- or PUFA-containing liposomes were prepared by adding 3.33 mg of cholesterol or PUFA-mixture (a

balanced mixture of linoleic acid 18:2(n-6), α-linolenic acid 18:3(n-3), arachidonic acid 20:4(n-6) and eicosapentaenoic acid 20:5(n-3)) from lipid stock solutions (2.5 mg/ml chloroform). The resulting suspensions were dried using a rotary evaporator, dissolved in 10 ml buffer (20 mmol/l NaPi, 150 mmol/l NaCL, pH 7) and incubated on a rotary shaker (100 revolutions/min) for 30 min. Subsequently, the liposome suspensions were sonicated in an ultrasonic bath and excess free cholesterol and PUFAs were removed by washing the liposomes in fresh buffer using an ultra-speed centrifuge (150.000 × g, 90 min, 4°C). Prior to the addition of liposomes to the experimental feeding solutions, the liposome stock suspensions were again sonicated for 2 min. Mean concentrations (± 1 SE) per 100µl liposome solution were: cholesterol: 12.6 ± 0.2 µg; PUFAs: linoleic acid 6.2 ± 0.1 µg; linolenic acid 6.3 ± 0.1 µg; arachidonic acid 7.8 ± 0.1 µg; eicosapentaenoic acid 7.3 ± 0.03 µg. Mean diameter of a liposome was 4.2 ± 0.2 µm. Thus, the intake of liposomes by the butterflies is not constrained by proboscis morphology (Molleman et al. 2005b).

In this experiment, egg hatching success was scored at the beginning of (days 1 - 4 of the oviposition period) and later in the oviposition period (days 8 - 11). Therefore, eggs of 32 - 44 females per treatment group were collected and placed, separated by female, in Petri dishes lined with moist filter paper and a small cutting of maize for hatching caterpillars. On average, 42.5 ± 0.9 and 26.4 ± 0.7 eggs per female (mean ± 1 SE) were collected at the beginning and later in the oviposition period, respectively. The number of hatching caterpillars was recorded daily until no more hatchlings were found on 2 consecutive days.

Data analysis

As the eggs of *B. anynana* are nearly perfect spheres, egg size was measured as cross-sectional area [mm²] using a digital camera (Leica DC300) connected to a binocular microscope. The resulting images were analysed using Scion Image public software (Scion Corporation 2000). Tight correlations between egg area (applying image analysis) and egg mass as well as hatchling size confirm that this method provides a highly reliable measurement of egg size in *B. anynana* (Fischer et al. 2002). To calculate egg size for individual females, the mean across all measurement days was used as between-day variation in egg size was negligible.

Data were analysed using one-way ANOVAs throughout; significant differences between treatment groups were identified using Tukey's HSD. As ANOVA

requirements were not met in all cases, data were re-analysed using the non-parametric Kruskal-Wallis-test, which did not reveal any qualitative differences from the ANOVA results (results not shown). Data on egg numbers and sizes over time were analysed using repeated measurements ANOVAs. Survival times were scored using Mantel's procedure (Mantel 1967) followed by a c^2-test (based on sum of scores) to test for significance. Hatching success was assessed using nominal logistic regressions on binary data, separately for early and late eggs. Females whose eggs did not hatch at all (assuming that eggs were not fertilized) and females without available data for both time periods (early and late eggs) were excluded from the analyses. All statistical tests were performed using Statistica 6.1 (StatSoft 2003). Throughout the text all means are given ± 1 SE.

Results

Experiment 1

Adult diet significantly affected survival probability (c^2_4 = 31.2, p < 0.001), longevity ($F_{4,264}$ = 10.0, p < 0.001), lifetime fecundity ($F_{4,264}$ = 6.1, p < 0.001) and mean egg size ($F_{4,263}$ = 10.7, p < 0.001) of *B. anynana* females. Longevity and survival were highest in banana-fed females, while females having had access to yeast exhibited the lowest longevity (Table 1, Fig. 1a). Lifetime fecundity was highest in banana-fed females, intermediate in the groups fed on sucrose or sucrose enriched with ethanol, and lowest in females fed on sucrose supplemented with yeast (Table 1). In contrast to the above results, a repeated-measurement ANOVA on egg numbers over time (until oviposition day 12) revealed no significant influence of dietary quality ($F_{4,228}$ = 1.0, p = 0.416), indicating that the difference in lifetime fecundity can be attributed to banana-fed females depositing higher egg numbers during the late oviposition period (significant interaction between time and diet: $F_{20,1140}$ = 2.1, p = 0.003). In all dietary groups egg numbers declined with increasing female age ($F_{5,1140}$ = 59.3, p < 0.001), with the decrease being more accentuated in the sucrose-based (i.e. a pure sucrose solution and sucrose solutions supplemented with liposomes, cholesterol, PUFAs, ethanol or yeast) than in both banana groups (Fig. 2a).

Egg size was largest in banana-fed females, followed by yeast- and ethanol-fed ones which were statistically indistinguishable, while females fed on a pure sucrose solution produced the smallest eggs (Table 1). A repeated-measurement ANOVA on

egg sizes over time (until oviposition day 12) revealed that egg sizes generally declined with increasing female age ($F_{5,650}$ = 5.3, p < 0.001), which was restricted to females feeding on sucrose-based solutions. Banana-fed females showed the opposite trend (significant interaction between time and diet: $F_{20,650}$ = 3.5, p < 0.001, see Fig. 3a).

Experiment 2

Similar to above, adult diet significantly affected survival probability (c^2_6 = 19.5, p = 0.003), longevity ($F_{6,277}$ = 3.8, p = 0.001), fecundity (until day 12 of the oviposition period: $F_{6,316}$ = 7.7, p < 0.001), and mean egg size ($F_{6,310}$ = 8.0, p < 0.001) of *B. anynana* females. Again, survival and longevity were higher in banana-fed females compared to the groups fed sucrose-based solutions (Table 1, Fig. 1b). Likewise, fecundity was significantly higher in females feeding on moist banana than on any other diet, with the advantage being accumulated throughout the oviposition period (Table 1, Fig. 2b). Throughout, egg numbers declined with increasing female age (repeated-measurement ANOVA: $F_{5,1410}$ = 102.7, p < 0.001), with the decrease being less pronounced in banana-fed females than in females fed on sucrose-based diets (significant interaction between time and dietary treatment: $F_{30,1410}$ = 1.6, p = 0.019, see Fig. 2b).

Egg size was largest in banana-fed females and smallest in females fed on sucrose-based solutions supplemented with liposomes or yeast (Table 1). Overall, differences in egg size among the sugar-based treatment groups were rather small (for significant differences between treatment groups see Table 1). Throughout, egg sizes declined with increasing female age (repeated-measurement ANOVA: $F_{5,740}$ = 30.1, p < 0.001) except for banana-fed females (significant interaction between time and dietary treatment: $F_{30,740}$ = 3.3, p < 0.001, Fig. 3b).

Hatching success did not differ significantly among treatment groups for first eggs (Wald c^2_6 = 11.5; p = 0.070;), but it did so for late eggs (Wald c^2_6 = 40.8; p < 0.001; see Fig. 4). At this stage, eggs from females fed on a pure or cholesterol-supplemented sucrose solution showed a clearly reduced hatching success. Throughout, hatching success was lower for late compared to first eggs.

Table 1. Effects of adult diet on female longevity and reproductive traits (means with 1 SE in parentheses) in *Bicyclus anynana* for *experiments 1* and *2*. Note that sucrose and ethanol concentrations differed between experiments (*experiment 1* versus *experiment 2*: 20 V% versus 40 V% sucrose, 2 V% versus 5 V% EtOH). Different superscript characters within rows indicate significant differences between treatment groups (Tukey HSD after ANOVA). EtOH: ethanol, PUFAs: polyunsaturated fatty acids.

Trait	Experiment	Sucrose only	EtOH	Yeast	Banana fresh	Banana decaying	Liposomes	Cholesterol	PUFAs
Longevity [days]	*experiment 1*	23.9 (1.2)^{a} *n* = 56	23.2 (1.2)^{a} *n* = 54	19.5 (1.2)^{b} *n* = 53	28.2 (1.2)^{c} *n* = 45	29.0 (1.2)^{c} *n* = 51			
	experiment 2	19.3 (1.3)^{a} *n* = 45	19.9 (1.6)^{a} *n* = 30	20.9 (1.3)^{a,b} *n* = 42	26.4 (1.3)^{b} *n* = 45		19.0 (1.3)^{a} *n* = 44	22.7 (1.4)^{a,b} *n* = 38	20.9 (1.4)^{a,b} *n* = 40
Lifetime fecundity	*experiment 1*	120.3 (8.7)^{a} *n* = 56	122.9 (8.8)^{a} *n* = 54	107.4 (8.9)^{b} *n* = 53	158.7 (8.8)^{c} *n* = 55	159.6 (9.1)^{c} *n* = 51			
	experiment 2	130.9 (10.4)^{a} *n* = 46	136.2 (11.3)^{a} *n* = 39	141.3 (10.7)^{a} *n* = 44	203.8 (10.1)^{b} *n* = 49		136.5 (9.9)^{a} *n* = 51	117.3 (10.6)^{a} *n* = 45	129.8 (10.1)^{a} *n* = 49
Egg size [mm²]	*experiment 1*	0.651 (0.006)^{a} *n* = 56	0.663 (0.006)^{b} *n* = 54	0.670 (0.006)^{b} *n* = 52	0.694 (0.006)^{c} *n* = 55	0.692 (0.006)^{c} *n* = 51			
	experiment 2	0.650 (0.006)^{a,c} *n* = 46	0.642 (0.006)^{a,b,d} *n* = 39	0.625 (0.006)^{b} *n* = 42	0.673 (0.006)^{c} *n* = 48		0.628 (0.006)^{a,b} *n* = 49	0.650 (0.006)^{a,c} *n* = 44	0.654 (0.006)^{c,d} *n* = 49

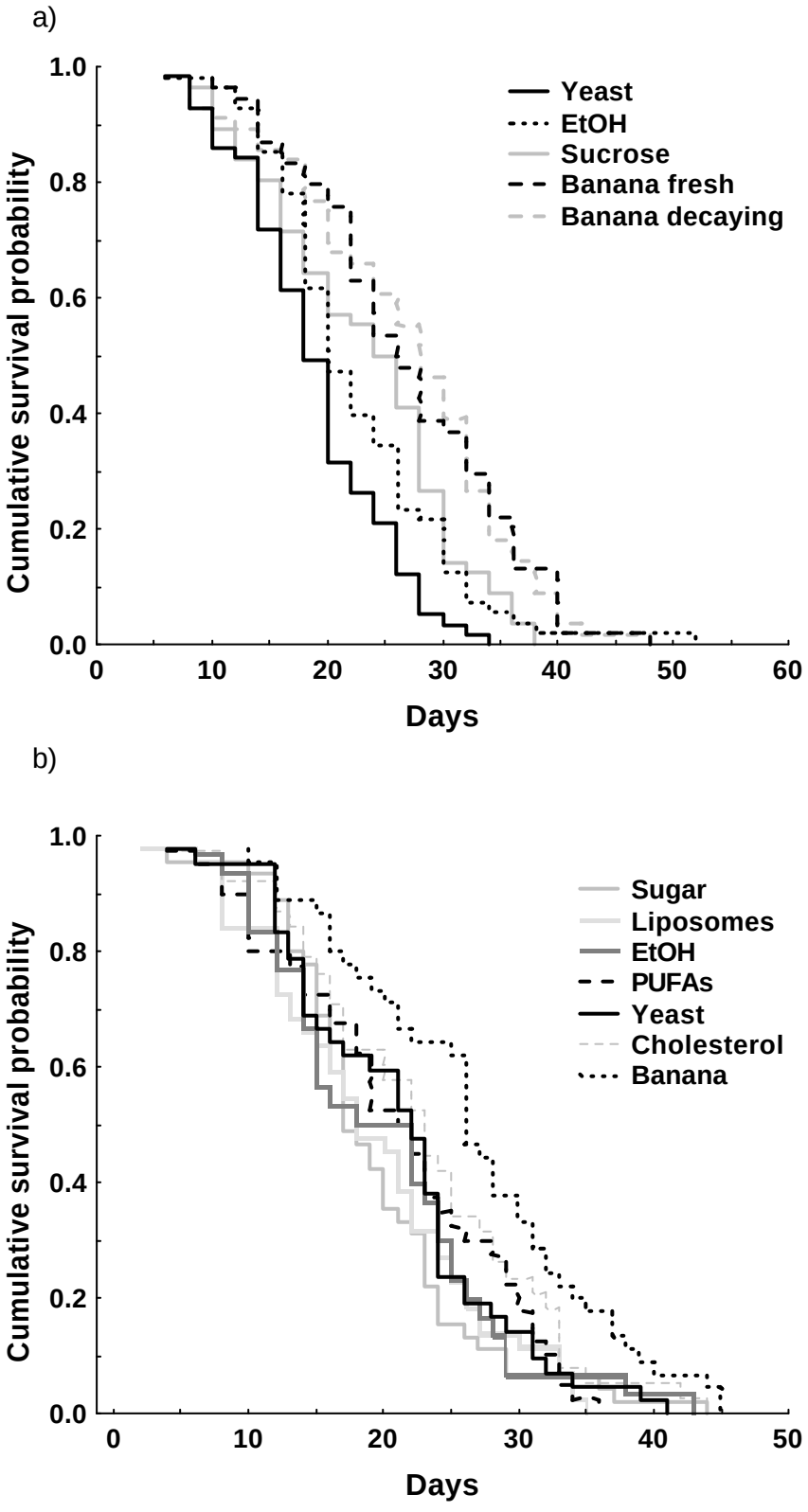

Fig. 1. Cumulative survival probabilities of female *Bicyclus anynana* fed on different diets for *experiments 1* (a) and *2* (b). EtOH: ethanol, PUFAs: polyunsaturated fatty acids.

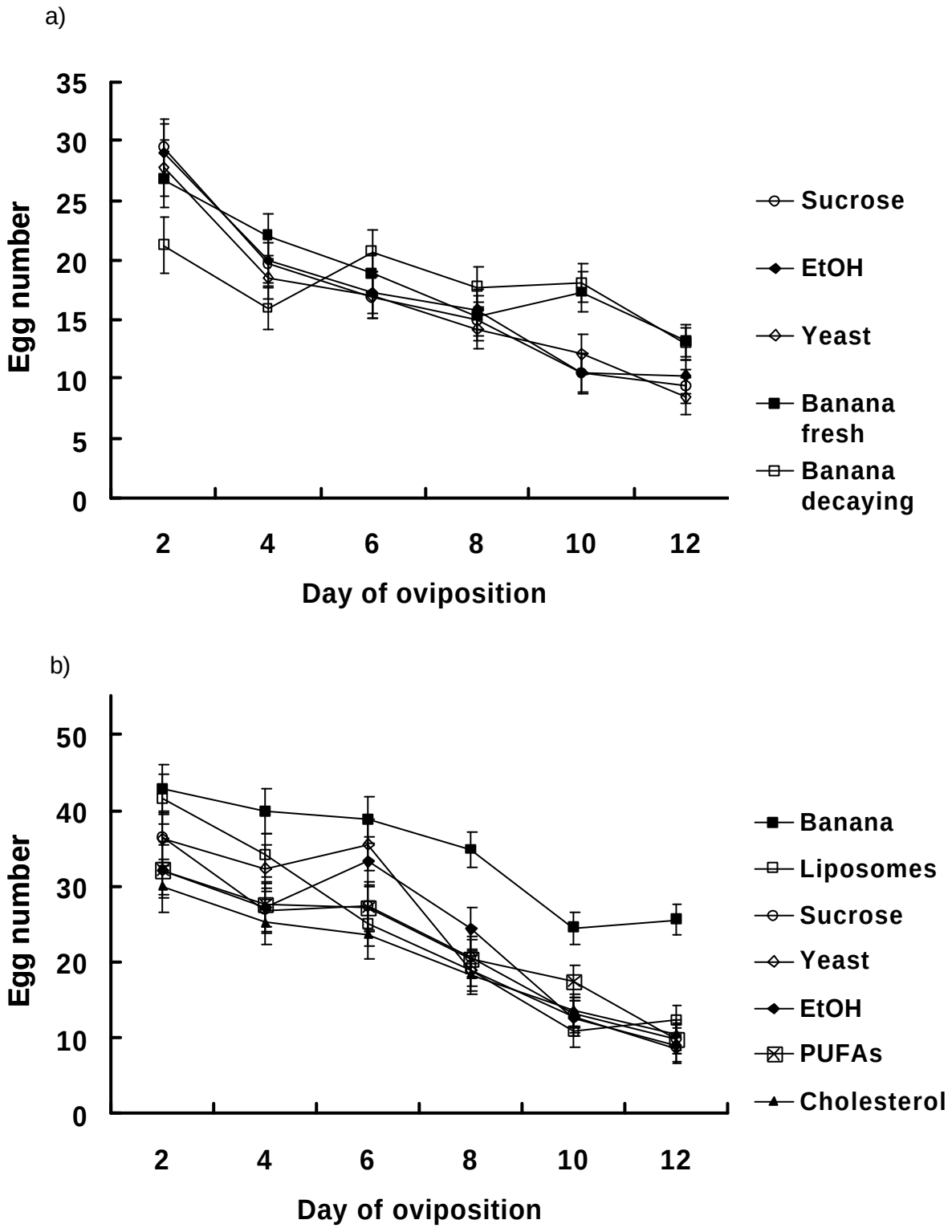

Fig. 2. Egg number over time (group means ± 1 SE) for female *Bicyclus anynana* fed on different diets for *experiments 1* (a) and *2* (b). EtOH: ethanol, PUFAs: polyunsaturated fatty acids.

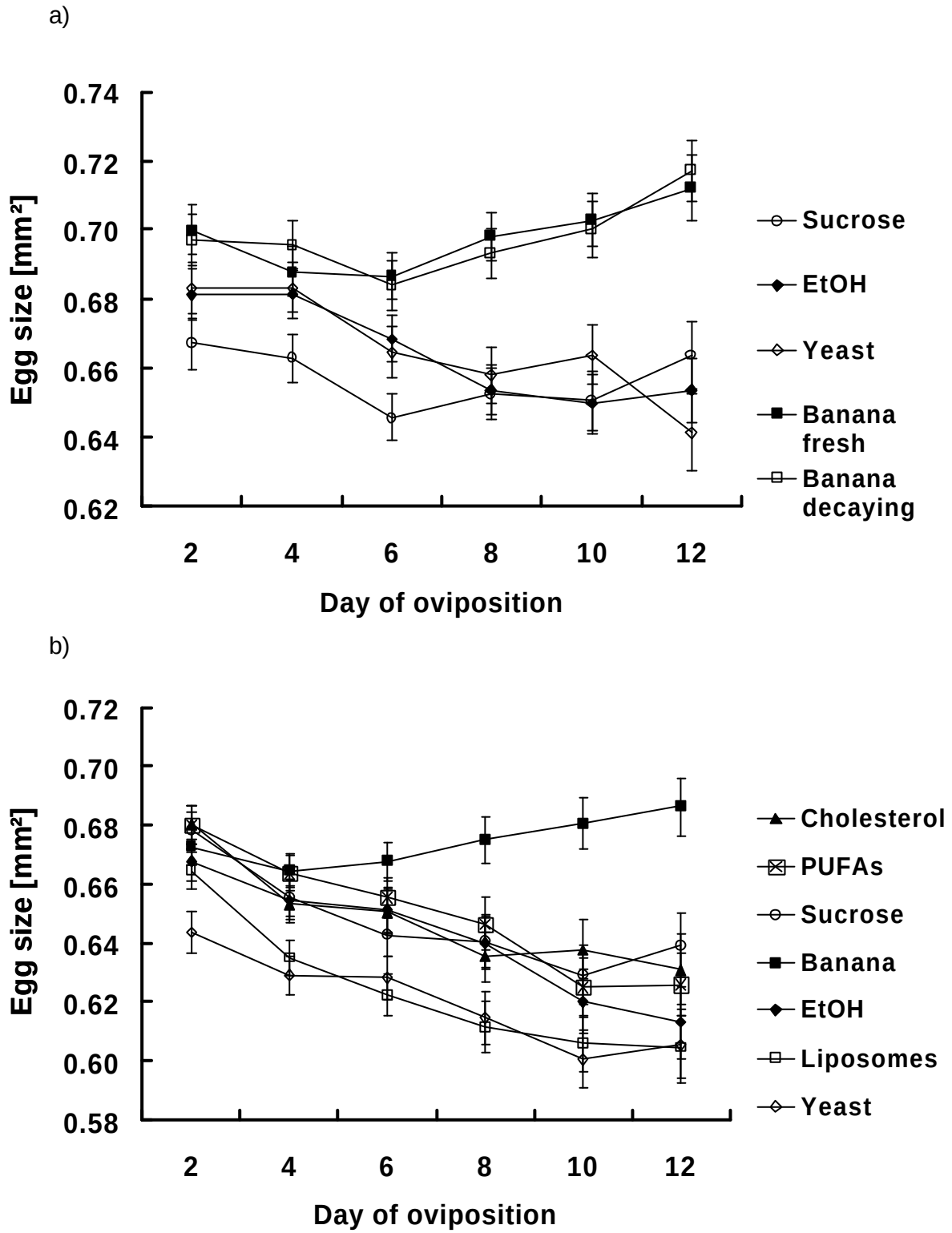

Fig. 3. Egg size over time (group means ± 1 SE) for female *Bicyclus anynana* fed on different diets for *experiments 1* (a) and *2* (b). EtOH: ethanol, PUFAs: polyunsaturated fatty acids.

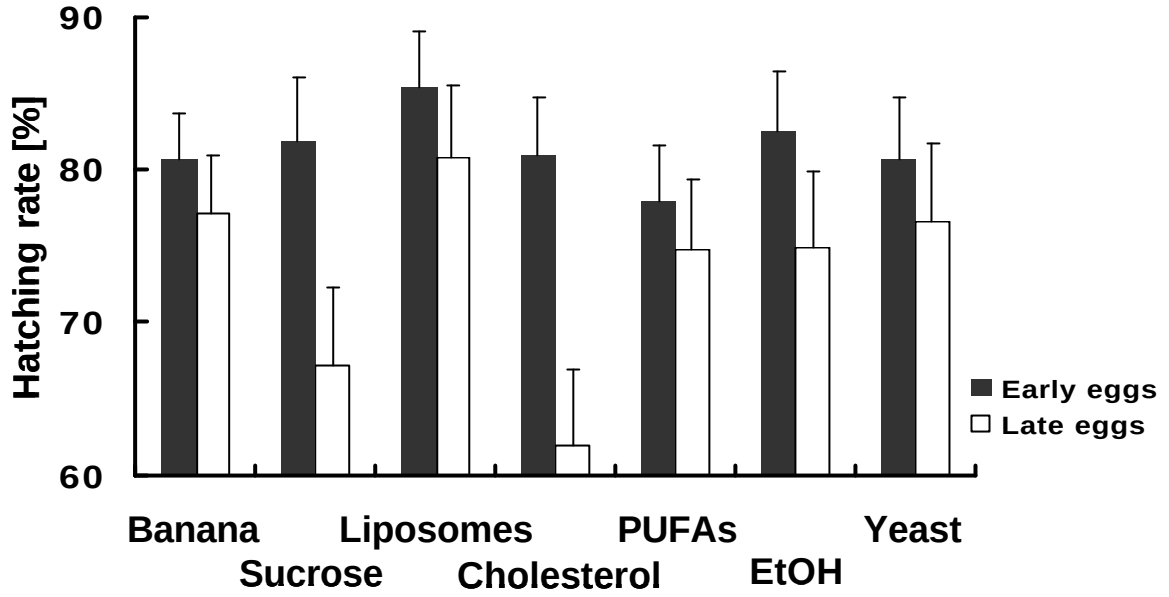

Fig. 4. Hatching success of early and late eggs (group means ± 1 SE) of *Bicyclus anynana* fed on different diets (*n* = 19 - 35). EtOH: ethanol, PUFAs: polyunsaturated fatty acids.

Discussion

Effects on longevity and reproduction

When feeding on banana, characterised by a complex nutritional composition, female *B. anynana* had longer life spans, larger egg sizes and a higher fecundity as compared to females fed on sucrose-based solutions with various admixtures (Table 1; see also Bauerfeind and Fischer 2005b). This study was aimed at disentangling the causes underlying this tremendous gain in reproductive output. As earlier experiments could largely rule out that amino acids or micro-nutrients such as minerals or vitamins play a decisive role (Bauerfeind and Fischer 2005b), we focussed in *experiment 1* on processes associated with the decay of fruit. Two lines of evidence suggest that the increased reproductive output in banana-fed females is not associated with such processes. First, performance was independent of the stage of fruit decay, as females feeding on fresh or decaying banana were statistically indistinguishable. Second, neither supplementation with yeast (mimicking fermentative products of proven value to insect frugivores) nor alcohol (as a likely volatile attractant) enhanced reproductive output or longevity as compared to a pure sugar solution.

These findings imply that at least during this early stage of decay fresh and decaying banana were of very similar quality for *B. anynana* female. Our data cannot rule out an effect of feeding on decaying fruit at later stages of fruit decomposition, characterised by a depletion of simple sugars and shifts in the community structure of microorganisms associated with increasing amounts of fermentative products like organic acids and alcohols (Morais et al. 1995; Dominy 2004; Dudley 2004). Yet, this remains to be experimentally tested, but appears rather unlikely based on the current results (cf. the results from yeast supplementation).

In principle, activities of microorganisms and fungi may provide additional resources that are able to enhance insect reproductive output. Accordingly, incorporation of yeast has been shown to dramatically increase egg production in various insect species, accompanied by a decreased life span (Chippindale et al. 1993; McEwen and Kidd 1995; Simmons and Bradley 1997; Good and Tatar 2001). Likewise, feeding on a sucrose solution supplemented with yeast resulted in a significant reduction in longevity in *B. anynana* (in *experiment 1*), though without any positive effect on reproduction. Interestingly, female survival was not reduced in *experiment 2* that included a higher sucrose concentration. This suggests that females feeding on a higher concentrated sucrose solution may need to imbibe less fluid and thus a lesser amount of presumably detrimental yeast-derived compounds in order to obtain the same amount of energy as compared to females that feed on a less concentrated sucrose solution. Yet, this needs to be verified in further experiments controlling for food consumption rates. Similarly, dietary amino acids led to a decrease in female longevity and survival probability without reproductive benefits (Bauerfeind and Fischer 2005b).

When interpreting these findings, it should be borne in mind, though, that both larval and adult diets seem to strongly interact with each other to fully meet all nutritional requirements of insects (Bauerfeind and Fischer 2005a) and all individuals in this experiment were abundantly fed during the larval stage. Under such conditions larval-derived (nitrogenous, but also lipid; see below) reserves seem to be already sufficient to realize full reproductive potential. This picture may change substantially when storage reserves are reduced due to larval food shortage. In that case, adult-derived amino acid intake has indeed been found to enhance reproduction in a butterfly (Jervis and Boggs 2005; Mevi-Schütz and Erhardt 2005). Thus, the nutritional status may yield striking effects on the use of and need for adult-derived

nitrogenous compounds. Similar considerations may hold for lipid stores under larval food-restriction (see below).

When feeding on decaying fruit, butterflies also imbibe fermentative products associated with the metabolic activities of the fungi, of which ethanol is the most prominent (Omura et al. 2000; Omura and Honda 2003; Leavey 2004). Depending on its concentration ethanol either serves as an energy source or as a toxin in *Drosophila* (Bokor and Pecsenye 2000; Heberlein et al. 2004). In *B. anynana* female survival and reproduction remained unaffected by dietary ethanol. This suggests that on the one hand the butterflies are able to deal with physiologically relevant concentrations of ethanol (see Bokor and Pecsenye 2000), but on the other hand do not gain from having access to this additional energy source. Further, the lack of effect suggests that ethanol, acting as a feeding cue in *B. anynana* and other Lepidoptera (e.g. Omura et al. 2000; Omura and Honda 2003), does not increase intake rates which in turn may affect reproductive output.

In *experiment 1* as well as in an earlier study (Bauerfeind and Fischer 2005b) we applied a sucrose concentration of 20 Vol%. However, maximum rates of energy gains have been reported for sugar concentrations of 30-50 % (Hainsworth et al. 1991; May 1985; Boggs 1988), implying that the lack of response to any additional nutritive compound might be due to a mere energetic constraint. Yet, butterflies were not limited in the amount of feeding solution offered, enabling them to imbibe adequate volumes of the dietary solution to fully meet their energetic requirements (Boggs 1988; Hainsworth et al. 1991). To entirely exclude the possibility of energetic restrictions we provided the butterflies with dietary sucrose solutions of 40 Vol% in *experiment 2* (being substantially higher than the carbohydrate content of banana), which did not change any of the results qualitatively.

Thus far, we could largely rule out that the enhanced reproductive output of fruit-fed *B. anynana* females is caused by processes associated with the decay of fruit, feeding preferences and associated changes in intake rates, energetic constraints imposed by artificial diets, or minerals and vitamins (current results and see Bauerfeind and Fischer 2005b). As insect eggs need to be provided with relatively large amounts of lipids stemming from exogenous sources (Kawooya and Law 1988; Ziegler and Van Antwerpen 2006), lipids may well be involved in triggering the increased reproductive output of fruit-fed females. Nevertheless, we could not detect

any beneficial effect of adult-derived cholesterol or PUFAs on female survival and reproductive output.

Cholesterol – as the major sterol found in insects – has been shown to meet the total sterol requirements of many insects with only a few exceptions (Dadd 1973; Canavoso et al. 2001 and references therein; Behmer and Nes 2003). Thus, dietary cholesterol is likely to be fully suitable for metabolic utilization by female *B. anynana*. Note that, despite of no effects on the quantity and size of eggs, feeding on cholesterol did affect egg hatching success (see below). Similarly, the lack of any effects of adult-derived PUFAs on female reproduction is surprising, as access to PUFAs during the larval stage is essential for normal development and realization of the full reproductive potential later in life (see Beenakkers et al. 1985; Stanley-Samuelson et al. 1988; Canavoso et al. 2001 for reviews). We here provided adult female butterflies with a mixture of linoleic acid, linolenic acid, arachidonic acid and eicosapentaenoic acid. Linoleic and linolenic acid are the most abundant PUFAs found in caterpillars and adult butterflies (Wang et al. 2006), and both are thought to adequately satisfy the nutritional need for PUFAs in most species (Canavoso et al. 2001 and references therein). Further, they are considered essential fatty acids for many insect species (Beenakkers et al. 1985). Eicosapentaenoic acid and arachidonic acid contribute to the synthesis of prostaglandins (Turunen and Pärnänen 1987) which are implicated in insect reproduction (Loher et al. 1981; Beenakkers et al. 1985).

The lack of response to cholesterol or PUFAs from adult feeding suggests that adult-derived lipids are not a determining factor for reproductive output in this species, even though lipids are likely to be of crucial importance during the larval stage. Thus, either the applied lipid concentrations were too small to elicit noticeable effects or – more likely – all lipid requirements of adult female *B. anynana* are usually met by stored resources derived from larval feeding.

The observed gain in reproductive output when feeding on banana could be the result of increased intake rates based on an intrinsic preference for bananas (due to e.g. odour, see e.g. Honda et al. 1998; Andersson 2003 for effects of floral odours on adult butterfly feeding response). Note, however, all females were facing no-choice situations with only one food source being provided, which was easily and abundantly available. Thus, it is not obvious why females should feed less than needed even if they would prefer a different diet when given a choice.

Effects on egg hatching success

Considering exclusively the size and number of eggs may be insufficient to assess fitness effects, which may also depend to a large extent on offspring viability. Therefore, survival rates among the offspring produced by females from different nutritional treatments were compared during early development, i.e. in the egg stage. In *B. anynana* as well as in many other insect species egg to adult survival is largely determined by egg hatching rates and neonate survival (e.g. Jann and Ward 1999; Fox et al. 2003). Hatching rates of eggs deposited early in the oviposition period were very similar across dietary treatment groups. Thus, shortly after eclosion all females were able to produce high quality eggs, ensuring a high hatching success throughout. With increasing female age hatching rates declined, with the decrease being more accentuated in females fed on a pure or on a cholesterol-supplemented sucrose solution.

This suggests that, when not adequately fed, females may run short of some specific nutrients initially available from larval stores, which are required to ensure maximum hatching success (see also Fox 1993; Yanagi and Miyatake 2002). Adequate nutrition is provided by banana, but more interestingly also by sugar solutions supplemented with liposomes (probably serving as a source of phospholipids), PUFAs (though it cannot be distinguished whether this effect is due to liposomes or the provided PUFAs *per se*), ethanol and yeast. Females fed on cholesterol-supplemented solutions, in contrast, had a rather low hatching success later in the oviposition period. This is odd, indicating an adverse effect of cholesterol which outweighs the beneficial effect of liposomes.

Conclusions

The high importance of adult income for reproduction in *B. anynana* does stress the complexity of reproductive resource allocation in holometabolous insects, which were formerly assumed to rely primarily on larval stores (Leather 1995; Telang et al. 2001; Mevi-Schütz and Erhardt 2003). The large advantage of feeding on fruit compared to sugar solutions with various admixtures seems extraordinary (Fischer et al. 2004; Bauerfeind and Fischer 2005b). Nevertheless, we could not identify a single pivotal substance (in addition to sucrose) that was able to elicit a comparably high reproductive performance as banana, although we tested all major substances known to be involved in insect egg production (cholesterol, PUFAs, nitrogenous

compounds; see Bauerfeind and Fischer 2005b for amino acids and micronutrients). Nevertheless, dietary quality may affect egg quality and thus hatching success (even without any obvious effects on egg numbers and sizes), though this issue needs further investigation. Additionally, we could largely rule out that processes associated with the decay of fruit are responsible for the increased reproductive output of fruit-fed females.

The available evidence clearly suggests that the greatly enhanced reproductive output in banana-fed females is due to a whole variety of substances contributing small effects each, rather than to one single pivotal substance (apart from sucrose; see O'Brien et al. 2004; Fischer et al. 2004). Thus, we conclude that resource congruence (the use of nutrient types in a specified ratio; Bazzaz 1996; see also O'Brien et al. 2004; Fischer et al. 2004) is the key to answer the question.

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Publication list

The subchapters of **Chapters 5 and 6** have been published or are accepted for publication in international peer-reviewed journals as follows:

Chapter 5 – Maternal body size and butterfly reproduction

Chapter 5.1

Bauerfeind, S.S. and Fischer, K. The role of maternal body size as a morphological constraint on egg size across butterfly species. Preliminarily accepted for publication in *Basic and Applied Ecology*.

Chapter 5.2

Bauerfeind, S.S. and Fischer, K. The role of maternal body size as an evolutionary constraint on egg size in a butterfly. Preliminarily accepted for publication in *Evolution*.

Chapter 6 – Maternal nutrition and butterfly reproduction

Chapter 6.1

Bauerfeind, S.S. and Fischer, K. 2005. Effects of food stress and density in different life stages on reproduction in a butterfly. *Oikos*, **111**, 514-524.

Chapter 6.2

Bauerfeind, S.S. and Fischer, K. 2005. Effects of adult-derived carbohydrates, amino acids and micronutrients on female reproduction in a fruit-feeding butterfly. *Journal of Insect Physiology*, **51**, 545-555.

Chapter 6.3

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Record of contributions to this thesis

All experiments (including experimental design, execution and analyses) and the survey of relevant literature were conducted by **myself** or under my direct supervision – if not stated otherwise (see below). Chapters 1 to 4 provide a general introduction, the synopsis and the summary of this thesis and were written by myself as were all published articles summarised in this thesis (Chapters 5 and 6).

All subchapters of Chapters 5 and 6 have been published or are accepted for publication in international peer-reviewed journals with the following co-authors:

Dr. Klaus Fischer is the supervisor of my thesis and co-author of all publications. He contributed support and supervision in all stages of the projects, discussion of experimental designs, analyses and results and critical comments on the first drafts of the chapters.

Chapter 6.3 Co-authors **Steffi Hartstein** and **Susann Janowitz** carried out *experiment 1* under my direct supervision (as a student research project); *experiment 2* was done in cooperation with **Dr. Dominik Martin-Creuzburg**.

Experiments presented in Chapters 5 and 6 were supported by the following student assistants and colleagues:

Chapter 5.1 Experiments were conducted and analysed by myself with the help of the following student assistants, who aided with the collection of the butterflies' eggs: Sven Arnold, Claudia Pflicke, Diana Pflücke, Anett Starkloff, Marc Steigenga and Katja Zimmer; Beth Longman carried out all experiments on *Araschnia levana* under my direct supervision (as a student research project).

Chapter 5.2 Experiments were conducted and analysed by myself, assistance was given by Volker Bornitzky, Anneke Dierks, Thorin Geister, Susann Janowitz, Isabell Karl, Claudia Pflicke, Marc Steigenga and Ilja Zeilstra, all of whom helped during the collection of butterfly eggs during different stages of the selection process.

Chapter 6.1 Experiments were conducted and analysed by myself, assistance was given by Sven Arnold, Claudia Pflicke, Anett Starkloff, Marc Steigenga and Ilja Zeilstra, who were involved in egg collection.

Chapter 6.2 *Experiment 1* was carried out by Carina Ferkau, Antje Rahnfeld and Doreen Schulze (as a student research project); *experiment 2* was carried out by myself.

Chapter 6.3 Experimental assistance was given by Anneke Dierks and Susann Janowitz who helped with egg collection.

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Bayreuth, May 2007

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Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

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