Hormonal regulation and environmental influences in the reproduction of the butterfly *Bicyclus anynana*

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Content

Summary

The production of offspring is arguably the most important event in the life of an insect, but its success depends on a multiplicity of intrinsic and environmental factors that may interact to various degrees. Factors being crucial include the hormonal regulation of reproduction, and the influence of the environment, especially temperature and nutrition. In this thesis, the hormonal regulation and environmental influences in reproduction were investigated for the tropical butterfly *Bicyclus anynana.*

Regarding hormonal control of reproduction, the butterfly *B. anynana* belongs to a group of the Lepidoptera, in which egg maturation starts after eclosion, and thus vitellogenesis and choriogenesis seem to depend exclusively on juvenile hormones (JH). Using a JH mimic (pyriproxyfen) and an antagonist of JH biosynthesis (MK-801), reproduction in *B. anynana* could be successfully manipulated towards either a higher or lower fecundity. Especially early fecundity responded to manipulations. Furthermore, both JH III and JH II were found in the hemolymph throughout lifetime. Nevertheless, fecundity and vitellogenin titres were not clearly related to JH titres, as both decreased continuously with age, although JH III titres stayed constant and JH II titres increased. Thus, reproduction in *B. anynana* is at least to some extent under hormonal control, with JH being probably an important signal especially for the initiation of reproduction.

Oviposition temperature induces a plastic response in egg size and number in *B. anynana*. While more but smaller eggs are laid at high temperatures representing wet season conditions, larger but fewer eggs are oviposited at lower temperatures, which are experienced in the dry season. This plasticity is thought to be adaptive in this species inhabiting seasonal environments. Vitellogenins are representing a major part of eggs and consequently, JH dynamics represents an obvious target for changes in egg size, as vitellogenin synthesis and/or incorporation into developing eggs might increase or decrease through changes in JH titres. Female *B. anynana* butterflies exposed to different oviposition temperatures showed the expected response to adult temperature, producing fewer but larger eggs at the colder temperature, but more and smaller eggs at the warmer temperature. However, no evidence was found that this striking example of phenotypic plasticity is under hormonal control, as JH III, JH II, vitellogenin titres and egg proteins showed no

significant variations across temperatures. Based on these similar patterns across temperatures, the results in this thesis suggest that the temperature-mediated reproductive plasticity is not mediated through JH in *B. anynana*.

The known fitness advantage of the larger eggs produced at lower temperatures in *B. anynana* may be related to size per se, to a larger absolute amount of nutrients or to relative changes in egg composition. Therefore, this thesis explored the consequences of temperature variation on egg and hatchling composition, and the associated use and turnover of energy and egg compounds in relation to temperature. Overall, larger eggs produced at the lower temperature were achieved by providing these eggs with larger quantities of all compounds investigated and thus more energy, whilst relative egg composition was rather similar to that of smaller eggs laid at the higher temperature. Turnover rates during embryonic development differed across developmental temperatures, suggesting an emphasis on hatchling quality (i.e. protein content) at the more stressful lower temperature, but on storage reserves (i.e. lipids) at the higher temperature. These observed differences may consequently represent adaptive maternal effects.

The availability of adequate adult nutrition is essential for successful reproduction in *B. anynana,* as without access to carbohydrates in the adult stage no eggs will be produced. A commonly used method for estimating the fitness effects of diets is determining the number and sometimes sizes of eggs produced and often not including offspring viability. Five different nutritional treatments were used for female *B. anynana* butterflies ranging from moist banana, plain sucrose solution, to sucrose solution enriched with lipids, yeast and finally minerals and vitamins. These treatments were analyzed with regard to their effects on egg composition as well as egg hatchling success. Adult diet was demonstrated to have pronounced effects on fecundity, egg composition and egg hatching success, with butterflies feeding on the complex nutrition of banana fruit performing best. Vitamins and minerals in a sucrose-based diet increased fecundity, but did not affect offspring hatching success. Effects of adult diet on egg composition were not straightforward, indicating complex interactions among specific compounds. Total egg energy and water content seemed to be related to hatching success of progeny. The results of this thesis demonstrate that egg composition should be taken into account in such studies, as egg size and number does not necessarily represent a reliable proxy for reproductive energetic investment.

Zusammenfassung

Die Erzeugung von Nachkommen ist einer der wichtigsten Schritte im Leben eines Insektes. Sein Erfolg hängt von einer Vielzahl von inneren und umweltbedingten Faktoren ab, die miteinander interagieren können. Besonders wichtig hierbei sind die hormonelle Regulation der Reproduktion und die Umwelteinflüsse von Temperatur und Ernährung auf die Fortpflanzung, welche in dieser Arbeit im tropischen Schmetterling *Bicyclus anynana* untersucht wurden.

B. anynana gehört zu der Gruppe von Schmetterlingen, in der die Eireifung erst nach dem Schlupf beginnt und Vitellogenese und Choriogenese ausschließlich von Juvenilhormonen (JH) gesteuert werden. Durch Einsatz eines JH Analogon (Pyriproxyfen) und eines Antagonisten der JH Biosynthese (MK-801), konnte die Fekundität weiblicher Schmetterlinge erfolgreich manipuliert werden. Dies war insbesondere in einem frühen Alter möglich, später hingegen nicht mehr. In der Hämolymphe adulter Weibchen wurden JH III und II identifiziert und über die gesamte Lebensdauer nachgewiesen, wobei sowohl die Fekundität als auch die Vitellogenin-Titer nicht mit den JH-Titern korrelierten. Während die Fekundität über die Lebenszeit abnahm, wurden für JH III konstante Titer und für JH II ansteigende Titer beobachtet. Somit wird die Reproduktion von *B. anynana* hormonell kontrolliert, wobei JH ein wichtiges Signal für die Initiation der Reproduktion darzustellen scheint.

Die Ovipositionstemperatur induziert bei *B. anynana* eine plastische Reaktion der Eigröße und –anzahl. Während bei wärmeren Temperaturen viele kleinere Eier abgelegt werden, werden größere aber weniger Eier bei niedrigeren Temperaturen abgelegt. Diese Plastizität wird dabei als adaptiv in der jeweiligen tropischen Umwelt, der wärmeren Regenzeit bzw. der kühleren Trockenzeit, angesehen. Da Vitellogenine ein Hauptbestandteil von Insekteneiern sind, könnten Änderungen des JH-Hämolymphtiters einen erwarteten Mechanismus darstellen wie Eigröße über die JH gesteuerte Vitellogeninsynthese und -einlagerung in Eier geregelt werden könnte. Die adulten Weibchen zeigten im Bezug zu ihrer Umgebungstemperatur das erwartete Muster mit weniger aber großen Eiern sowie kleinen aber vielen Eiern. Allerdings konnte nicht nachgewiesen werden, dass JH diese Plastizität steuert, da sowohl der JH III-Titer, JH II-Titer, und Vitellogenintiter, als auch die Eiproteine keinerlei signifikanten Variationen zwischen den Umgebungstemperaturen

aufzeigten. Aus diesen Ergebnissen kann man somit folgern, dass die über Temperatur induzierte reproduktive Plastizität von *B. anynana* nicht über JH gesteuert wird.

Die bekannte höhere Fitness von größeren Eiern bei niedrigen Temperaturen bei *B. anynana* könnte von der Größe der Eier bestimmt sein, der Menge der Eiinhaltstoffe oder auch von relativen Änderungen in der chemischen Eizusammensetzung. Daher wurde untersucht, inwiefern unterschiedliche Temperaturen Auswirkungen auf die Ei- und L1-Larven-Zusammensetzung haben, und wie verschiedene Stoffe während der Embryonalentwicklung genutzt oder beibehalten werden. Grundsätzlich waren größere Eier, die bie der niedrigeren Temperatur abgelegt worden waren, dadurch charakterisiert, dass sie größere Mengen aller Komponenten und damit auch mehr Energie enthielten. Die relative Zusammensetzung war über die Temperaturen hinweg sehr ähnlich. Während bei der niedrigeren Temperatur vor allem Qualitätskomponenten wie Proteine in den L1-Larven wiedergefunden wurden, wurden bei der wärmeren Temperatur in höherem Maße Speicherkomponenten wie Lipide festgestellt. Diese Muster stellen vermutlich adaptive maternale Effekte dar.

Die Verfügbarkeit einer entsprechenden adulten Ernährung ist essentiell für eine erfolgreiche Reproduktion bei *B. anynana*, da ohne Zugang zu Kohlenhydraten als Adulte keine Eier produziert werden können. Oft wird bei Fragen der Fitness im Zusammenhang mit der Ernährung die reine Eizahl, teils die Eigrösse, und nur sehr selten der Schlupferfolg der Nachkommen betrachtet. Hier wurden fünf unterschiedliche Ernährungen benutzt, nämlich Banane, reine Zuckerlösung und Zuckerlösung mit Lipiden, Hefe sowie Mineralien und Vitaminen. In Abhängigkeit von der Adulternährung wurde untersucht, wie Eiinhaltstoffe und Schlupferfolg der Nachkommen zueinander in Relation stehen. Die unterschiedlichen Ernährungsarten hatten deutliche Effekte auf Fekundität, Eizusammensetzung und Schlupferfolg. Schmetterlinge mit Zugang zu Banane waren dabei anderen in allen Belangen überlegen. Vitamine und Mineralien in einer Zuckerlösung konnten signifikant die Fekundität erhöhen, beeinflussten den Schlupferfolg aber nicht. Generell waren die Effekte auf Eiinhaltstoffe nicht korreliert mit dem zugehörigen Futter, so dass z. B. nicht mehr Lipide bei Zusatz der Lipidernährung zur Zuckerlösung gefunden wurden. Tendenziell war Wasserinhalt als auch der absolute Energiegehalt der Eier für die Fitness der Nachkommen von Bedeutung. Diese Resultate zeigen, dass es generell wichtig ist, die Eizusammenstellung bei solchen Fragestellungen zu beachten, da

Größe und Anzahl von Eiern nicht prinzipiell ein guter Prädiktor für das reproduktive Investment der Mutter sind.

Introduction

The production of offspring is arguably the most important event in the life of an insect (Leather, 1995). However, reproduction of a species depends on a multiplicity of intrinsic and environmental factors that may interact to various degrees. Factors, which are crucial for successful reproduction of a female insect, include the inherent capacity of the ovaries to produce eggs of a certain number and size, the hormonal control of reproduction, and the acquisition of all vital resources for eggs, which are utilized by the developing progeny (Engelmann, 1999).

Insects are generally characterized by having two ovaries with several ovarioles each. Here, oocytes are produced through oogenesis, with the respective mechanisms being largely under hormonal control (Finch and Rose, 1995). This accounts especially for vitellogenesis, the process by which yolk is build outside the ovaries and subsequently incorporated into the oocytes. Along with female-specific proteins, lipids are taken up by the oocytes, and finally, when vitellogenesis is nearly finished, glycogen is build in the oocyte itself (Eggert et al., 2003). While lipids and glycogen are representing energy resources for the developing embryo, proteins are mainly structural components, but may additionally serve as energetic resource (Beenakkers et al., 1985). Thus, nutrition, i.e. the quality and amount of food available for a female insect during larval and adult life, is a critical environmental factor that ensures successful oogenesis and thereby reproduction (Wheeler, 1996). Additionally, other environmental factors may affect reproduction. Of these, temperature is one of the most important ones, influencing almost every life history parameter. Generally, fecundity scales to an optimal point between the lower and upper temperature thresholds, at which no reproduction can occur at all, making temperature a vital factor for an organism’s potential fecundity (Leather, 1995).

Reproduction in the tropical butterfly *Bicyclus anynana*

The tropical butterfly *Bicyclus anynana* Butler, 1879 used in this thesis as a model organism, ranges from Southern Africa to Ethiopia inhabiting sub-Saharan, highly seasonal environments such as savannahs and dry forests (Larsen, 1991). Generally, egg maturation in *B. anynana* starts after eclosion to the imago, with the first eggs oviposited at days 3 or 4 of adult life. These first oviposition days represent

the maximum of daily egg numbers, followed by a continuous decrease with female age (Bauerfeind and Fischer, 2005). Regarding hormonal control of reproduction, the butterfly *B. anynana* belongs to a group of the Lepidoptera, in which egg maturation starts after eclosion, and thus vitellogenesis and choriogenesis seem to depend exclusively on juvenile hormones (Ramaswamy et al., 1997). The life history of *B. anynana* closely matches all criteria for inclusion in this particular group (Ramaswamy et al., 1997), as this species is polyandrous (Brakefield et al., 2001) and because adult-derived carbohydrates are needed for the onset of oviposition (Bauerfeind and Fischer, 2005; Fischer et al., 2004). Indeed, juvenile hormone mimics were shown to increase (early) fecundity in this butterfly (Steigenga et al., 2006), making reproductive control through juvenile hormones likely.

Regarding its temperature environment, the tropical butterfly *B. anynana* shows a striking phenotypic plasticity in wing color patterns (two seasonal morphs) as an adaptation to alternate wet- and dry-seasons and the associated changes in resting background and predation (Brakefield, 1997; Lyytinen et al., 2004). Further, it shows a striking reproductive plasticity: *B. anynana* females produce numerous small eggs under warm wet season conditions, but fewer and larger eggs at cool temperatures typical of the dry season (Fischer et al., 2003b). Such temperature-mediated plasticity is a common feature in ectothermic animals (Atkinson, 1994; Blanckenhorn, 2000; Ernsting and Isaaks, 2000; Yampolski and Scheiner, 1996). Based on differential survival probabilities with a high hatching success at warm temperatures favouring many small eggs, and a lower hatching success at colder temperatures favouring increased investment per offspring at the expense of egg number, this reproductive plasticity is thought to represent an example of adaptive phenotypic plasticity (Fischer et al., 2003b).

Apart from temperature, the availability of adequate adult nutrition is of high importance in *B. anynana,* which feeds on a variety of fallen and decaying fruit, including e.g. that from *Ficus* trees (Brakefield, 1997; Larsen, 1991). Access to carbohydrates in the adult stage is essential for egg production in this species, as without carbohydrates no eggs will be produced ("income breeder"; Bauerfeind and Fischer, 2005; Fischer et al., 2004). Thus, adult diet here is even more important than in many nectivorous butterflies, which do lay eggs if fed only water (Hill and Pierce,

1989; Karlsson and Wickman, 1990; Murphy et al., 1983; Stern and Smith, 1960). Consequently, even the first eggs laid contain considerable amounts of adult-derived nutrients, followed by a quick shift towards even higher ratios (Fischer et al., 2004).

Using *B. anynana*, this thesis focuses on two main subjects with regard to reproduction in this butterfly: the general hormonal regulation of reproduction, and the influence of the environment, especially temperature and nutrition, on reproductive traits.

Hormonal control of reproduction

Juvenile hormones, sequiterpenoid-like compounds secreted by the corpora allata (CA), a pair of epithelial glands, are well-known for preventing metamorphosis in the insect juvenile stages and for having pronounced effects on reproduction in the adults (Gilbert et al., 2000; Nijhout, 1994; Ramaswamy et al., 1997). Regarding reproduction, their primary function is to initiate vitellogenin synthesis in the fat body and to regulate the uptake of yolk by the ovary (Hoffmann, 1995). Juvenile hormone biosynthesis is a tightly regulated process involving stimulating (allatotropins) and inhibiting (allatostatins) neuropetides secreted by brain neurosecretory cells (Kataoka et al., 1989; Stay and Tobe, 2007), but also classical neurotransmitters (Chiang et al., 2002; Granger et al., 2000; Liu et al., 2005; Rachinsky and Tobe, 1996). While the above described reproductive strategies in response to prevailing temperatures have been extensively studied in *B. anynana*, its hormonal control is hitherto only poorly understood (Steigenga et al., 2006). Consequently, this issue serves as starting point for this thesis.

Is reproduction in *B. anynana* controlled by juvenile hormone and if so, how dependent is egg maturation on juvenile hormones over time?

Environmental effects on reproduction

Temperature-mediated plasticity and its hormonal control

Environmental effects on the expression of the phenotype, called phenotypic plasticity, are widespread in nature (Endler, 1986; Ghalambor et al., 2007; Miner et al., 2005; Nussey et al., 2007; Pigliucci, 2005). Such phenotypic plasticity may allow an organism to adjust its physiological, behavioural and morphological phenotype to environmental needs, and indeed many such cases are considered adaptive (see Miner et al., 2005; Pigliucci, 2005; Stearns, 1992). One of the most important abiotic factors for ectotherms is temperature, as it has vital consequences for biochemical and physiological processes and induces a variety of plastic responses (Eckert et al., 2002; Nijhout, 1999; Stearns, 1992). Understanding the regulation of plasticity poses an exciting challenge though, as environmental effects need to trigger different developmental pathways present within the same genotype (Flatt et al., 2005; Nijhout, 1999; Pigliucci, 2005; Sinervo and Svensson, 1998; Zera, 2007).

In insects, juvenile hormones and 20-hydroxy-ecdysone are important regulators of key aspects of their life histories, and are therefore good candidates for the regulation of phenotypic plasticity (Gäde et al., 1997; Nijhout, 1994). Indeed, traits known to be under hormonal control include metamorphosis, behaviour, caste determination, reproduction and polymorphisms (e.g. de Wilde and Beetsma, 1982; Dingle and Winchell, 1997; Emlen and Nijhout, 1999; Gäde et al., 1997; Gilbert et al., 2000; Hoffmann, 1995; Nijhout, 1994). Regarding the latter, seasonal wing polyphenism of *B. anynana* is a prominent example, being regulated through ecdysteroids during pupal development (Koch et al., 1996; Zijlstra et al., 2004). Hormones, therefore, provide a mechanistic link between environments, genes and trait expression (Finch and Rose, 1995; Flatt et al., 2005; Sinervo and Svensson, 1998).

To test for the influence of juvenile hormones on temperature-mediated plasticity in reproductive traits, egg number, egg size and longevity were scored at 20 and 27°C. The temperatures chosen for experiments are similar to the ones experienced by the butterflies in the field during the dry and wet season, respectively (Brakefield and Mazzotta, 1995; Brakefield and Reitsma, 1991). Thus, no marginal temperatures

were included, but ones the butterflies should be well adapted to. The effects of both temperatures on JH, vitellogenin and protein titres in the females' haemolypmh as well as on the final biochemical composition of eggs were investigated in addition to the above traits. In doing so, this thesis investigates, whether the striking reproductive plasticity in *B. anynana* is mediated through hormones.

Is there evidence that the reproductive plasticity in dependence of temperature is mediated through hormones in *B. anynana*?

Egg provisioning and resource utilization: effects of temperature on egg and hatchling composition

In general, eggs need to be provisioned with nutrients for successful embryonic development, with lipids and proteins comprising the main components of insect eggs (Beenakkers et al., 1985; Diss et al., 1996; Van Handel, 1993; Ziegler and Van Antwerpen, 2006). Although we know that the larger eggs produced at the lower temperature exhibit a higher hatching success in *B. anynana* (Fischer et al., 2003b), it is unclear whether this fitness advantage is related to size per se, to a larger absolute amount of nutrients or to relative changes in egg composition. The interplay between the effects of environmental variation on egg size, egg composition and in turn on offspring fitness is generally largely under-explored in insects (Casas et al., 2005; Giron and Casas, 2003; Jann and Ward, 1999; Karl et al., 2007; Kyneb and Toft, 2006), at least partly so because most studies exclusively rely on egg numbers and/or egg size as fitness measures (Azevedo et al., 1997; Bernardo, 1996; McIntyre and Gooding, 2000).

Furthermore, the use of specific egg compounds during embryonic development may partly depend on the environment in which development takes place and/or the environment experienced by the parents. The latter, called maternal effects, may cause differences in offspring provisioning or the activation of different developmental programs that tune the offspring's phenotype for the environment experienced by the mother (Fox and Czesak, 2000; Mousseau and Fox, 1998; Wolf and Brodie, 1998). Environmental experience can be transmitted to offspring via cytoplasmic egg

factors, e.g. yolk amount, egg composition, hormones or mRNA (Mousseau and Dingle, 1991; Mousseau and Fox, 1998; Sakwínska, 2004).

While the general influence of temperature on ectotherm growth and development is exceedingly well studied (e.g. Angilletta and Dunham, 2003; Atkinson, 1994; Jarošík et al., 2004; Liu et al., 1995; Robinson and Partridge, 2001; Wagner et al., 1984), the question of associated changes in energy budgets and turn-over rates has rarely been addressed (Booth and Kiddell, 2007; Guisande and Harris, 1995; Van Handel, 1993). Therefore, against the background of reproductive plasticity in the butterfly *B. anynana*, this study explores the consequences of temperature variation on egg and hatchling composition, and the associated use and turnover of energy and egg compounds.

Does temperature variation affect egg composition along with egg size?

What are the effects of temperature on the use and turnover of egg compounds and energy by the developing embryo, and are there indications of maternal effects?

The importance of nutrition and egg provisioning for progeny fitness

Reproduction is a nutrient-limited process, so that availability of adequate nutrition is of crucial importance for successful reproduction (Wheeler, 1996). A multitude of studies documented pronounced effects of diet quality and quantity on female reproductive output and thereby fitness (e.g. Braby and Jones, 1995; Leather, 1995; Willers et al., 1987). Fitness, however, is composed of various components, such that determining individual fitness is a challenging enterprise. Although frequently used, simply counting offspring (egg) numbers or using proxies only vaguely related to survival (such as egg size) might be misleading (Bernardo, 1996; McIntyre and Gooding, 2000). In order to gain a more integrated understanding of reproductive resource allocation, we need to shed more light on the interplay between reserves originating from storage versus income, between diet quality / quantity and egg composition, and the associated consequences for offspring viability.

Holometabolous insects are interesting models for studying reproductive resource allocation, because diets and energetic needs change dramatically between life stages, warranting integrated strategies for timed nutrient accumulation, storage and release (e.g. Boggs, 1981; Braby and Jones, 1995; Pan and Telfer, 2001; Stearns, 1992; Wheeler, 1996). This seems particularly important for female insects, since nutrient investment into eggs constitutes a major expenditure of energy (Boggs, 1981; Braby and Jones, 1995; Parker and Begon, 1986). The Lepidoptera, feeding as larvae on protein-rich plant foliage, were historically believed to primarily rely on larval-derived nutrients for reproduction and somatic maintenance (Jervis and Boggs, 2005; Leather, 1995; Mevi-Schütz and Erhardt, 2005; O'Brien et al., 2002; Telang et al., 2001). However, recent studies highlight the complex interactions between storage reserves and adult income, and that the adult diet may contribute significantly to reproductive output (e.g. Fischer et al., 2004; Hill, 1989; Mevi-Schütz and Erhardt, 2005; O'Brien et al., 2004).

While substantial progress was made in some of these areas in recent years (especially in relation to use of income versus storage; Fischer et al., 2004; Fischer et al., 2002; Jervis and Boggs, 2005; Mevi-Schütz and Erhardt, 2005; O'Brien et al., 2004; O'Brien et al., 2002), others remain poorly understood. This is particularly true for the effects of adult nutrition on egg composition and in turn on offspring fitness (Casas et al., 2005; Giron and Casas, 2003; Jann and Ward, 1999; Karl et al., 2007; Kyneb and Toft, 2006), which will be investigated here.

Does adult nutrition of female *B. anynana* butterflies affect egg composition and if so, what are the consequences for progeny fitness?

Synopsis

Hormonal control of reproduction in *B. anynana*

LC-MS analyses revealed that two different juvenile hormones, JH II and JH III, occurred in the hemolymph of *B. anynana* females (manuscript 2). While JH III occurs in all insect orders and consequently also in the Lepidoptera, the occurrence of JH II and I is more restricted, but both have been found in addition to JH III in other lepidopteran species (Gäde et al., 1997; Nijhout, 1994). Regarding vitellogenins, two sub-units, a larger and a smaller one, were identified, following the pattern typical for most insects (large > 150 kDa, small < 65 kDa; Raikhel and Dhadialla, 1992). No vitellogenins could be detected in freshly eclosed *B. anynana* females, supporting the notion that egg maturation starts only after metamorphosis in *B. anynana* (Ramaswamy et al., 1997).

To further investigate how JHs may affect reproduction in *B. anynana*, MK-801 as a high-affinity antagonist of ionotropic NMDA receptors in the Corpora allata (Begum et al., 2004; Chiang et al., 2002; Wong et al., 1986) was used to artificially reduce JH biosynthesis (manuscript 1). Additionally, JH mimics (pyriproxifen, methoprene) were applied to increase JH active compounds in the hemolymph. For comparative purposes, those experiments used additionally the hemimetabolous insect *Gryllus bimaculatus* de Geer. In the mediterranean field cricket *G. bimaculatus*, reproduction strongly depends on JH, representing a well studied organism in this respect (Hoffmann et al., 1996; Lorenz, 2003; Lorenz et al., 1995a; Lorenz et al., 1995b).

In both species studied, the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 clearly reduced lifetime reproductive output. Fecundity was reduced by up to 40% in *G. bimaculatus*, and by up to 24% in *B. anynana*. In the latter, egg size was negatively affected by about 4% during the first days by MK-801, which was not the case in *G. bimaculatus*. While in *G. bimaculatus* egg production was reduced throughout the oviposition period, inhibitory effects of MK-801 were restricted to the first days of the oviposition period in *B. anynana*. To infer causality, the combined effects of JH mimics and MK-801 were tested on initial fecundity in both insect species: in accordance with lifetime fecundity data, initial egg numbers were clearly

reduced in the MK-801 treated groups, but significantly increased in groups treated with a JH mimic. Most interestingly, egg numbers were very similar to controls in the groups treated with both compounds. Consequently the negative effects of MK-801 on fecundity were restored by adding JH active compounds. These data, together with the detection of JH III and II in the butterfly hemolymph, suggest that JH does affect reproduction in both species.

Despite the overall similarity of effects in both species used, there were also some interesting differences on fecundity. While in *G. bimaculatus* egg production was reduced throughout the oviposition period (at least until day 11 following ecdysis), in *B. anynana* the inhibitory effects of MK-801 were restricted to the first days of the oviposition period. Furthermore, the dose dependence of effects was more pronounced in *B. anynana* in comparison to *G. bimaculatus*. These findings may suggest some differences in the effects of JH on egg maturation across species. In *G. bimaculatus* JH biosynthesis and fecundity can be manipulated throughout the entire oviposition period by allatostatins and JH (mimic) injections administered early in life (Koch and Hoffmann, 1985; Lorenz, 2001), which does not seem to be the case in *B. anynana*.

The insect ionotropic NMDA receptor is found in the Corpora allata and is part of JH biosynthesis regulation by stimulating the flux of calcium and so JH biosynthesis (Chiang et al., 2002). For the antagonist MK-801 used here, which is a high-affinity antagonist in vertebrate NMDA receptors (Wong et al., 1986), it was unclear however, whether its mode of action was indeed related to insect JH biosynthesis, as combined *in vitro* and *in vivo* data on JH biosynthesis were missing (Begum et al., 2004). Due to the small size of the Corpora allata and very small amounts of hemolymph in *B. anynana*, such experiments were restricted to *G. bimaculatus*. MK-801 clearly reduced JH biosynthesis in single cricket corpora allata by up to 57.4%, and JH hemolymph titers by 48% in a dose-dependent manner (manuscript 1). In combination with the prior results, this clearly suggests that JH biosynthesis in the Corpora allata is at least in part controlled by an NMDA receptor with calcium as second level messenger. Based on these findings, NMDA receptor antagonists can be important tools for manipulating juvenile hormone biosynthesis and, therefore, for

gaining a better understanding of the mechanistic basis of reproduction, as here demonstrated in *B. anynana*.

Thus, manuscript 1 and 2 shows that reproduction in *B. anynana* is under hormonal control, with JH being an important signal especially for the initiation of reproduction.

Environmental effects on reproduction

Temperature-mediated plasticity and its hormonal control

Vitellogenins are representing a major part of eggs (as vitellins, Ziegler and Van Antwerpen, 2006) and are essential for successful larval development (Diss et al., 1996; Van Handel, 1993). Therefore, JH dynamics represents an obvious target for changes in egg size (Fox and Czesak, 2000; Ramaswamy et al., 1997), as vitellogenin synthesis and/or vitellogenin incorporation into developing eggs might easily increase or decrease through changes in JH titres (Flatt and Kawecki, 2007; Hoffmann, 1995). Thus, the pronounced temperature-mediated plasticity in egg size of *B. anynana*, producing larger eggs at lower temperatures and vice versa, might be related to variation in JH and consequently vitellogenin titres. Accordingly, temperature may also affect egg protein content.

Female *B. anynana* butterflies exposed to different oviposition temperatures showed the expected response to adult temperature, producing fewer but larger eggs at the colder 20℃ temperature, but more and smaller eggs at the warmer 27℃ temperature (manuscript 2, Fischer et al., 2003a; Fischer et al., 2003b). However, no evidence was found that this striking example of phenotypic plasticity is under hormonal control, as neither JH III nor JH II showed significant variation across temperatures throughout much of the oviposition period (manuscript 2). In line with the lack of variation in JH, hemolymph vitellogenin titres also showed no significant variation across temperatures. Vitellogenin levels showed a strong decrease with female age, which was similar for total hemolymph proteins, indicating again a depletion of resources with increasing fecundity (see above). In contrast, total hemolypmph protein titres were lower at 27℃ than at 20℃, potentially indicating a

more rapid depletion of other protein classes than female specific proteins with age at 27℃ (manuscript 2). As it is generally the case for insects (Van Handel, 1993; Ziegler and Van Antwerpen, 2006; Lorenz, 2003), *B. anynana* eggs were primarily composed of water (84%), lipids and protein (each 6-7%) followed by glycogen (3%) and free carbohydrates (0.3%, manuscript 2 and 3). Proteins were found generally in higher absolute quantities in large compared to small eggs. With regard to relative investment, protein content was higher in the smaller eggs produced at 27℃, at the expense of lipids (manuscript 2, but see manuscript 3). A relatively higher amount of protein in smaller eggs at the expense of lipids has also been found across eggs selected for large and small egg size in *B. anynana*, indicating that there might be a minimum threshold for protein below which successful embryonic development is not possible (Karl et al., 2007). Overall, however, egg composition was fairly similar across temperatures.

Based on similar patterns of JH, vitellogenins and egg proteins across temperatures, the results obtained from manuscript 2 suggest that temperature-mediated plasticity in egg size and number is not mediated through JH in *B. anynana*.

Egg provisioning and resource utilization: effects of temperature on egg and hatchling composition

In this experiment, two maternal and developmental (embryonic) temperature environments were used to explore the consequences of that variation for egg composition, hatchling composition and the associated use and turnover of energy and egg compounds. Differences in maternal (i.e. oviposition) temperature induced the above mentioned patterns of phenotypic plasticity with regard to egg size and number (manuscript 2, Fischer et al., 2003a; Fischer et al., 2003b). The larger size (about 28% in dry weight) of the eggs produced at the lower oviposition temperature was associated with higher absolute amounts of water, lipids, protein, glycogen and free carbohydrates. In relative terms, eggs produced at 20°C and 27°C showed a fairly similar composition, suggesting that the larger size of the eggs produced at the lower temperature is achieved by a considerably higher energetic investment per offspring. This is particularly true for the major egg components, lipids, protein, and glycogen which showed only minor differences across oviposition temperatures. Consequently, although egg size may be uncorrelated with energy content (Azevedo et al., 1997; Baur and Baur, 1997; Giron and Casas, 2003; Guisande and Harris, 1995; Jaeckle, 1995; McIntyre and Gooding, 2000), in *B. anynana* larger eggs did contain more energy throughout (manuscript 2 and 3).

Differences induced by oviposition temperature were not restricted to egg size and numbers, but were also present in the hatchlings resulting from these eggs (manuscript 3). Similar to eggs, hatchlings from eggs produced at the colder temperature were ca. 25% larger in dry weight than those produced at the warmer temperature. Relative amounts found in hatchlings originating from eggs produced at 20°C were characterized by a reduced water and free carbohydrate content compared to hatchlings originating from 27°C. The differences in free carbohydrates might be connected to lower metabolic rates at the lower temperature (Jarošík et al., 2004; Liu et al., 1995). Water content is considered important for fitness in *B. anynana* with higher amounts decreasing desiccation risk (Fischer et al., 2003a; Fischer et al., 2006), which may account also for the relatively higher water amounts in hatchlings from eggs produced at the warmer temperature.

Development at the lower compared to the higher temperature resulted in significantly higher absolute amounts of protein and glycogen per individual in the resulting hatchlings, but left the remaining compounds unaffected. While protein and lipid content was higher in hatchlings compared to eggs, glycogen was highly reduced, suggesting that it represents an important energy source for the developing embryo (Guisande and Harris, 1995; Van Handel, 1993). Contrary to the mosquito *Culex quinquefasciatus* (Van Handel, 1993), protein content in *B. anynana* did not remain constant during development from egg to hatchling, indicating an incomplete transformation into structural components due to metabolic losses. As embryonic development at the lower temperature resulted in larger hatchlings regardless of oviposition temperature and therefore egg size (thus following the temperature-size rule; Angilletta and Dunham, 2003; Atkinson, 1994), this was achieved by higher conversion rates of protein and glycogen into hatchlings.

Regarding relative hatchling composition in dependence of developmental temperature, hatchlings having developed at 20°C were characterized by reduced relative lipid, and free carbohydrate, but increased protein and glycogen content compared to 27°C hatchlings. Significant interactions between oviposition and developmental temperature for relative hatchling lipid and protein content indicate that in both cases effects of the developmental temperature were much more pronounced for hatchlings originating from eggs produced at 20°C. This interaction was additionally supported by the data on absolute amounts of glycogen and proteins, as also here higher protein and glycogen amounts were found in 20°C developed hatchlings which originated from that temperature. Furthermore, the relative conversion of protein from egg to hatchling was higher for eggs produced at 20°C. The overall different turnover rates of protein/glycogen and lipids/free carbohydrates suggest an emphasis on hatchling quality (i.e. protein content) at the more stressful low temperature, and therefore the presence of adaptive maternal effects. These patterns might be related to the different survival probabilities during the wet (warm) and the dry (cold) season in *B. anynana's* natural environment. Under wet season conditions, survival probability of eggs and hatchlings is generally high, while it is much lower under dry season conditions (Brakefield, 1997; Fischer et al., 2003a; Fischer et al., 2003b). As egg/hatchling protein content is at least often closely related to fitness (Diss et al., 1996), an increased preservation of proteins

(through an increased consumption of other compounds, mainly lipids) should be more beneficial under more stressful conditions (i.e. low ambient temperatures). Under warm wet season conditions, in contrast, survival probability is high regardless of egg quality (Fischer et al., 2003a; Fischer et al., 2003b), such that an enhanced preservation of protein should be less important. Under this adaptive scenario, effects would be expected to be more pronounced when females had the chance to tune their offspring's development through maternal effects, which indeed seems to be the case: effects are much more pronounced for eggs produced at 20℃ compared to 27℃. Such maternal effects may evolve for cross-ge nerational phenotypic plasticity with mothers passing on their experience to the offspring to increase offspring fitness in predictable environments (Mousseau and Dingle, 1991; Mousseau and Fox, 1998; Rossiter, 1996). Additionally, a significant interaction between oviposition and developmental temperature for absolute hatchling water content showed that absolute water content was highest when the developmental temperature matched the oviposition temperature. This finding indicates furthermore that mothers tuned their offspring's phenotype for the environment experienced during oviposition, suggesting beneficial acclimation (Wilson and Franklin, 2002; Woods and Harrison, 2002).

Overall, manuscript 2 and 3 show that larger eggs produced at the lower temperature were achieved by providing these eggs with larger quantities of all compounds investigated, and thus more energy, whilst relative egg composition was rather similar to that of smaller eggs laid at the higher temperature. Turnover rates during embryonic development, which were investigated in manuscript 3, differed across developmental temperatures, suggesting an emphasis on hatchling quality (i.e. protein content) at the more stressful lower temperature, but on storage reserves (i.e. lipids) at the higher temperature. These differences may represent adaptive maternal effects.

The importance of nutrition and egg provisioning for progeny fitness

Individual fitness is a complex trait that is difficult to measure. A commonly used method for estimating the fitness effects of dietary treatments (and other factors) is determining the number (and sometimes size) of eggs produced (see Azevedo et al., 1997; Bernardo, 1996). Studies directly measuring offspring viability, in contrast, are much less frequent (Capinera et al., 1977; Diss et al., 1996; McIntyre and Gooding, 2000; Quickenden and Roemhild, 1969). Not accounting for differences in offspring viability is obviously problematic, and very little is known to date on the interplay between diet quality, age, egg content, and offspring viability (Casas et al., 2005; Karl et al., 2007; McIntyre and Gooding, 2000). Five different nutritional treatments were used for female *B. anynana* butterflies ranging from moist banana, plain sucrose solution, to sucrose solution enriched with lipids, yeast and finally minerals and vitamins. Eggs oviposited early (days 3-4) and late (days 16-20) within the oviposition period were analyzed with regard to their composition as well as egg hatchling success.

Clearly, the complex nutritional composition of banana fruit was superior compared to every other diet, not only increasing reproductive output compared to sugar-based diets, but also positively affecting egg hatching success (manuscript 4). While fecundity at days 3-4 was fairly similar across treatment groups, variation in later fecundity was higher with the banana group showing the highest fecundity, followed by the sucrose solution enriched minerals and the vitamin group. Further, hatching success of progeny was similar in the beginning but dropped tremendously from about 75% down to 30% in all sucrose-based diets, while staying high for eggs produced by banana-fed mothers. As these differences were particularly pronounced later in life, the results suggest the depletion of essential nutrients in sucrose-fed females with age (Giron and Casas, 2003; Karlsson and Wiklund, 1984), being on the one hand important for realizing higher fecundity levels, but also for successful embryonic development. Positive effects of additionally providing minerals or vitamins, though not necessarily in combination with each other, on fecundity have been reported also for some other insects (Engelmann, 1999; Pappas and Fraenkel, 1977), but were clearly not connected to offspring survival. General reproductive output might therefore be a consequence of resource congruence, as sugar enriched with minerals and vitamins increased fecundity (though not reaching the levels of the

banana group), while either adding protein or lipids had no detectable effect (Bauerfeind and Fischer, 2005; Bauerfeind et al., 2007).

In contrast to fecundity, egg size was not affected by nutritional treatment, but a general decline in egg provisioning was found. Egg size and thus energy content decreased with age, which is in agreement with other studies (Braby and Jones, 1995; Giron and Casas, 2003; Karlsson and Wiklund, 1984; McIntyre and Gooding, 2000; Mousseau and Dingle, 1991). Effects of adult diet on egg composition were not straightforward, so that e.g. lipids in female butterfly diet were not connected to higher amounts of lipids in eggs. Protein content declined with female age, but only in the two groups exhibiting highest fecundity (banana, sucrose solution enriched minerals and vitamins) suggesting that *B. anynana* reproduction generally strongly depends on nitrogenous resources accumulated during the larval phase (see also Jervis and Boggs, 2005; O'Brien et al., 2002; Wheeler, 1996). This development was counter-balanced by an increased investment of lipids into later eggs, presumably synthesised from the adult diet (Bauerfeind and Fischer, 2005; Fischer et al., 2004). As lipids represent a higher energetic resource than all other content classes (39.1 vs. 17.2 kJ/g, Ganong, 1974; Silbernagel and Despopolos, 1991), this had the consequence that in the high fecundity groups relative investment (per mg dry mass) as well as absolute investment in reproduction remained unaffected, although egg size declined with female age (see also Karl et al., 2007). Thus, caution is needed when trying to draw general conclusions from studies measuring egg size and number only, and studies on reproductive resource allocation should take into account variation in egg quality (McIntyre and Gooding, 2000).

Overall total egg energy and water content showed relations to egg hatching success, while egg protein, lipid, glycogen and free carbohydrate content did not seem to limit successful development. There is some evidence already that a high water content, presumably reducing desiccation risk, may be important for successful egg development in *B. anynana* (Fischer et al., 2003a; Fischer et al., 2006). Consequently, water should not be exclusively considered a cheap filler, especially since probably some energy is needed to incorporate water into eggs. The importance of adult diet for different components of *B. anynana* fitness exemplifies

the complexity of reproductive resource allocation in insects, which were formerly assumed to rely primarily on larval stores (Leather, 1995; Telang et al., 2001).

To conclude, manuscript 4 demonstrates that adult diet has pronounced effects on fecundity, egg composition and egg hatching success, with butterflies feeding on the complex nutrition of banana fruit performing best. Vitamins and minerals in a sucrose-based diet increased fecundity, but did not affect offspring hatching success. Effects of adult diet on egg composition were not straightforward, indicating complex interactions among specific compounds. There was some evidence that total egg energy and water content were related to hatching success. The results demonstrate that egg composition should be taken into account in such studies, as egg size and number does not necessary represent a good proxy for reproductive energetic investment.

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Record of contributions to this thesis

All experiments, including its design, execution and analyses were conducted by myself or followed my direct instructions. In manuscript 1 PD Dr. Matthias W. Lorenz performed the *in vitro* experiments in *Gryllus bimaculatus* due to his expertise, in manuscript 3 Susann Janowitz and Jana Perlick helped two nights collecting larvae and in manuscript 4 Ina Thamke helped for 30 hours by supporting me in egg collection and analysis of egg composition. All other work, as well as all manuscripts and this thesis were solely done by me.

Prof. Dr. Klaus Fischer, Prof. Dr. Klaus. H. Hoffmann and PD Dr. Matthias W. Lorenz are the supervisors of this thesis and consequently co-authors of all publications. They contributed support and supervision throughout. Supervisor was also PD Dr. Martina Meyering-Vos for the project presented in manuscript 2 by supporting and introducing me to SDS-PAGE analysis.

Manuscript 1

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Effects of the NMDA receptor antagonist MK-801 on female reproduction and juvenile hormone biosynthesis in the cricket *Gryllus bimaculatus* and the butterfly *Bicyclus anynana.*

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Abstract

Apart from regulating insect development, juvenile hormones (JHs) play an important role in insect reproduction, where they initiate vitellogenin synthesis and regulate the uptake of yolk by the ovary. JH synthesis is a tightly regulated process controlled by neurons and peptidergic neurosecretory cells. One of the known stimulatory regulators of JH biosynthesis is glutamate, and its N-methyl-D-aspartate (NMDA) receptor has been recently found in the cockroach *Diploptera punctata*. In this study we demonstrate a strong reduction in reproductive output in the tropical butterfly *Bicyclus anynana* and the Mediterranean field cricket *Gryllus bimaculatus* caused by the NMDA receptor antagonist MK-801. Such inhibiting effects on reproduction could be overruled by the application of JH mimics. In *G. bimaculatus*, MK-801 inhibits *in vitro* JH biosynthesis in the corpora allata and reduces *in vivo* JH hemolymph titres in a dose-dependent manner. These results suggest that JH biosynthesis in the corpora allata is at least in part controlled by an NMDA receptor with Ca^{2+} as second level messenger. Based on our findings we consider NMDA receptor antagonists as important tools for manipulating juvenile hormone biosynthesis and therefore for gaining a better understanding of the mechanistic basis of reproduction.

Keywords

fecundity, corpora allata, glutamate receptor, insects

Abbreviations

CA= Corpora allata, JH= Juvenile hormone, NMDA= N-methyl-D-aspartate

Introduction

In insects, hormones are the main regulators of larval development, metamorphosis, behaviour, caste determination, diapause, polymorphism, and reproduction (Flatt et al., 2005; Gäde et al., 1997; Nijhout, 1994). Among the different hormones, the juvenile hormones (JHs) and the ecdysteroids are generally considered the most important ones in affecting these processes in insects. JHs are sequiterpenoid lipid-like compounds secreted by the corpora allata (CA), a pair of epithelial glands, and are well-known for preventing metamorphosis in the insect juvenile stages and for having pronounced effects on reproduction in the adults (Gilbert et al., 2000; Nijhout, 1994; Ramaswamy et al., 1997). Their primary function in reproduction is to initiate vitellogenin synthesis in the fat body and to regulate the uptake of yolk by the ovary (Hoffmann, 1995). JH biosynthesis is a tightly regulated process involving stimulating (allatotropins) and inhibiting (allatostatins) neuropetides secreted by brain neurosecretory cells (Kataoka et al., 1989; Stay and Tobe, 2007), but also classical neurotransmitters (Chiang et al., 2002a; Granger et al., 2000; Liu et al., 2005; Rachinsky and Tobe, 1996).

At least in the cockroach *Diploptera punctata*, CA function is controlled by either stimulating ionotropic (Chiang et al., 2002a) or inhibiting metabotropic receptors (Liu et al., 2005). The identified ionotropic N-methyl-D-aspartate (NMDA) glutamate-gated receptor in the CA of *D. punctata* has striking similarities with the vertebrate NMDA receptor, especially regarding its structure, high Ca^{2+} permeability, and its response to typical antagonists like MK-801, conantokin and Mg^{2+} (Chiang et al., 2002a). Interestingly, NMDA receptors in mammals are important for reproductive control through their effects on the release of the gonadotropin-releasing hormone required for the initiation of puberty, the maintenance of reproductive capability and reproductive behaviour (Mahesh and Brann, 2005). Similar effects have been described in other vertebrates (Flynn et al., 2002), but also in a protochordate species, *Ciona intestinalis*, suggesting a highly conserved reproductive function in chordates (D'Aniello et al., 2003; Di Fiore et al., 2000).

MK-801, as a high-affinity antagonist of NMDA receptors (Wong et al., 1986), has been intensively studied in mammalian models, in particular in connection with its effects on neuronal plasticity and its neurotoxicity-reducing effects in ischaemia, epilepsia, brain hypoxia, and hypoglycemia (Ellison, 1995; Williams et al., 1991; Wolf,

1998). Furthermore, this antagonist was used to study NMDA receptor mediated effects on reproduction in other vertebrates (Flynn et al., 2002; Luderer et al., 1993; Mahesh and Brann, 2005; Melis et al., 2004). By contrast, in insects MK-801 was thus far used in three species. MK-801 was found to inhibit NMDA triggered JH biosynthesis *in vitro* in the cockroach *D. punctata* via reduced levels of free cytosolic calcium in the CA (Chiang et al., 2002a; Chiang et al., 2002b). Furthermore, Begum and co-workers (Begum et al., 2004) used MK-801 as an efficient blocker of vitellogenesis in the flesh fly *Neobellieria bullata* and the locust *Schistocerca gregaria*. However, studies on long term effects of MK-801 on reproduction and on JH biosynthesis, involving *in vitro* and *in vivo* measurements, are apparently missing (Begum et al., 2004). We address these issues here using two insect species: the hemimetabolous cricket *Gryllus bimaculatus* and the holometabolous butterfly *Bicyclus anynana*, in order to validate the above supposed mode of action and to test for its generality (Zera, 2007).

Gryllus bimaculatus represents has been extensively used to study the hormonal control of reproduction, which strongly depends on JH (Hoffmann et al., 1996; Lorenz, 2003; Lorenz et al., 1995a; Lorenz et al., 1995b), thus making it a highly suitable target for JH manipulation. The butterfly *B. anynana* belongs to a group of the Lepidoptera in which vitellogenesis and choriogenesis seem to depend exclusively on JH (Ramaswamy et al., 1997). Reproduction, including different strategies in response to prevailing temperatures, has been extensively studied in *B. anynana* (Fischer et al., 2003a; Fischer et al., 2004; Fischer et al., 2003b; Steigenga et al., 2005), while its hormonal control is hitherto only poorly understood (Steigenga et al., 2006), making this species another suitable model. Given the dependence of reproduction on JH in both species, we here examine the effect of the NMDA receptor antagonist MK-801 on lifetime fecundity and egg size, on *in vitro* and *in vivo* JH biosynthesis, and its interactions with JH mimics.

Material and methods

Animals and experimental populations

For this study two species of insects, the tropical butterfly *Bicyclus anynana* Butler, 1879 (Lepidoptera, Satyrinae) and the Mediterranean field cricket *Gryllus bimaculatus* de Geer, 1773 (Ensifera, Gryllidae) were used. *B. anynana* is a fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen, 1991). 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 over 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof et al., 2005). *G. bimaculatus* has a global distribution, spanning Africa, Asia and southern Europe (Harrison and Bogdanowicz, 1995; Ragge, 1972). The laboratory colony at Bayreuth University was established with field-caught animals from Italy and Spain in 1995. Regularly, individuals (also originating from Mediterranean areas) from commercial suppliers were added to the stock population to maintain high levels of heterozygosity (Lorenz et al., 2004).

Insect rearing

Bicyclus anynana was reared in a climate cell at 27℃, 70% relative humidity, and a photoperiod of 12 h light: 12h dark. Larvae were fed on young maize plants in population cages (50x 50x 80 cm). The resulting pupae were collected from the plants and transferred to cylindrical hanging cages. Throughout all experiments, butterflies had access to moist banana for adult feeding. *G. bimaculatus* was reared at 27℃, 30-40% relative humidity and a photoperiod of 16 h light: 8 h dark. Larvae were reared in population cages (45x 40x 65 cm), fed on a mixture of commercial rat/mouse, rabbit and cat diet (Altromin GmbH, Lage, Germany), and supplied with drinking water *ad libitum*. Newly ecdysed adults (day 0) were collected daily and transferred to population cages (22x 20x 37 cm).

Experimental design

To investigate the effects of the NMDA receptor antagonist MK-801 on female reproduction and juvenile hormone biosynthesis, five different experiments were performed as outlined below.

Experiment 1: Effects of MK-801 on *B. anynana* reproduction

On the day of eclosion, female butterflies were randomly divided among four treatment groups, being treated with 0 (control), 10, 20 or 30 µg MK-801 in 4 µl Ringer solution. These solutions were repeatedly injected into the females' thorax, using a Hamilton syringe, on days 0, 2 and 6 of adult life. All females were kept together with male butterflies for mating until day 2 of adult life. After the mating period, females were placed individually in translucent plastic containers (1 L, covered with gauze) containing a fresh cutting of maize for egg-laying. Eggs of ~40 females per group were collected and counted daily until the death of the females. Egg size was measured as cross-sectional area (mm^2) using a digital camera (Leica DC300, Leica Microsystems, Wetzlar, Germany) connected to a stereo microscope (Leica MZ 7.5). The resulting images were analysed using Scion Image public software (Scion Corporation 2000, Frederick, Maryland, USA). 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).

Experiment 2: Effects of MK-801 on *G. bimaculatus* reproduction

Adult female crickets were randomly divided among three treatment groups, being injected with 0 (control), 50, or 150 µg MK-801 in 4 µl DMSO / Ringer (1:1 v/v) solution (note that MK-801 is not soluble in pure Ringer at high concentrations). This solution was injected into the lateral intersegmental membrane between the third and fourth abdominal segment, using a Hamilton syringe, took place on days 0 and 3 of adult life. Females were housed together with males for mating from day 2 until day 4 following ecdysis. Thereafter, females were placed individually in plastic boxes (18x 13.5x 6 cm) and provided moist sand as egg laying substrate. Eggs were collected, counted and measured (as outlined above) daily for the following 8 days. For each group about 35 females were used.

Experiment 3: Interactive effects between MK-801 and JH mimics in *B. anynana* and *G. bimaculatus*

To investigate whether any potential effects of MK-801 on reproductive traits are mediated through variation in JH titres, JH mimics were applied to artificially increase JH active compounds in the hemolymph. As mimics, pyriproxyfen (Dr. Ehrenstorfer GmbH, Germany) was used for *B. anynana*, and methoprene (Fluka, Taufkirchen, Germany) for *G. bimaculatus*. The compounds are known to work well as JH mimics in these species (Hoffmann et al., 1996; Steigenga et al., 2006). Both species were randomly divided among four treatment groups, being treated with MK-801, a JH mimic, MK-801 plus JH mimic or the pure solvent (control). *B. anynana* females (70-77 per group) were treated on days 0 and 2 with either 10 µg MK-801 in 6µl Ringer (being injected), 0.1 µg pyriproyfen in 2 µl aceton (applied topically on the abdomen using a Hamilton syringe), both compounds or 6 µl Ringer / 2 µl acetone (control). Females were kept together with males for mating until day 2, and were afterwards placed individually in plastic containers as described above. Egg numbers were determined for day 3 of adult life only. *G. bimaculatus* females (33-36 per group) were treated on days 0 and 3 with either 150 µg MK-801 in 4 µl DMSO:Ringer (1:1), 30 µg methoprene in 4 µl isooctane (applied topically on to the abdomen), both compounds or 4 µl DMSO/Ringer / 4µl isooctane (control). Females were kept together with males for mating until day 3 and were then placed individually in plastic containers as described above. Egg numbers were determined for days 4 to 6 following ecdysis. The respective concentrations and treatment days were chosen on the basis of experiments 1 and 2 as well as pilot studies.

Experiment 4: Effects of MK-801 on *in vitro* JH biosynthesis in *G. bimaculatus*

Due to the small size of the CA and very small amounts of hemolymph in *B. anynana*, experiments 4 and 5 were restricted to *G. bimaculatus*. Single CA from *G. bimaculatus* females were used in a rapid partition assay (Feyereisen and Tobe, 1981). Methods essentially followed Lorenz et al. (1995b, 1997) with some modifications: the TC 199 incubation medium (M 7653, Sigma, Deisenhofen, Germany) with Hank's salts and sodium bicarbonate, without L-glutamine, buffered with 25 mmol l^{-1} HEPES, supplemented with $CaCl_2$ to a final concentration of 3 mmol l^{-1}, L-methionine to a final concentration of 0.28 mmol l^{-1} and sodium acetate to a final concentration of 2.5 mmol l^{-1}, fortified with 1% Ficoll 400, was adjusted to pH 7.2. As

radio-labelled precursor, 2-^{14}C-acetate (MC 213; Hartmann Analytic, Braunschweig, Germany) was added to a final specific activity of 64 MBq mmol^{-1}. The resulting total acetate concentration in the radio-labelled incubation medium was 2.58 mmol l^{-1}. Single glands without MK-801 were pre-incubated for 90 min for stabilizing JH synthesis in the *in vitro* setup, then transferred to the first incubation for 120 min and finally assigned to the second incubation with the respective treatments for 120 min. JH release was examined in untreated control animals and at 6 MK-801 concentrations ranging from 10^{-3} to 10^{-6} mol l^{-1}. Changes in JH release were referred to the first incubation to correct for release differences between single CA (N = 20-35, but for 10^{-3} and 10^{-6} mol l^{-1} N = 10).

Experiment 5: Effects of MK-801 on *in vivo* JH titres in *G. bimaculatus*

The JH titres in the hemolymph of *G. bimaculatus* females were quantified by liquid chromatography-mass spectrometry (LC-MS; Westerlund and Hoffmann, 2004). The experimental design followed the one described for experiment 2 with 21-24 females for each treatment. Three and 24 h after the second injection at day 3, 20 µl of hemolymph were collected per female and extracted (Westerlund and Hoffmann, 2004). The samples were separated on a C18 reverse-phased column (ReproSil-Pur ODS-3, 5 µm; Dr. Maisch GmbH, Germany), protected by a guard column (C18 cartridge; Phenomex, Aschaffenburg, Germany) with differing gradients of water/methanol. MS analysis was accomplished by using electrospray ionization (ESI) in positive ion mode using a Shimadzu LCMS-2010A. As only the relative differences between treatments were of importance, no additional calibration to estimate the absolute amount of juvenile hormone was applied.

Data analysis

Differences in egg numbers over time were analyzed using two-way repeated measurements ANOVAs, with treatment and time (i.e. oviposition day) as factors. Data on total fecundity, mean egg size (averaged over the oviposition period), and JH titres were analyzed with standard ANOVAs. As treatment with MK-801 frequently resulted in the production of zero eggs per day, no repeated measurements ANOVAs could be calculated for egg sizes. Differences among treatment groups were located using Tukey's HSD. The fecundity data from *G. bimaculatus* were square-root transformed prior to analyses to meet ANOVA requirements. Survival probabilities of

B. anynana females over time were analyzed by survival analyses for multiple groups, based on Gehan's generalized Wilcoxon test. The dose response curve for the release of JH by the CA of *G. bimaculatus* with regard to MK-801 treatment was calculated by a sigmoidal 5-parameter fit of SigmaPlot 9.1. All statistical tests were performed using Statistica 6.1 and values are given as means ± 1 s.e.m.

Results

Experiment 1: Effects of MK-801 on *B. anynana* reproduction

The number of eggs laid over time differed significantly across treatment groups (repeated measurements ANOVA: $F_{3,1370}$ = 5.78, P < 0.001). Differences were particularly pronounced during the first days of the oviposition period (Fig. 1A). Following an initial increase, egg numbers generally declined with female age ($F_{10,1370}$ = 53.17, P < 0.001). A significant treatment by time interaction ($F_{30,1370}$ = 5.31, P < 0.001) probably reflects the above mentioned pronounced differences in early fecundity, whereas egg numbers were more similar later in life. Note that the third injection of MK-801 on day 6 of adult life had only a minor effect on egg production (Fig. 1A). In line with the above results on daily fecundity, lifetime fecundity differed significantly across treatment groups, being reduced by ca. 24% in the 30 µg MK-801 group as compared to the control ($F_{3,156}$= 3.10, P = 0.028, Tab. 1A). Again, differences were most pronounced during the first days of the oviposition period ($F_{3,152}$ = 10.43, P < 0.001; Tab. 1B).

Egg size generally decreased with increasing female age, but (if averaged over the whole oviposition period) did not differ significantly between treatment groups ($F_{3,155}$ = 1.83, P = 0.14; Tab. 1A, Fig. 1B). However, restricting the analysis to days 3-7 of adult life shows that mean egg size tended to decrease with increasing MK-801 concentration ($F_{3,155}$= 4.00, P = 0.009; Tab. 1B). MK-801 treatment did not affect female survival probability (χ^2_4 = 2.5, P = 0.47; Fig. 2).

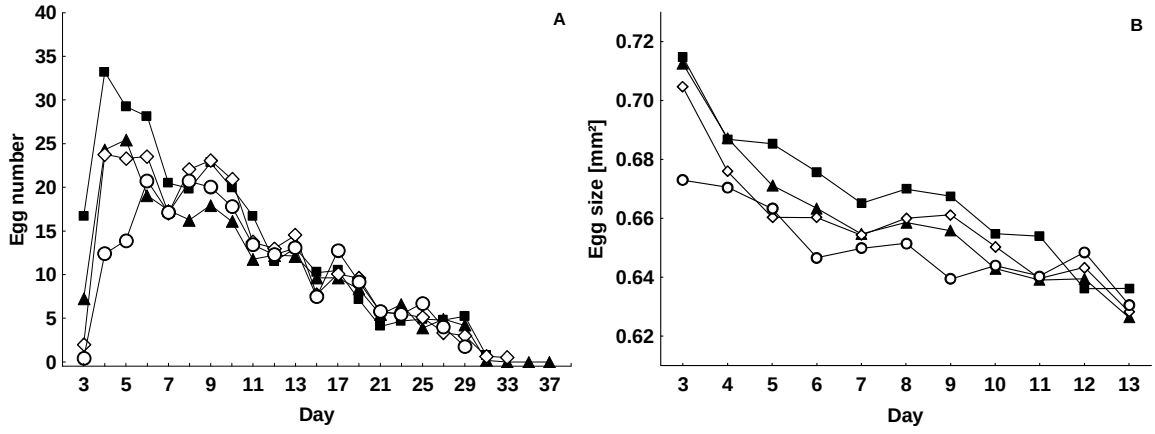

Fig. 1: Number (A) and size (B) of eggs produced over time by female *Bicyclus anynana* treated with different concentrations of MK-801. Injections of MK-801 (controls were injected with Ringer solution only) were given on days 0, 2 and 6 of adult life. To improve clarity, no standard errors are presented (N = 38-40, square= control, triangle= MK-801 10µg, diamond= MK-801 20µg, circle= MK-801 30µg).

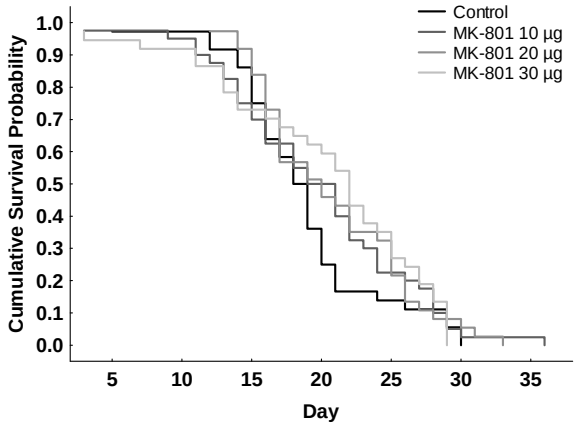

Fig. 2: Effects of MK-801 on the cumulative survival probability of *Bicyclus anynana* females. Injections of MK-801 (control: Ringer solution) were applied on days 0, 2 and 6 of adult life (*N* = 38-40).

Tab. 1: Effects of MK-801 treatment on fecundity and mean egg size in *Bicyclus anynana* and *Gryllus bimaculatus*. A) Lifetime fecundity (i.e. total number of eggs produced throughout the live of a given female) and mean egg size for *B. anynana* (experiment 1); B) egg numbers and mean egg size for days 3-7 after eclosion for *B. anynana* (experiment 1); C) fecundity and mean egg size until day 12 after ecdysis for *G. bimaculatus* (experiment 2).

		Fecundity		**Mean Egg size**	
A) *Bicyclus anynana*	**N**	**Mean**	**s.e.m.**	**Mean**	**s.e.m.**
Control	40	275.9	15.3	0.658	0.007
MK-801 10 µg	40	226.1	19.5	0.650	0.006
MK-801 20 µg	38	252.6	13.2	0.649	0.004
MK-801 30 µg	38	208.6	18.2	0.639	0.006
B) *Bicyclus anynana*					
Control	40	126.5	7.8	0.684	0.007
MK-801 10 µg	40	91.1	6.6	0.675	0.006
MK-801 20 µg	38	89.6	6.8	0.667	0.005
MK-801 30 µg	38	59.4	6.5	0.656	0.006
C) *Gryllus bimaculatus*					
Control	34	386.5	61.9	0.932	0.007
MK-801 50 µg	37	286.9	39.0	0.942	0.007
MK-801 150 µg	34	233.4	38.6	0.941	0.009

Experiment 2: Effects of MK-801 on *G. bimaculatus* reproduction

Daily egg numbers differed significantly across treatment groups, being generally lower in the groups treated with MK-801 (repeated measurements ANOVA: $F_{2,642}$ = 5.35, P = 0.006; treatment by time interaction $F_{12,642}$ = 0.77, P = 0.686; Fig. 3A). Egg numbers peaked on day 6 of adult life, followed by a constant decline with female age ($F_{6,642}$ = 67.79, P < 0.001). Accordingly, lifetime fecundity was significantly reduced (by ca. 40%) in the group treated with 150 µg MK-801 as compared to the control group ($F_{2,104}$ = 3.10, P = 0.04; Tab. 1C). Egg size was not significantly affected by MK-801 ($F_{2,103}$ = 0.54, P = 0.59; Fig. 3B). As this experiment was terminated on day 12 following ecdysis (coinciding with the end of egg-laying), no longevity data are available, but at least during this phase mortality rates were very similar (Control: 0 individuals; 50 µg MK-801: 2; 150 µg MK-801: 0).

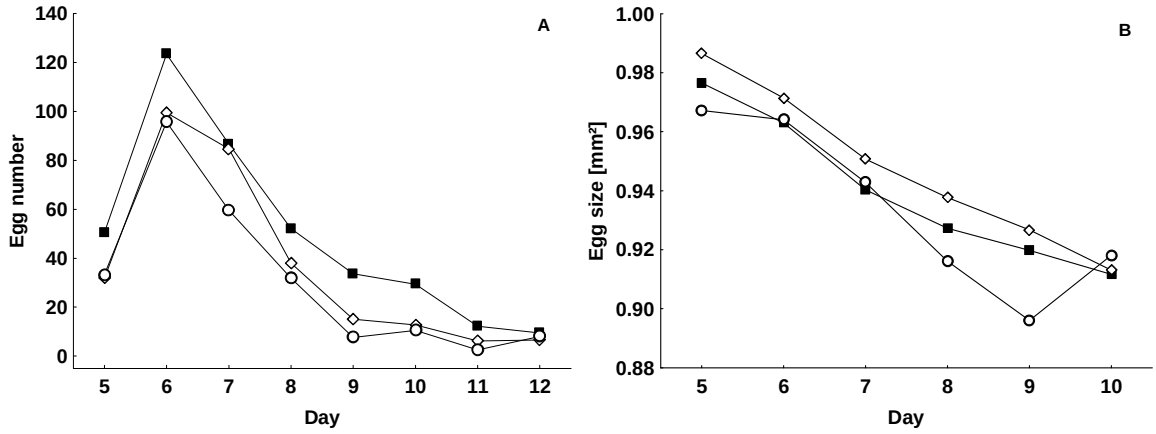

Fig. 3: Number (A) and size (B) of eggs produced over time by female *Gryllus bimaculatus* treated with different concentrations of MK-801. Injections of MK-801 (controls were injected with Ringer:DMSO 1:1 v/v) were given on days 0 and 3. To improve clarity, no standard errors are presented (N = 34-37, square= control, diamond= MK-801 50µg, circle= MK-801 150µg).

Experiment 3: Interactive effects between MK-801 and JH mimics in *B. anynana* and *G. bimaculatus*

Egg numbers varied significantly across treatment groups in both species (*B. anynana*: $F_{3,270} = 9.99$, $P < 0.001$; *G. bimaculatus*: $F_{3,139} = 10.55$, $P < 0.001$; Figs 4A, B). They were reduced in the MK-801 treated groups, but increased in the groups treated with a JH mimic. Most interestingly, egg numbers were very similar to controls in the groups treated with both compounds.

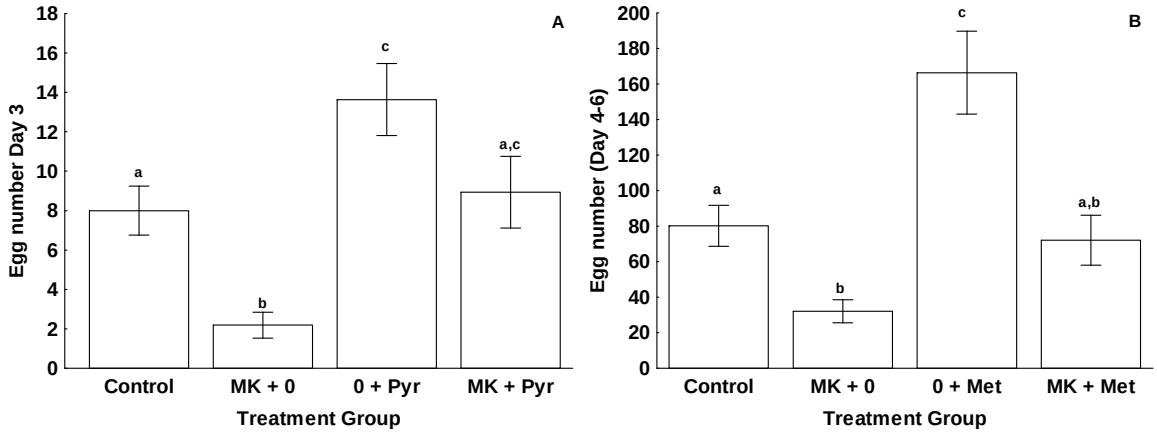

Fig. 4: Effects of MK-801 (MK), juvenile hormone mimics [pyriproxyfen (Pyr) or methoprene [Met]), both compounds or the pure solvent (control) on early fecundity in *Bicyclus anynana* (A, *N* = 70-77) and *Gryllus bimaculatus* (B, *N* = 33-36). Given are means ± 1 s.e.m. Letters above bars indicate significant differences between groups (Tukey HSD after ANOVA).

Experiment 4: Effects of MK-801 on *in vitro* JH biosynthesis in *G. bimaculatus*

JH synthesis in single *G. bimaculatus* CA decreased significantly with increasing amounts of MK-801 ($F_{5,135}$ = 13.81, P < 0.001; Fig. 5). Maximal inhibition of -57.4% occurred at the highest concentration (10^{-3} mol l^{-1} MK-801), 50% inhibition was reached at about $1.5*10^{-4}$ mol l^{-1}. The control groups, untreated in the second incubation, showed a JH synthesis of -1.9% compared to the first incubation, suggesting that JH synthesis remained stable over time (paired t-test: t = 1.86, n = 42, P = 0.13).

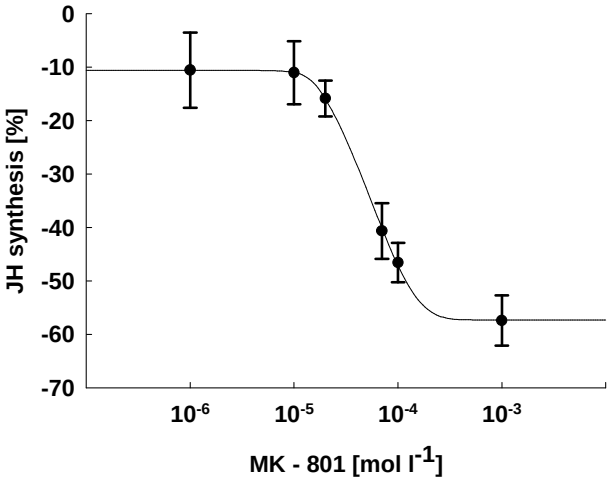

Fig. 5: Dose response curve for *in vitro* inhibition of juvenile hormone (JH) synthesis by MK-801 in single *Gryllus bimaculatus* corporus allatum. Values are given relative to the first incubation rates of each corporus allatum (N = 20-35, but for 10^{-3} and 10^{-6} mol l^{-1} N = 10). Controls released JH III in the second incubation at a rate of 26.8 ± 1.8 pmol h^{-1}.

Experiment 5: Effects of MK-801 on JH titres *in vivo* in *G. bimaculatus*

Only JH III was detected in the hemolymph of *G. bimaculatus* females. Injection of MK-801 significantly decreased JH titres *in vivo,* maximally by 48.4% (repeated measurements ANOVA: $F_{2,65}$ = 4.07, P = 0.022; Fig. 6). Furthermore, JH III titres increased significantly by ca. 74% from day 3 to 4 ($F_{1,65}$ = 29.1, P < 0.001; treatment by time interaction $F_{2,65}$ = 1.93, P = 0.15).

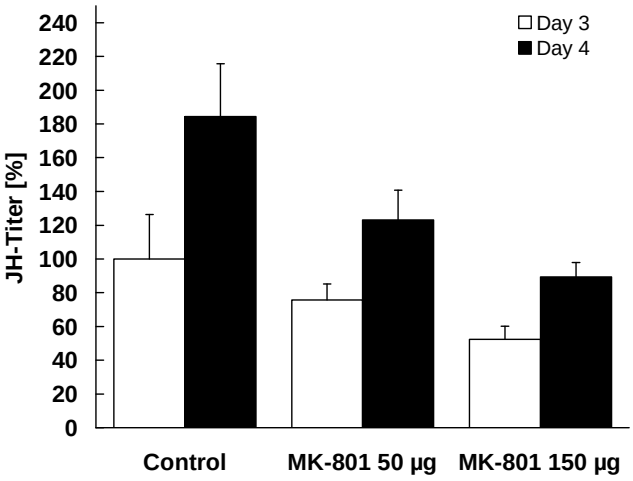

Fig. 6: Effects of MK-801 and time on *in vivo* juvenile hormone titres in the hemolymph of *Gryllus bimaculatus* females (N = 21-24). Females were injected on days 0 and 3 following ecdysis. Hemolymph was sampled 3 and 24 hours after the last injection. The control group at day 3 was set to 100%. Given are means ± 1 s.e.m.

Discussion

In both species studied, the NMDA receptor antagonist MK-801 clearly reduced reproductive output. Fecundity was reduced by up to 40% in *G. bimaculatus*, and by up to 24% in *B. anynana*. In the latter, egg size was additionally negatively affected by MK-801 (significantly so during the first days of oviposition), which was not the case in *G. bimaculatus*. For *B. anynana* detailed information on JH biosynthesis in the CA is lacking, but JHs generally play a key role for egg maturation in this group of lepidopterans (Ramaswamy et al., 1997). Furthermore, Steigenga et al. (2006) demonstrated that applications of the JH mimic pyriproxifen significantly increased fecundity but decreased longevity, supporting the notion that in *B. anynana* JH has pleiotropic effects on key life-history traits, as has been found for other insects (Flatt et al., 2005; Ramaswamy et al., 1997; Zera et al., 1998). For *G. bimaculatus* much more detailed information on JH biosynthesis and the hormonal control of reproduction, especially egg maturation, is available (Hoffmann et al., 1996; Koch and Hoffmann, 1985; Lorenz et al., 1997).

However, we propose that the overall similar reduction in reproductive output found in both species is causally related to the inhibitory effects of MK-801 on JH biosynthesis (Chiang et al., 2002a). Accordingly, we predicted that negative effects of MK-801 on fecundity can be restored by adding JH active compounds. Indeed this was found when treating females with both, MK-801 and JH mimics, yielded fecundity data for both species that were statistically indistinguishable from those of the control groups. Similarly, Begum et al. (2004) showed that JH treatment could overrule the blocking effect of MK-801 on vitellogenesis in *S. gregaria*. Although these findings strongly suggest that the NMDA receptor is involved in JH biosynthesis, a proof can only be obtained by *in vitro* and *in vivo* analyses (Begum et al., 2004; Zera, 2007).

Corresponding analyses in *G. bimaculatus* (performing the same measurements in *B. anynana* was not possible for practical reasons) showed a reduction of *in vitro* JH biosynthesis and *in vivo* hemolymph JH titres (by up to 48%) in MK-801 treated compared to control females. JH biosynthesis was inhibited successfully in *G. bimaculatus* CA by up to 60%, resembling the results of *in vitro* measurements of active CA glands in *D. punctata* (Chiang et al. 2002a). In the experiments of Chiang et al. (2002a) CA glands were incubated with NMDA to compensate for the missing glutamate stimulus from the severed nerves, with much lower concentrations of MK-

801 needed for this degree of inhibition. The rise of JH III titres in the hemolymph between days 3 and 4 was expected, as in *G. bimaculatus* JH III titres reach their maximum shortly before the onset of egg laying (Koch and Hoffmann, 1985). Taken together, the available evidence leaves little doubt that MK-801 affects JH biosynthesis and concomitantly JH titres in both species.

The effects of MK-801 on JH biosynthesis in *G. bimaculatus* are possibly mediated through a glutamatergic NMDA receptor, acting on the Ca^{2+} flux and thereby on JH biosynthesis. In adults of the cockroach *D. punctata*, JH biosynthesis in the CA is initially sensitive to allatostatins but insensitive to ionotropic glutamate stimulation, resulting in low rates of JH synthesis. Mating changes this pattern towards insensitivity of the CA to allatostatins and a high response to glutamate stimulation, resulting in high rates of JH synthesis (Chang et al., 2005). *G. bimaculatus* allatostatins, in contrast, seem to act less age dependent throughout the life cycle, although on days with maximum JH synthesis allatostatic inhibition is slightly lowered (Lorenz, 2001). However, calcium ions (with their influx being regulated by the NMDA receptor in *D. punctata*; Chiang et al., 2002a), stimulate JH synthesis also in *G. bimaculatus* (Klein et al., 1993; Woodring and Hoffmann, 1994), and there is no interaction between allatostatins (or allatotropins) and Ca^{2+} mediated effects on JH biosynthesis (Lorenz, 2001). A further target of glutamate might also be a Na^{+}-dependent transporter (Kosakai and Yoshino, 2001).

There is no indication of any toxic side effects of the compounds or solvents used that may have affected our results. For *B. anyana*, MK-801 was dissolved in Ringer solution, thereby minimizing any potential solvent effects. Indeed, lifetime fecundity in the control group was very similar to values obtained from other studies not involving injections or applications (Bauerfeind and Fischer, 2005; Bauerfeind et al., 2007). Furthermore, survival data revealed no difference among control and MK-801 treated groups, suggesting that MK-801 is a highly specific compound without any toxic side-effects. For *G. bimaculatus* it was necessary to use DMSO as solvent because of the much higher concentrations of MK-801 employed. Concomitantly, lifetime fecundity was generally lower than in other studies (Koch and Hoffmann, 1985; Lorenz, 2007; Meyering-Vos et al., 2006), but again, there was no detectable effect on mortality rates, although data were restricted to the egg laying period in this case.

Despite the overall similarity of effects in both species used, there were also some interesting differences in the effects of MK-801 on fecundity. In *G. bimaculatus* egg

production was reduced throughout the oviposition period (at least until day 11 following ecdysis), but in *B. anynana* the inhibitory effects of MK-801 were restricted to the first days of the oviposition period. Furthermore, the dose dependence of effects seems more pronounced in *B. anynana* in comparison to *G. bimaculatus*. These findings may suggest some differences in the effects of JH on egg maturation across species. In *G. bimaculatus* JH biosynthesis and fecundity can be manipulated throughout the entire oviposition period by allatostatins and JH (mimic) injections administered early in life (Koch and Hoffmann, 1985; Lorenz, 2001). Therefore, egg maturation seems to depend on a constant input of JH mediated signals in *G. bimaculatus*. In *B. anynana*, in contrast, JH seems to be an important signal for the initiation of egg maturation, which might not be needed later on (Steigenga et al., 2006).

In conclusion, the NMDA receptor antagonist MK-801 reduced fecundity in *G. bimaculatus* and *B. anynana*, two species not being phylogenetically closely related. This effect could be reversed by concurrent applications of JH mimics. Furthermore, MK-801 inhibited *in vitro* JH biosynthesis in the CA and reduced *in vivo* JH hemolymph titres in a dose-dependent manner in *G. bimaculatus*. These results suggest that in *G. bimaculatus* JH biosynthesis in the CA is at least in part controlled by an NMDA receptor with Ca^{2+} as a second level messenger, as has been found in the cockroach *D. punctata* (Chiang et al., 2002a). As MK-801 is readily available commercially, is fairly soluble in water and can be used orally, it obviously represents a convenient tool for manipulating JH biosynthesis in insects. With the growing knowledge on NMDA receptors in insects (Chiang et al., 2002a; Chiang et al., 2002b; Locatelli et al., 2005; Xia et al., 2005), such antagonists may yield new insights into the mechanistic basis of reproduction and associated trade-offs in insects.

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Manuscript 2

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Effects of temperature on reproductive output, egg provisioning, juvenile hormone and vitellogenin titres in the butterfly *Bicyclus anynana*

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Abstract

Environmentally induced phenotypic plasticity is common in nature. Hormones, affecting multiple traits and signaling to a variety of distant target tissues, provide a mechanistic link between environments, genes and trait expression, and may therefore well be involved in the regulation phenotypic plasticity. Here we investigate whether in the tropical butterfly *Bicyclus anynana* temperature-mediated plasticity in egg size and number, with fewer but larger eggs produced at lower temperatures and vice versa, is under control of juvenile hormone, and whether different temperatures cause differences in egg composition. Female *B. anynana* butterflies showed the expected response to temperature, however, we found no evidence for an involvement of juvenile hormone. Neither haemolypmh JH II and JH III titres nor vitellogenin levels differed across temperatures. The smaller eggs produced at the higher temperature contained relatively higher amounts of water, free carbohydrates and proteins, but relatively lower amounts of lipids. While these smaller eggs had a lower absolute energy content, total reproductive investment was higher at the higher temperature (due to a higher fecundity). Overall, our study indicates that temperature-mediated plasticity in reproduction in *B. anynana* is mechanistically related to a biophysical model, with oocyte production (differentiation) and oocyte growth (vitellogenesis) having differential temperature sensitivities.

Keywords

phenotypic plasticity, egg composition, egg size, hormonal control, fecundity, insect, reproductive investment

Introduction

Environmental effects on the expression of the phenotype, called phenotypic plasticity, are widespread in nature (Endler, 1986; Ghalambor et al., 2007; Miner et al., 2005; Nussey et al., 2007; Pigliucci, 2005). Such plastic changes to the phenotype may either comprise merely biochemical or physiological interactions of the organism with its environment, or may be adaptations to spatially heterogeneous or temporarily varying environments (Bradshaw, 1965; Levins, 1963). Consequently, much effort has been devoted to distinguishing between both scenarios over recent decades (e.g. Blanckenhorn, 2000; Fischer et al., 2003a; Gotthard and Nylin, 1995; Pigliucci, 2005). Comparably less effort, in contrast, has been dedicated to disentangling the mechanistic basis of phenotypic plasticity (Brakefield et al., 1998; Hodin and Riddiford, 2000; Zera, 2003). Understanding the regulation of plasticity poses an exciting challenge, though, as environmental effects need to trigger different developmental pathways present within the same genotype (Flatt et al., 2005; Nijhout, 1999; Pigliucci, 2005; Sinervo and Svensson, 1998; Zera, 2007).

In insects, juvenile hormones (JHs) and 20-OH ecdysone are important regulators of key aspects of their life histories, and are therefore good candidates for the regulation of phenotypic plasticity (Gäde et al., 1997; Nijhout, 1994). Indeed, traits known to be under hormonal control include metamorphosis, behaviour, caste determination, reproduction and polymorphisms (e.g. de Wilde and Beetsma, 1982; Dingle and Winchell, 1997; Emlen and Nijhout, 1999; Gäde et al., 1997; Gilbert et al., 2000; Hoffmann, 1995; Nijhout, 1994). Hormones therefore provide a mechanistic link between environments, genes and trait expression (Finch and Rose, 1995; Flatt et al., 2005; Sinervo and Svensson, 1998). In the tropical butterfly *Bicyclus anynana*, for instance, seasonal wing polyphenism is under hormonal control, being induced during pupal development (Koch et al., 1996; Zijlstra et al., 2004).

The same species shows pronounced temperature-mediated plasticity in egg size, producing larger eggs at lower temperatures and vice versa (Fischer et al., 2003a; b; c), which is a common feature in ectothermic animals (Atkinson, 1994; Blanckenhorn, 2000; Ernsting and Isaaks, 2000; Yampolski and Scheiner, 1996). Several lines of evidence indicate that in *B. anynana* this plastic response comprises an adaptation to

the alternate wet-dry seasonal environments experienced in nature (Fischer et al., 2003a; b). Regarding its mechanistic basis, however, our understanding is far from being complete. All we know thus far is that oocyte growth seems to be less sensitive to temperature than is oocyte production, resulting in a lower number of larger eggs at lower temperatures (Steigenga and Fischer, 2007).

Based on the arguments raised above and because insect reproduction is generally under strong hormonal control, we here explore whether temperature affects juvenile hormone, vitellogenin and total protein titres in *B. anynana*. Due to differences in the timing of the onset of egg maturation the Lepidoptera can be distinguished into four groups (Ramaswamy et al., 1997). Among these, *B. anynana* belongs to the group in which egg maturation does not start before adult eclosion. In this group JH is necessary for the synthesis of vitellogenins, inducing patency of ovarioles, uptake of vitellogenin and choriogenesis (Hoffmann, 1995; Ramaswamy et al., 1997). Vitellogenins are female-specific proteins synthesized in the fat body (Sappington and Raikhel, 1998), representing a major part of eggs (as vitellins; Ziegler and Van Antwerpen, 2006) and being essential for successful larval development (Diss et al., 1996; Van Handel, 1993). Thus, environmentally induced changes in egg number and size could be well under the control of JH in *B. anynana*.

In extension to previous studies we here not only investigate the effects of temperature on egg number, egg size and longevity, but also on JH, vitellogenin and protein titres in the females' haemolypmh. Another unresolved issue being addressed here is whether temperature affects the biochemical composition of eggs. Although we know that the larger eggs produced at the lower temperature exhibit a higher hatching success (Fischer et al., 2003a; b), it is unclear whether this fitness advantage is related to size per se, to a larger absolute amount of nutrients or to relative changes in egg composition. The interplay between the effects of environmental variation on egg size, egg composition and in turn on offspring fitness is generally largely under-explored in insects (Casas et al., 2005; Giron and Casas, 2003; Jann and Ward, 1999; Karl et al., 2007; Kyneb and Toft, 2006), at least partly so because most studies exclusively rely on egg numbers and/or egg size as fitness measures (Azevedo et al., 1997; Bernardo, 1996; McIntyre and Gooding, 2000). This approach is obviously problematic, as egg size and composition are not necessarily

tightly correlated, and as variation in egg composition can be ecologically and evolutionarily more important than variation in egg size (Azevedo et al., 1997; Fox and Czesak, 2000; Giron and Casas, 2003; McIntyre and Gooding, 2000).

Methods

Study organism and experimental population

For this study the tropical butterfly *Bicyclus anynana* Butler, 1879 (Lepidoptera, Nymphalidae, Satyrinae) was used. *B. anynana* is a fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen, 1991). Reproduction is essentially confined to the warmer wet season when oviposition plants are abundantly available, and in which 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). 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 over 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof et al., 2005).

Experimental Design

All individuals used in this study were reared within the same environmental cabinet at a constant temperature of 27°C, a high relative humidity (70%) and a photoperiod of L12:D12. Larvae were reared on young maize plants in population cages (50 x 50 x 80 cm), with plants being regularly replaced. The resulting pupae were collected from the plants and transferred to cylindrical hanging cages (30 x 38 cm). Males and females were kept together for mating until day 3 of adult life, after which females were randomly divided among two environmental cabinets set at 27°C and 20°C respectively (70% relative humidity and L12:D12 throughout). The temperatures chosen for our experiments are similar to the ones experienced by the butterflies in the field during the dry and wet season, respectively (Brakefield, 1997; Windig, 1994). Thus, we did not include marginal temperatures, but ones the butterflies should be well adapted to. Throughout all experiments, butterflies had access to

moist banana for adult feeding ad libitum. Four different experiments were performed as detailed below.

Effects of temperature on reproduction and longevity

Per temperature treatment, about 50 females were placed individually into translucent plastic pots (1 l, covered with gauze), containing a fresh cutting of maize for egg-laying. Eggs were collected and counted daily until the death of the females. Egg size was measured as cross-sectional area (mm^2) using a digital camera (Leica DC300, Leica Microsystems, Wetzlar, Germany) connected to a stereo microscope (Leica MZ 7.5). The resulting images were analysed using Scion Image public software (Scion Corporation 2000, Frederick, Maryland, USA).

Effects of temperature on juvenile hormone (JH) titres

After the mating period, *B. anynana* females were separated from males and transferred group-wise to cylindrical hanging cages (30 x 38 cm; at both temperatures), containing maize leaves for egg laying. Starting on the day after the temperature transfer (day 4 of adult life), haemolypmh samples were taken from random females with micropipettes for hormone determination (1-5 µl, BRAND, Wertheim, Germany). Additional samples were collected on days 7, 10, 13 and 16. Per time point and temperature group, ca. 10 samples were taken, each including the haemolypmh of 9 females (resulting in 15.8 ± 0.3 µl haemolypmh on average). In total, ca. 180 females were used per time point. Micropipette volumes were recorded for later calculation of the total amount of haemolypmh for each sample.

Haemolypmh samples were immediately placed into a bi-phasic isooctane : MeOH solution (150 µl : 150 µl) in flint glass culture tubes (6 x 50 mm, Fisher Scientific, U.S.A). Samples were vortexed, left for ~20 minutes, vortexed, and then centrifuged for 15 min at 10.000 g to remove precipitated proteins. The extracted solution was transferred into a new culture tube using a Hamilton syringe, and afterwards stored at -80℃ for later analysis. Haemolypmh JH titres were quantified by liquid chromatography-mass spectrometry (LC-MS) following Westerlund and Hoffmann (2004). The samples were separated on a C18 reversed-phase column (ReproSil-Pur ODS-3, 5 µm; Dr. Maisch GmbH, Germany), protected by a guard column (C18 cartridge; Phenomex, Germany). Mass spectrometry was accomplished by using

electrospray ionization (ESI) in the positive ion mode using a Shimadzu LCMS-2010A.

Effects of temperature on vitellogenin and protein titres

The experimental design used here was very similar to the one described above (experiment 2). At the same 5 time points, again ca. 10 samples per temperature group were collected. One sample consisted of haemolypmh from 3 different females (1 µl each), collected with micropipettes. Samples were transferred to Eppendorf tubes with 3 µl insect Ringer solution and a few crystals of phenylthiourea to avoid melanization, and then stored at -80℃ for later an alysis. Haemolypmh protein was quantified with the RotiNanoquant assay (Roth, Karlsruhe, Germany), by using 1 µl of each sample. Vitellogenin was measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a vertical slab gel apparatus (Biometra Minigel, Biometra Göttingen, Germany) under denaturing conditions (cf. Laemmli, 1970; Sorge et al., 2000).

Female specific vitellogenins were identified by comparing the polypeptide profiles of freshly eclosed females, 4 day old females, 4 day old males, 6 day old pupae of unknown sex (for all of which haemolypmh was collected as described above), and 1 day old eggs (10 homogenized eggs in 40 µl Ringer solution). For SDS-PAGE, 1 µl of the haemolypmh sample and 20 µl of egg extract were used. Samples were combined with 20 µl sample buffer (86 mM NaCl, 0.4 mM KCl, 3 mM $CaCl_2$). The relative molecular masses of electrophoretically separated proteins were determined using the Amersham Biosciences molecular weight calibration kit (Amersham Biosciences Europe, Freiburg, Germany). Differences according to treatments and age were compared using relative differences in the optical density of the respective vitellogenin lanes. On each gel, all 10 groups were represented (2 temperature groups by 5 time points), and the 4 day old females from 27℃ were arbitrarily set to 100% relative vitellogenin amount.

Effects of temperature on egg composition

For this experiment, females from experiment 1 were used. Starting on day 14 of adult life, 20-30 eggs were collected per female (after egg size measurements) and stored at -20℃ for egg content analyses. Females n ot having laid ≥ 20 eggs within

the following 4 days were excluded from further analyses. Egg water content was estimated as mass difference between egg fresh and dry mass (after drying the eggs for 24 h at 70°C). The extraction and separation of egg lipid, protein, glycogen, and free carbohydrate from the same samples followed Lorenz (2003). Colorimetric determination of total lipid, glycogen, and free carbohydrate was performed using modified sulphophosphovanillin and anthrone methods. Protein was measured with an EL 808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, Vermont, USA) using the RotiQuant Universal assay (Roth, Karlsruhe, Germany) and bovine serum albumin as a standard (Karl et al., 2007; Lorenz, 2003). All data were corrected using measured recovery rates (free carbohydrate: 96.5 ± 2.4%, lipid: 91.1 ± 2.1%, protein: 72.7 ± 2.6%, glycogen: 83.4 ± 1.9%, Lorenz, 2003). To correct for different recovery rates between treatments, data were standardized to 100% dry mass. From the resulting values for egg components, energy investment per mg egg dry mass was calculated using average caloric values of 17.2 kJ g^{-1} for free carbohydrates, proteins and glycogen and 39.0 kJ g^{-1} for lipids (Ganong, 1974; Silbernagel and Despopolos, 1991).

Statistical analyses

Temperature-related differences in egg number and size over time were analyzed using repeated measures ANOVAs, with temperature treatment and oviposition day (time) as factors. For egg size only the data until day 14 of adult life were included, as later the frequency of females ovipositing no eggs on specific days increased substantially. Longevity data were analyzed using Cox's proportional hazards, with lifetime fecundity and mean egg size as covariates and temperature treatment as fixed factor. Differences across temperatures in lifetime fecundity, mean egg size, egg composition and egg energy content were analyzed by standard t-tests. Differences in JH, vitellogenin and protein titres were analyzed using 2-way ANOVAs with temperature treatment and age as fixed factors. All statistical tests were performed using STATISTICA 6.1. Throughout the text, means are given ± 1 SE.

Results

Effects of temperature on reproduction, and longevity

Daily egg numbers were significantly higher at 27 than at 20℃ (repeated measures ANOVA: $F_{1,90}$ = 42.42, P < 0.001). Concomitantly, lifetime fecundity was by 23.9% higher at 27 than at 20℃ (264.7 ± 12.8 versus 201. 4 ± 17.3: t = -2.93, N = 105, P < 0.001). Further, egg numbers generally decreased with female age, the decline being more pronounced at 27 compared to 20℃ ($F_{10,900}$ = 18.12, P < 0.001; treatment x time interaction: $F_{10,900}$ = 2.54, P = 0.005; Fig. 1A).

Over time, the eggs produced at 20℃ were significa ntly larger than the eggs produced at 27℃ (repeated measures ANOVA: $F_{1,46}$ = 17.30, P < 0.001), a difference also found when egg sizes were averaged over the whole oviposition period (20℃: 0.703 ± 0.006 mm², 27℃: 0.654 ± 0.006 mm²; t = 5.86, N = 105, P < 0.001). Overall, egg size tended to decrease with female age ($F_{10,460}$ = 1.96, P = 0.036). While this was especially pronounced at 27℃, females oviposit ing at 20℃ showed the opposite pattern of an increase in egg size with time (treatment x time interaction: $F_{10,460}$ = 11.57, P < 0.001; Fig. 1B).

Survival probability was significantly higher at 20℃ than at 27℃ (longevity at 20℃: 28.3 ± 1.2, at 27℃: 20.9 ± 0.8; χ^2_1 = 17.6, N = 105, P < 0.001; Fig. 1C). Both covariates, lifetime fecundity (χ^2_1 = 10.1, P = 0.002) and mean egg size (χ^2_1 = 4.8, P = 0.028) significantly affected longevity, tending to show positive associations (Pearson correlations; fecundity – longevity: r = 0.152, p = 0.121; egg size – longevity: r = 0.410, p < 0.001).

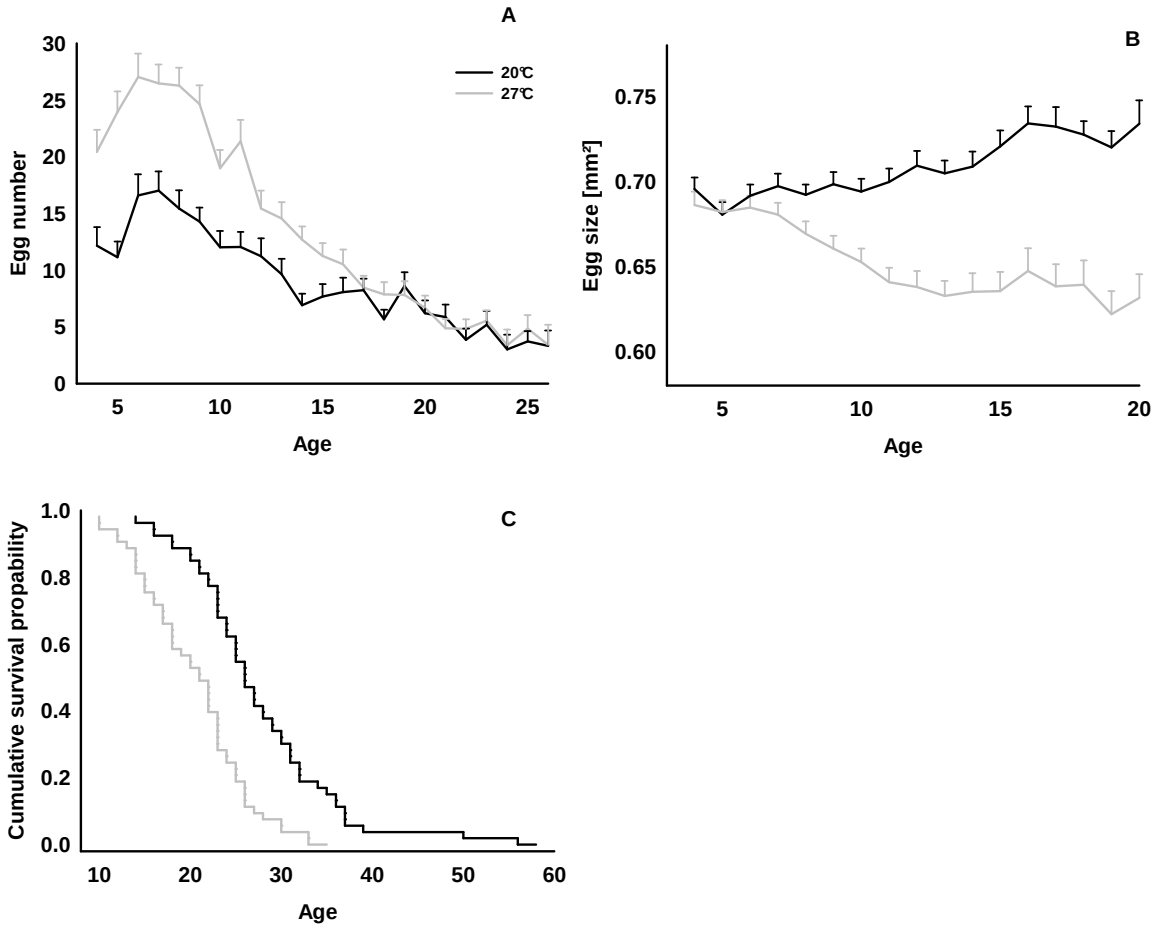

Fig. 1: Effects of temperature on daily fecundity (A), daily egg size (B; means + 1 SE) and cumulative survival probability (C) for *Bicyclus anynana* females. Data for egg number and egg size are restricted to the first 26 and 20 days of adult life, respectively, owing to low sample sizes afterwards. Initial sample size was 51-52 females per temperature group.

Effects of temperature on JH titres

In the haemolypmh of *B. anynana* females JH III and JH II were detected. JH III titres did not vary significantly across temperatures ($F_{1,88}$ = 0.33, P = 0.57) or with female age ($F_{4,88}$ = 0.79, P = 0.54; treatment x time interaction: $F_{4,88}$ = 0.24, P = 0.93; Fig. 2A). JH II titres also showed no significant variation across temperatures ($F_{1,88}$ = 0.38, P = 0.54), but increased significantly with female age ($F_{4,88}$ = 20.7, P < 0.001; treatment x time interaction: $F_{4,88}$ = 0.71, P = 0.59; Fig. 2B).

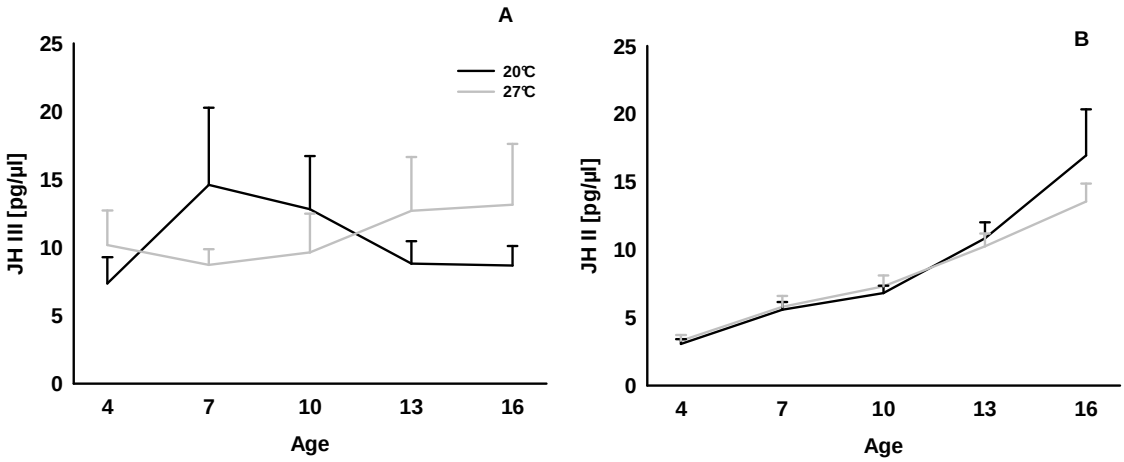

Fig. 2: Effects of temperature and age on juvenile hormone III (A) and II (B) titres in the haemolypmh of *Bicyclus anynana* females (means + 1 SE).

Effects of temperature on vitellogenin and protein titres

Two vitellogenin bands at about 136 and 46 kDa ('female specific proteins') were present in the haemolypmh of ovipositing (4 days old) females and in egg extract, but absent from freshly eclosed females, 4 days old males and pupae (Fig. 3A). Relative vitellogenin titres did not differ between temperatures ($F_{1,80}$ = 0.38, P = 0.54), but decreased significantly with female age by about 50% ($F_{4,80}$ = 5.7, P < 0.001; treatment x time interaction: $F_{4,80}$ = 0.34, P = 0.85; Fig. 3B). Total haemolypmh protein showed a comparable decrease with female age ($F_{4,89}$ = 38.8, P < 0.001). Protein titres were additionally significantly higher at 20 than at 27℃, except at the beginning of the oviposition period ($F_{1,89}$ = 20.6, P < 0.001; treatment x time interaction: $F_{4,89}$ = 2.34, P = 0.06; Fig. 3C).

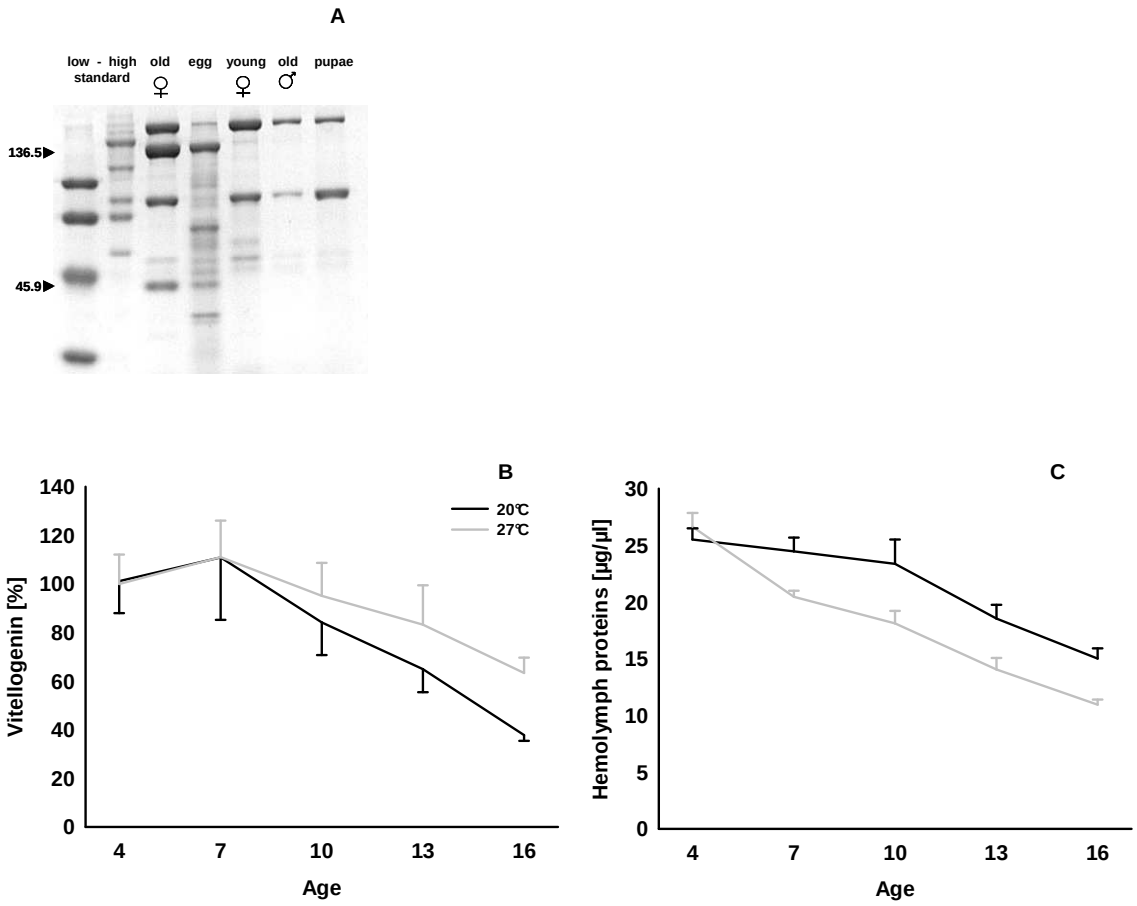

Fig. 3: Protein profiles for 4 day old females (old ♀), egg extract (egg), freshly eclosed females (young ♀), 4 day old males (old ♂), and male/female pupae analyzed by SDS-PAGE (A) as well as relative haemolypmh vitellogenin (B) and total protein titres (C; means + 1 SE) in relation to temperature and age in *Bicyclus anynana* females.

Effects of temperature on egg composition

Across temperatures, eggs consisted primarily of water (83.4 ± 0.16%), followed by lipids (7.62 ± 0.12%), proteins (5.12 ± 0.09%), glycogen (3.52 ± 0.07 %) and free carbohydrates (0.35 ± 0.01%). Eggs produced at 27℃ were significantly lighter in fresh (-15.2%; t = 7.82, N = 57, P < 0.001) and in dry mass (-27.3%; t = 11.99, N = 59, P < 0.001) compared to those produced at 20℃. On an absolute basis, the smaller eggs from 27℃ contained significantly smal ler amounts of each compound analyzed: -20.7% water (t = 7.57, N = 61, P < 0.001), -31.1% lipids (t = 2.99, N = 61, P < 0.001), -21.7% proteins (t = 10.66, N = 61, P < 0.001), -27.9% glycogen (t = 5.57, N = 61, P < 0.001) and -15.8% free carbohydrates (t = 8.30, N = 61, P < 0.001; Fig. 4A). Regarding relative egg composition, the smaller eggs produced at 27℃ contained relatively higher amounts of water (+ 9.0%; t = -3.09, N = 61, P < 0.001), free carbohydrates (+16.4%; t = -4.41, N = 61, P < 0.001) and proteins (+7.5%; t = -2.43, N = 61, P = 0.018), but relatively lower amounts of lipids (-5.3%; t = 2.33, N =

61, $P < 0.001$; Fig. 4B). Glycogen did not differ across temperature groups ($t = 0.05$, $N = 61$, $P = 0.958$).

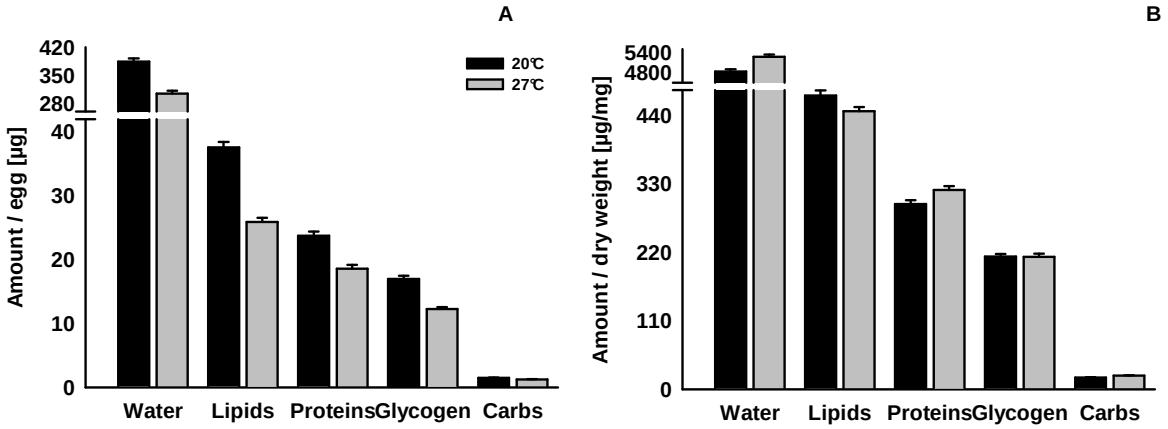

Fig 4: Effects of temperature on *Bicyclus anynana* absolute (i.e. per egg; A) and relative (i.e. per 1 mg egg fresh mass; B) egg composition (means + 1 SE; Carbs: Free carbohydrates).

The larger eggs produced at 20℃ contained, in absolute terms, 28.7% more energy compared with to those produced at 27℃ ($t = 12.26$, $N = 58$, $P < 0.001$). In relative terms this difference was less pronounced (+2.0% energy per 1 mg egg dry mass; $t = 2.32$, $N = 58$, $P = 0.023$; Fig. 5). Energy content per egg was significantly positively related to egg size (in mm²) (27℃: $r = 0.80$, $P < 0.001$, $N = 32$; 20℃: $r = 0.78$, $P < 0.001$, $N = 27$) and egg dry mass (27℃: $r = 0.96$, $P < 0.001$, $N = 32$; 20℃: $r = 0.93$, $P < 0.001$, $N = 29$). Using the former estimate, lifetime energy expenditure into reproduction was significantly higher at 27 (524.6 ± 30.7 J) than at 20℃ (415.2 ± 31.0 J; $t = -2.50$, $N = 110$, $P = 0.014$).

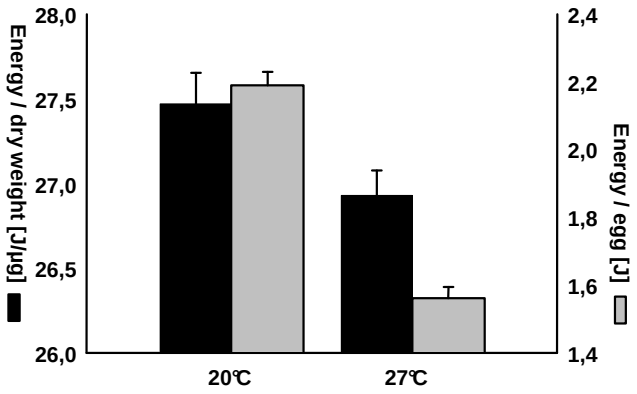

Fig. 5: Effects of temperature on relative and absolute egg energy content in *Bicyclus anynana* (means + 1 SE).

Discussion

Effects of temperature on reproduction and longevity

As expected, temperature induced a plastic response in egg size and number. Females produced fewer but larger eggs at the lower compared to the higher temperature, confirming earlier results on *B. anynana* (Fischer et al., 2003a; b; Steigenga et al., 2005) and other insects (e.g. Avelar, 1993; Blanckenhorn, 2000; Huey et al., 1995). As all females were reared in a common environment, only differences in adult (i.e. acclimation) temperature can explain the pattern observed. The decline in egg numbers over time, being stronger at 27°C, is a common feature for insects, probably reflecting the depletion of resources for reproduction (Braby and Jones, 1995; Fox and Czesak, 2000; Giron and Casas, 2003; Karlsson and Wiklund, 1984). As has been found before, butterflies lived longer at the colder than at the warmer temperature (Fischer et al., 2003b). Longevity and lifetime fecundity were positively correlated and did thus not trade off against each other, as is typical for income breeders (Fischer, 2007; Fischer et al., 2006; Jervis and Ferns, 2004). Thus far all results resemble previous findings, allowing to investigating associated changes in hormone and vitellogenin titres as well as in egg composition.

Effects of temperature on JH, vitellogenin and protein titres

JH dynamics represents an obvious target for changes in egg size (Fox and Czesak, 2000; Ramaswamy et al., 1997), as vitellogenin synthesis and/or vitellogenin incorporation into developing eggs might easily increase or decrease through changes in JH titres (Flatt and Kawecki, 2007; Hoffmann, 1995). While JH III occurs in all insect orders, JH II is typical for lepidopteran species (Nijhout, 1994). However, both hormones found did not show significant variation across temperatures throughout much of the oviposition period, rendering a decisive role in mediating plasticity in egg size unlikely. This, however, does not rule out different rates of JH biosynthesis and degradation at both temperatures, leading to similar haemolypmh titres at any given time. However, recent work on *B. anynana* using the JH mimic pyriproxyfen and the antagonist MK-801 revealed that JH seems to primarily function as a signal initiating egg maturation and has thus pronounced effects on fecundity, but not on egg size (Geister et al., 2008; Steigenga et al., 2005).

In line with the lack of variation in JH, haemolypmh vitellogenin titres also showed no significant variation across temperatures. In contrast, vitellogenin levels showed a strong decrease with female age, probably again indicating a depletion of resources with an increasing number of eggs laid. Having two vitellogenin (female-specific protein) sub-units, a larger and a smaller one, is typical for most insects (large > 150 kDa, small < 65 kDa; see Raikhel and Dhadialla, 1992). The fact that no vitellogenins could be detected in freshly eclosed *B. anynana* females supports that indeed egg maturation starts only after metamorphosis (Ramaswamy et al., 1997). Consequently, adult-derived carbohydrates, without which no eggs will be laid, are essential for egg production in *B. anynana* (Bauerfeind and Fischer, 2005; Fischer et al., 2004).

In contrast to vitellogenins, total hemolypmph protein titres were lower at 27 than at 20℃ and also showed a steeper decline with female age at the higher temperature, potentially indicating a more rapid depletion of other protein classes than female specific proteins with age at 27℃. Furthermore, as JH titres were either stable (JH III) or increased with age (JH II) while vitellogenin titres decreased, vitellogenesis might not be tightly related to JH in older *B. anynana* females. JH might be more important for the onset of egg maturation than for the control of reproduction throughout lifetime (see Geister et al., 2008).

Effects of temperature on egg composition

As is generally the case for insects (Van Handel, 1993; Ziegler and Van Antwerpen, 2006), *B. anynana* eggs were primarily composed of water, lipids and protein (Karl et al., 2007). Although egg size and composition are not necessarily tightly correlated (Azevedo et al., 1997; Fox and Czesak, 2000; Giron and Casas, 2003), very few studies thus far examined variation in egg composition (Casas et al., 2005; Giron and Casas, 2003; Karl et al., 2007; McIntyre and Gooding, 2000). As expected, all compounds analyzed were found in higher absolute quantities in large compared to small eggs. More interesting are differences in relative egg composition, with the smaller eggs produced at 27℃ containing relatively more water, free carbohydrates, glycogen and proteins, but less lipids.

Variation in egg water content, which is important for successful embryonic development in *B. anynana* (Fischer et al., 2003a; Fischer et al., 2006), was almost negligible (ca. 1% lower in the larger eggs produced at the lower temperature). Moreover, previous analyses revealed contradicting evidence, with (genetically) larger eggs having a higher rather than a lower egg water content (Karl et al., 2007), suggesting that there is no firm association between egg size and water content. Free carbohydrates, being present in eggs in small amounts only, probably reflect metabolic activity such as enhanced gluconeogenesis or lipid degradation (Lorenz, 2003; Thompson et al., 2003), and are consequently higher at 27°C (Jarošík et al., 2004).

Apart from water, lipids and proteins are the most important egg compounds, with lipids covering the energetic demands of the developing progeny, while proteins are mainly structural components, but can additionally serve as energetic resource (Beenakkers et al., 1985; Diss et al., 1996; Van Handel, 1993). A relatively higher amount of protein in smaller eggs at the expense of lipids has also been found across eggs selected for large and small egg size in *B. anynana*, indicating that there might be a minimum threshold for protein below which successful embryonic development is not possible (Karl et al., 2007). The fact that eggs produced at the lower temperature contained relatively less protein (mainly vitellins) is generally in line with the results on JH and vitellogenin haemolypmh titres regarding the fact that JH does not control egg size plasticity in *B. anynana*.

Effects of temperature on reproductive investment

Reproductive investment is an essential feature in the study of life histories, as the available resources need to be allocated among offspring of different sizes and numbers, and because resources allocated to reproduction cannot be used for somatic maintenance (Einum and Fleming, 2000; Giron and Casas, 2003; Smith and Fretwell, 1974). Consequently, the total amount of energy available for reproduction is a critical element of reproductive resource allocation. Although estimating reproductive investment exclusively by measuring egg numbers and/or egg size might be problematic (Azevedo et al., 1997; Bernardo, 1996; Jaeckle, 1995; McIntyre and Gooding, 2000), our results demonstrate that the larger eggs produced at 20°C had a higher energy content than the smaller eggs from 27°C, and that egg size and

egg dry mass were strongly positively related to egg energy content at both temperatures.

When considering the total energy of all eggs produced, however, reproductive investment was much higher at 27℃ compared to 20℃ , obviously caused by a much higher fecundity. This finding cautions against the notion that reproductive investment is a fixed amount of energy, as has been assumed traditionally (see Caley et al., 2001; Czesak and Fox, 2003; Einum and Fleming, 2000; Schwarzkopf et al., 1999; Smith and Fretwell, 1974; Winkler and Wallin, 1987). The higher investment in females ovipositing at 27 rather than 20℃ is likel y caused by the higher temperature being more beneficial to this tropical butterfly than the lower one (Brakefield, 1997; Fischer et al., 2003a; b).

Conclusions

As hormones affect multiple traits and signal to a variety of distant target tissues, evolutionary or environmental changes of endocrine systems are thought to be an especially important mechanism causing variation in insect life histories (Dingle and Winchell, 1997; Flatt et al., 2005; Fox and Czesak, 2000; Nijhout, 1999; Ramaswamy et al., 1997; Sinervo and Svensson, 1998; Zera et al., 2007). In our study, female *B. anynana* showed the expected response to adult temperature, producing fewer but larger eggs at the colder temperature, but more and smaller eggs at the warmer temperature. However, we found no evidence that this striking example of phenotypic plasticity is under hormonal control, as neither haemolypmh JH titres nor vitellogenin levels differed across temperatures. Additionally, energy investment per 1 mg dry mass was similar across treatments, suggesting oocyte growth proceeded overall in a rather similar manner, although there was some variation in egg composition across temperatures.

Thus, our study further supports the notion that temperature-mediated plasticity in egg size and number is mechanistically related to a biophysical model, proposing a change of oocyte production rate relative to oocyte growth (e.g. Ernsting and Isaaks, 2000; van der Have and de Jong, 1996; Van Voorhies, 1996). In essence it is assumed that oocyte production (differentiation) and oocyte growth (vitellogenesis)

have differential temperature sensitivities. Recent work on ovarian dynamics in *B. anynana* is well in agreement with this model (Steigenga and Fischer, 2007).

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Manuscript 3

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Energetics of embryonic development: Effects of temperature on egg and hatchling composition in a butterfly

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Abstract

Phenotypic plasticity may allow an organism to adjust its phenotype to environmental needs. Little, however, is known about environmental effects on offspring biochemical composition and turnover rates, including energy budgets and developmental costs. Using the tropical butterfly *Bicyclus anynana* and employing a full-factorial design with two oviposition and two developmental temperatures, we here explore the consequences of temperature variation on egg and hatchling composition, and the associated use and turnover of energy and egg compounds. At the lower temperature larger eggs were produced, achieved by provisioning these eggs with larger quantities of all compounds investigated (and thus more energy), whilst relative egg composition was rather similar to that of smaller eggs laid at the higher temperature. Turnover rates during embryonic development differed across developmental temperatures, suggesting an emphasis on hatchling quality (i.e. protein content) at the more stressful lower temperature, but on storage reserves (i.e. lipids) at the higher temperature. These differences may represent adaptive maternal effects. Embryonic development was much more efficient at the lower temperature, providing a possible mechanism underlying the temperature-size rule.

Keywords

egg size, phenotypic plasticity, *Bicyclus anynana*, maternal effect, temperature-size rule

Introduction

One of the most important abiotic factors for ectotherms is temperature as it has vital consequences for biochemical and physiological processes (Eckert et al., 2002). The temperatures experienced in the natural environment are, therefore, of particular importance for an organism's life-history, including developmental and growth strategies (Jarošík et al., 2004; Stearns, 1992). Further, temperature induces a variety of plastic responses (Nijhout, 1999; Stearns, 1989). Such phenotypic plasticity may allow an organism to adjust its physiological, behavioural and morphological phenotype to environmental needs, and indeed many such cases are considered adaptive (see Miner et al., 2005; Pigliucci, 2005; Stearns, 1989). While the general influence of temperature on ectotherm growth and development is exceedingly well studied (e.g. Angilletta and Dunham, 2003; Atkinson, 1994; Jarošík et al., 2004; Liu et al., 1995; Robinson and Partridge, 2001; Wagner et al., 1984), the question of associated changes in energy budgets and turn-over rates has rarely been addressed (Booth and Kiddell, 2007; Guisande and Harris, 1995; Van Handel, 1993).

Regardless of the specific temperature environment, eggs need to be provisioned with nutrients for successful embryonic development, with lipids and proteins comprising the main components of insect eggs (Beenakkers et al., 1985; Diss et al., 1996; Van Handel, 1993; Ziegler and Van Antwerpen, 2006). Egg proteins consist mainly of vitellins, which are mostly transformed into structural elements during embryonic development, while rarely being used as energy sources (Chapman, 1998; Gillot, 2005). In the mosquito *Culex quinquefasciatus*, for instance, no protein loss occurred during embryonic development (Van Handel, 1993). Lipids, in contrast, mainly cover the energetic demands of the developing progeny (Beenakkers et al., 1985).

The use of specific egg compounds during embryonic development may partly depend on the environment in which development takes place and/or the environment experienced by the parents. The latter, called maternal effects, may cause differences in offspring provisioning or the activation of different developmental programs that tune the offspring's phenotype for the environment experienced by the mother (Fox et al., 1997; Gilchrist and Huey, 2001; Mousseau and Fox, 1998a; Wolf

and Brodie, 1998). Environmental experience can be transmitted to offspring via cytoplasmic egg factors, e.g. yolk amount, egg composition, hormones or mRNA (Mousseau and Dingle, 1991; Mousseau and Fox, 1998a, b; Sakwínska, 2004). However, studies concerned with such physiological aspects of environmental variation are rare (Booth and Kiddell, 2007; Garcia-Guerrero et al., 2003). The question of physiological developmental costs is of particular interest in this context, as such costs can be quantified and thereafter referred to functions or environmental variables (Bernardo, 1996; Rose and Bradley, 1998). To this end eggs comprise ideal models, as they have initially a fixed composition and thus amount of energy, and because energy budgets can be easily determined by analysing the resulting hatchlings. Further, there are no energetic costs of different behaviours or the like besides the energy use for embryonic development and somatic maintenance, otherwise potentially masking consequences of maternal and environmental factors.

The tropical butterfly *Bicyclus anynana* used here as model organism shows striking phenotypic plasticity in wing color patterns (two seasonal morphs) as an adaptation to alternate wet- and dry-seasonal environments and the associated changes in resting background and predation (Brakefield, 1997; Lyytinen et al., 2004). Further, *B. anynana* females produce numerous small eggs under warm wet season conditions, but fewer and larger eggs at cool temperatures typical of the dry season (Fischer et al., 2003a, b). Based on differential survival probabilities, with a high hatching success at warm temperatures favouring many small eggs, and a lower hatching success at colder temperatures favouring increased investment per offspring at the expense of egg number, this plasticity is thought to represent an example of adaptive phenotypic plasticity (Fischer et al., 2003a, b). Egg size is in general a particular interesting life-history trait, as it is subject to a trade-off with fecundity, often affects the fitness of progeny, and is consequently subject to selection in both the parental and progeny generation (Bernardo, 1996; Marshall and Uller, 2007; Mousseau and Fox, 1998a).

We here use variation in the maternal and developmental (embryonic) temperature environment to explore the consequences of that variation for egg composition, hatchling composition and the associated use and turnover of energy and egg compounds. Using a full-factorial design including different maternal oviposition

temperatures (resulting in eggs of different size) and embryonic developmental temperatures enables us to distinguish between maternal and developmental effects as well as their interactions on turnover rates. Furthermore, energy efficiency and its relation to hatchling size can be explored in relation to temperature, which plays an important role in disentangling the mechanistic basis of the temperature-size rule (Atkinson, 1994; Atkinson et al., 2006; Karl and Fischer, 2008).

Material and Methods

Study organism and experimental population

Bicyclus anynana Butler, 1879 (Lepidoptera, Nymphalidae, Satyrinae) is a tropical butterfly with a distribution ranging from Southern Africa to Ethiopia, which feeds on a variety of fallen and decaying fruit (Brakefield, 1997; Larsen, 1991). It inhabits a highly seasonal environment with a favorable wet season and a rather adverse dry season (Brakefield, 1997; Larsen, 1991). 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; Brakefield and Reitsma, 1991; Windig, 1994). 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 over 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, 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.

Experimental design

All individuals used in this study were reared within a single environmental cabinet at a constant temperature of 27℃, a high relative hum idity (70%) and a photoperiod of L12 : D12 (24 h light cycle). Larvae were reared in population cages (50 x 50 x 80 cm) on young maize plants, which were regularly replaced. Resulting pupae were collected from the plants and transferred to cylindrical hanging cages (30 x 38 cm). After adult eclosion, males and females were kept together for mating until day 3 of adult life, after which the females were randomly divided among two temperatures

(20 and 27°C; identical humidity and photoperiod throughout; see above). The temperatures chosen for our experiments are similar to the ones experienced by the butterflies in the field during the dry and wet season, respectively (Brakefield and Mazzotta, 1995; Brakefield and Reitsma, 1991). Thus, we did not include marginal temperatures, but ones the butterflies should be well adapted to. Throughout all experiments adult butterflies had access to moist banana for adult feeding.

Per temperature, ca. 70 females were placed individually into translucent plastic pots (1 l, covered with gauze), containing a fresh cutting of maize for egg-laying. Eggs were removed and the maize cutting was replaced on a daily basis. Starting on day 14 of adult life, the eggs produced by a given female per day were randomly divided among 3 different treatment groups, until an overall number of ca 40-60 eggs was reached. One third of the eggs collected was stored at -80°C for later egg content analysis, another third was kept at the oviposition temperature for embryonic development, and the last third was transferred to the alternative temperature for embryonic development. Developing eggs were kept separated per female in petri dishes and checked every 6 hours for hatchlings. All hatchlings were removed and immediately stored at -80°C for later analysis of their biochemical composition. To reach adequate sample sizes, all eggs and larvae belonging to an individual female were pooled across oviposition days for further analyses.

Analysis of egg and hatchling biochemical composition

Egg and hatchling water content was estimated as mass difference between egg fresh and dry mass, after drying the samples for 24 hours at 70°C. The extraction and separation of lipid, protein, glycogen, and free carbohydrates from the same sample followed Lorenz (2003). Colorimetric determination of total lipid, glycogen, and free carbohydrate was performed using modified sulphophosphovanillin and anthrone methods. Protein was measured with an EL 808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Bad Friedrichshall, Germany) using the RotiQuant Universal assay (Roth, Karlsruhe, Germany) and bovine serum albumin as a standard (Karl et al., 2007; Lorenz, 2003). All data were corrected using measured recovery rates (free carbohydrate: 96.5 ± 2.4%, lipid: 91.1 ± 2.1%, protein: 72.7 ± 2.6%, glycogen: 83.4 ± 1.9%; Lorenz, 2003). To correct for different recovery rates between treatments, data were standardized to 100% dry mass. From the resulting values for egg and hatchling compounds, energy investment per 1 mg dry mass was

calculated using average caloric values of 17.2 kJ g^{-1} for free carbohydrates, proteins and glycogen and 39.0 kJ g^{-1} for lipids (Ganong, 1974; Silbernagel and Despopolos, 1991).

Statistical analysis

Differences in egg components across temperatures were analyzed using t-tests. As the eggs of individual females were divided between two developmental temperatures, the respective hatchling data are not independent from each other, as they are siblings. Therefore, repeated measures ANOVAs were used to analyze hatchling data with oviposition temperature as fixed factor and developmental temperature as repeated measure. All statistical tests were performed using STATISTICA 6.1. Throughout the text, means are given ± 1 SE.

Results

Egg and hatchling size and embryonic development

Eggs produced at 20℃ were significantly heavier, and thus larger than those produced at 27℃ (by 28.8 % in dry mass; t = 14.9, N = 137, P < 0.001; Fig. 1). Likewise, the hatchlings resulting from the eggs produced at 20℃ were by 25.1 % heavier than from eggs produced at 27℃ (repeated measures ANOVA; $F_{1,126}$= 111.9, P < 0.001). Further, embryonic development at the lower temperatures resulted in 7.2 % heavier hatchlings compared to the higher temperature ($F_{4,126}$= 25.1, P < 0.001; oviposition temp. x developmental temp.: $F_{4,126}$= 3.2, P = 0.078). Embryonic development lasted significantly longer at 20℃ than at 27℃ (20℃: 6.3 days, 27℃: 2.8 days; $F_{1,131}$ = 1627.2, P < 0.001), and was 0.9 days shorter for eggs oviposited at 27℃ compared to 20℃. A significant interaction between oviposition and developmental temperature indicates that the eggs produced and reared at 20℃ had particularly long development times, and those produced and reared at 27℃ particularly short development times ($F_{1,131}$ = 38.4, P < 0.001).

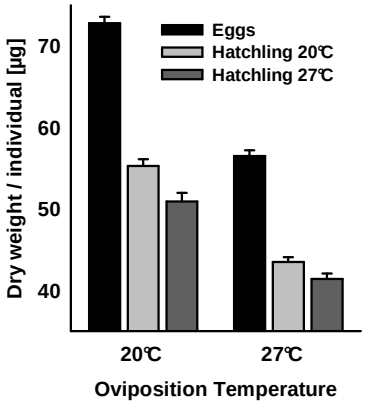

Fig. 1: Effects of oviposition (and developmental) temperature on egg and hatchling dry mass (means + 1 SE) in *Bicyclus anynana*. Sample sizes range between 57 and 77.

Composition of individual eggs and hatchlings

Owing to their high weight, the larger eggs produced at 20℃ contained significantly higher absolute amounts of water (+19.3%; t = 6.36, N = 135, P < 0.001), lipids (+27.9%; t = 9.34, N = 135, P < 0.001), proteins (+23.7%; t = 8.35, N = 134, P < 0.001) and glycogen (+37.1%; t = 8.20, N = 135, P < 0.001), but not of free

carbohydrates (t = 1.7, N = 135, P = 0.091) compared to eggs produced at 27℃ (Fig. 2). Similarly, hatchlings from eggs produced at 20 compared to 27℃ had significantly higher absolute amounts of water (+11.1%), lipids (+20.4%) and proteins (+35.7%), while glycogen and free carbohydrate content did not differ among those groups (Tab. 1, Fig. 2). Embryonic development at the lower compared to the higher temperature resulted in significantly higher amounts of protein (+17.7%) and glycogen (+10.5%) in resulting hatchlings, but left the remaining compounds unaffected. A significant interaction between oviposition and developmental temperature for hatchling water content indicates that water content was highest when the developmental temperature matched the oviposition temperature, but was somewhat lower when eggs were transferred to the alternative temperature for embryonic development (Fig. 2A). For protein and glycogen, the difference between developmental temperatures was larger for hatchlings originating from eggs produced at 20℃ than at 27℃, causing in both case s significant interactions (Fig. 2C, D; Tab. 1).

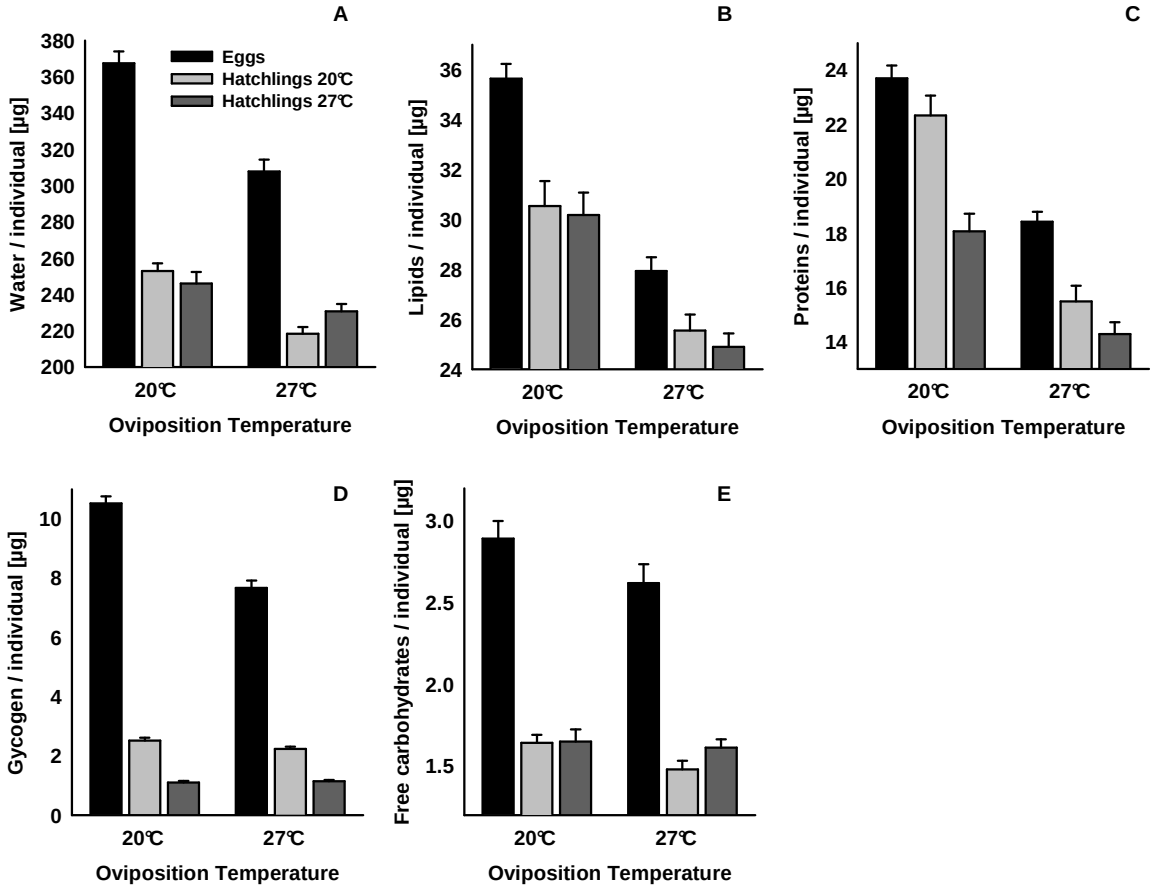

Fig. 2: Effects of oviposition (and developmental) temperature on egg and hatchling water (A), lipid (B), protein (C), glycogen (D) and free carbohydrate (E) content per individual (means + 1 SE) in *Bicyclus anynana*. Sample sizes range between 55 and 70.

Tab. 1: Effects of oviposition temperature (Ovi. temp.) and developmental temperature (Dev. temp.) on hatchling composition, as tested by repeated measures ANOVAs.

Compound	Effect	*MQ*	*F*	*P*
Water	Ovi. temp.	38143	15.4	**< 0.001**
	Dev. temp.	467	0.9	0.355
	Ovi. temp. x Dev. temp.	5706	10.5	**0.001**
Lipid	Ovi. temp.	1624	31.7	**0.001**
	Dev. temp.	16	0.4	0.509
	Ovi. temp. x Dev. temp.	2	0.1	0.841
Protein	Ovi. temp.	1737	66.7	**< 0.001**
	Dev. temp.	458	18.7	**< 0.001**
	Ovi. temp. x Dev. temp.	145	5.9	**0.016**
Glycogen	Ovi. temp.	0.8	2.4	0.125
	Dev. temp.	95.8	303.7	**< 0.001**
	Ovi. temp. x Dev. temp.	1.6	5.2	**0.024**
Free Carbohydrates	Ovi. temp.	0.6	2.4	0.123
	Dev. temp.	0.3	1.7	0.197
	Ovi. temp. x Dev. temp.	0.2	1.3	0.250

Relative composition of eggs and hatchlings (per 1 mg dry mass)

Across temperatures, eggs consisted primarily of water (83.7 ± 0.2%), followed by lipids (8.0 ± 0.12%), proteins (5.3 ± 0.09%), glycogen (2.3 ± 0.04%) and free carbohydrates (0.7 ± 0.02%). Similarly, hatchlings were composed mainly of water (82.8 ± 0.21%), followed by lipids (9.8 ± 0.14%), protein (6.2 ± 0.11%), glycogen (0.6 ± 0.02%) and free carbohydrates (0.6 ± 0.01%). Eggs produced at 20 compared to 27℃ had relatively less water (-6.9%; t = -2.81, N = 135, P < 0.001) and free carbohydrates (-13.9%; t = -2.72, N = 135, P < 0.001) but more glycogen (+6.4%; t = 2.03, N = 135, P < 0.001), while lipid (t = -0.53, N = 135, P = 0.60) and protein (t = 0.29, N = 135, P = 0.76) content did not differ across oviposition temperatures (Fig. 3). All hatchling compounds were significantly affected by oviposition and developmental temperature, except for a lack of a significant effect of oviposition temperature on hatchling lipid content (Tab. 2; Fig. 3). Hatchlings originating from eggs produced at 20℃ were characterized by a reduced relative water (-10.3%), free

carbohydrate (-14.7%) and glycogen (-15.3%) but an increased protein (+8.9%) content compared to hatchlings originating from eggs produced at the higher temperature. Development at 20 compared to 27℃ red uced relative water (-8.7%), lipid (-6.8%), and free carbohydrate (-11.8%), but increased protein (+6.3%) and glycogen (+90.3%) content of hatchlings. Significant interactions between oviposition and developmental temperature for hatchling lipid and protein content indicate that in both cases effects of the developmental temperature were much more pronounced for hatchlings originating from eggs produced at 20℃ (Tab. 2).

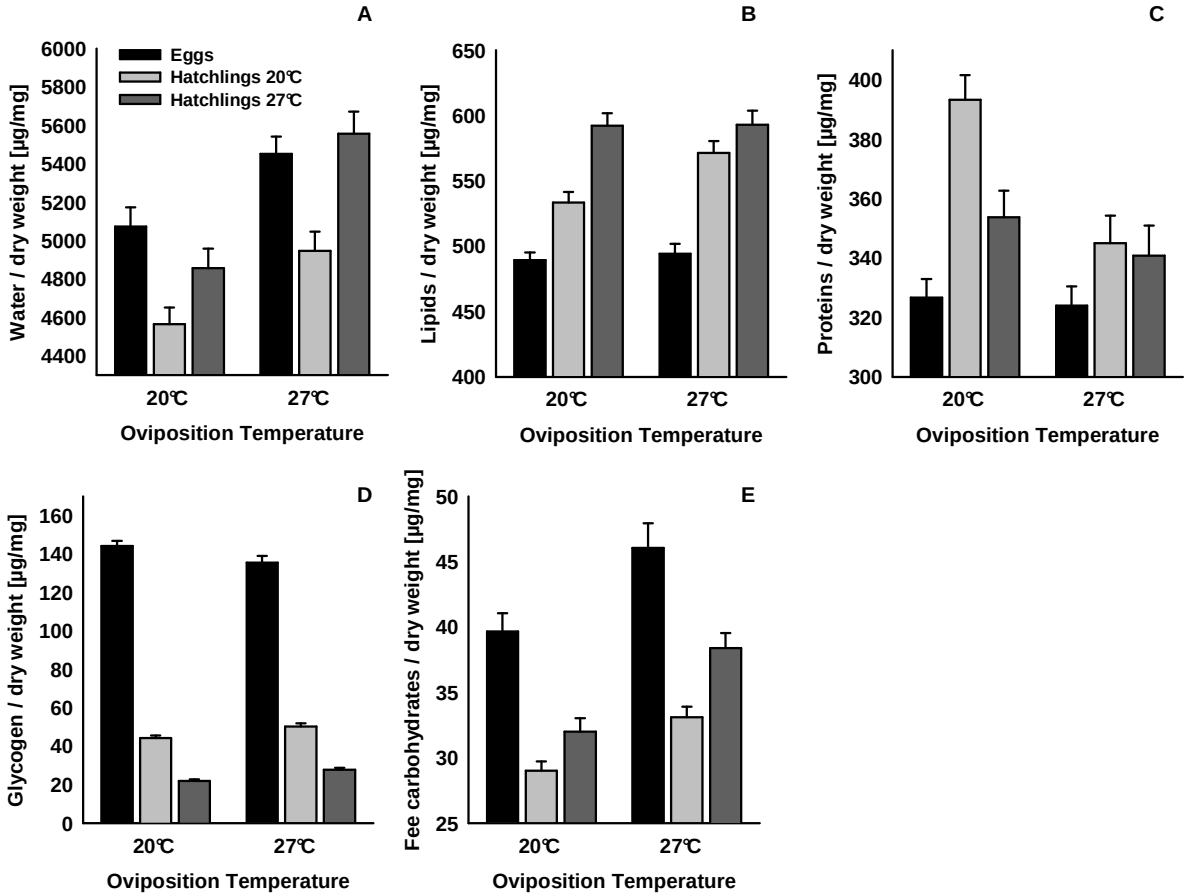

Fig. 3: Effects of oviposition (and developmental) temperature on egg and hatchling water (A), lipid (B), protein (C), glycogen (D) and free carbohydrate (E) content per 1 mg dry mass (means + 1 SE) in *Bicyclus anynana*. Sample sizes range between 55 and 70.

Tab. 2: Effects of oviposition temperature (Ovi. temp.) and developmental temperature (Dev. temp.) on hatchling composition per 1 mg dry mass, as tested by repeated measures ANOVAs.

Compound	Effect	*MQ*	*F*	*P*
Water	Ovi. temp.	1.8E+07	23.7	**< 0.001**
	Dev. temp.	1.3E+07	23.3	**< 0.001**
	Ovi. temp. x Dev. temp.	1.6E+06	3.1	0.079
Lipid	Ovi. temp.	23091	3.2	0.077
	Dev. temp.	99401	27.2	**< 0.001**
	Ovi. temp. x Dev. temp.	21549	5.9	**0.017**
Protein	Ovi. temp.	57668	8.3	**0.005**
	Dev. temp.	29515	8.8	**0.004**
	Ovi. temp. x Dev. temp.	19289	5.8	**0.018**
Glycogen	Ovi. temp.	2209	18.7	**< 0.001**
	Dev. temp.	30993	328.8	**< 0.001**
	Ovi. temp. x Dev. temp.	1	0.1	0.909
Free Carbohydrates	Ovi. temp.	1696	25.3	**< 0.001**
	Dev. temp.	1060	26.1	**< 0.001**
	Ovi. temp. x Dev. temp.	81.4	2.0	0.159

Energy content and turn-over

Due to their higher weight the eggs produced at 20°C had a significantly higher total energy content (by 28.5%; t = 13.1, N = 135, P < 0.001) than the eggs produced at 27°C, though relative energy investment (per 1 mg e gg dry mass) was similar and not significantly different across groups (t = -0.5, N = 135, P = 0.60; Fig. 4). Concomitantly, the hatchlings originating from 20°C eggs showed a higher absolute energy content (repeated measures ANOVA; $F_{1,124}$= 58.3, P < 0.001), while relative energy content did not differ significantly across groups (though there was a trend towards higher values in hatchlings from 27°C eggs; repeated measures ANOVA; $F_{1,125}$= 3.2, P = 0.076). Developing at 27°C compared to 20°C yiel ded a significantly lower absolute energy content ($F_{1,124}$= 7.9, P < 0.001; oviposition temp. x developmental temp.: $F_{1,124}$= 0.1, P = 0.934), but a considerably higher relative energy content ($F_{1,125}$ = 27.2, P < 0.001). The latter was particularly pronounced in hatchlings originating from eggs produced at 20°C (ovipositon temp. x developmental temp.: $F_{1,125}$ = 5.9, P = 0.017; Fig. 4).

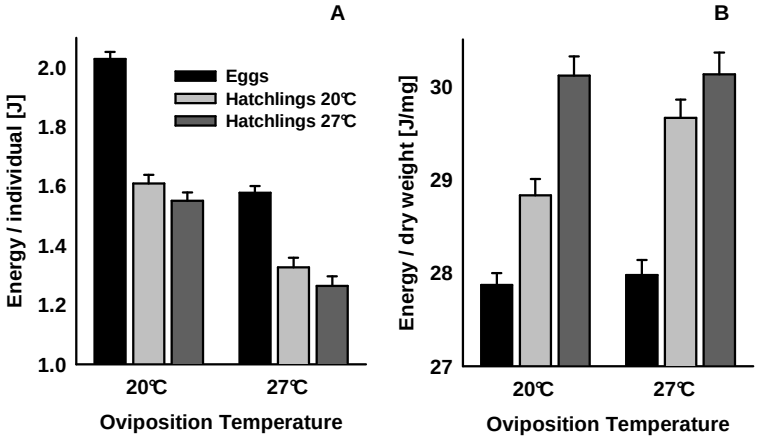

Fig. 4: Effects of oviposition (and developmental) temperature on egg and hatchling energy content per individual (A) and per 1 mg dry mass (B) (means + 1 SE) in *Bicyclus anynana*. Sample sizes range between 55 and 69.

Relative loss in dry mass during embryonic development (from egg to hatchling) was significantly higher (by 2.7%) for eggs produced at 20℃ compared to 27℃ (repeated measures ANOVA; $F_{1,128}$ = 5.6, P = 0.020), and was higher (by 4.8%) when developing at 27℃ compared to 20℃ ($F_{1,128}$ = 27.5, P < 0.001; oviposition temp. x developmental temp.: $F_{1,128}$ = 1.3, P = 0.260; Fig. 5). Results on relative energy loss yielded qualitatively identical results, with a 4.1% higher loss for eggs produced at 20℃ (repeated measures ANOVA, $F_{1,122}$ = 6.4, P = 0.013), and a 3.4% higher loss for eggs developing at 27℃ ($F_{1,122}$ = 8.6, P = 0.005; ovipositon temp. x developmental temp.: $F_{1,128}$ = 0.1, P = 0.728) compared to the alternative groups. Accordingly, per 1 mg hatchling mass relatively more energy was needed for eggs produced at 20℃ (repeated measures ANOVA; $F_{1,113}$ = 4.7, P = 0.032), and more during development at 27℃ compared to 20℃ ($F_{1,113}$ = 4.7, P = 0.032, oviposition temp. x developmental temp.: $F_{1,113}$ = 0.1, P = 0.959; Fig. 5).

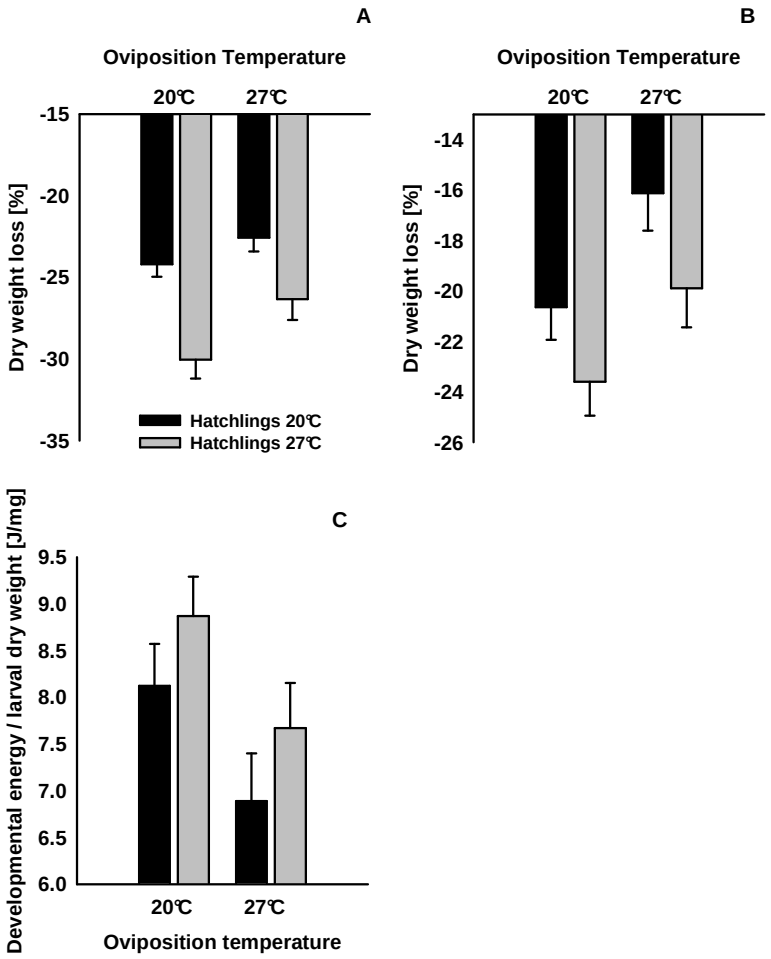

Fig. 5: Effects of oviposition and developmental temperature on relative loss in dry mass (relative to egg dry mass; A) and energy content (relative to egg energy content; B), and on the amount of energy consumed per 1 mg hatchling mass (i.e. energetic efficiency, C) during embryonic development (means + 1 SE) in *Bicyclus anynana*. Sample sizes range between 49 and 71.

Discussion

Composition of individual eggs and hatchlings

Temperature induced plastic changes in egg size and number in *B. anynana,* with fewer but larger eggs produced at the colder temperature and more but smaller eggs at the warmer, confirming known patterns of phenotypic plasticity (Fischer et al., 2003a, b; Steigenga et al., 2005). Not surprisingly, the larger size of eggs produced at the lower temperature was associated with higher absolute amounts of water, lipids, protein, glycogen and free carbohydrates (though the latter not significantly; cf. Karl et al., 2007). Generally, water, lipids and protein represented the major components of eggs followed by glycogen and free carbohydrates, as is typically found in insects (Lorenz, 2003; Van Handel, 1993; Ziegler and Van Antwerpen, 2006). The above temperature-induced differences in size and quantities were not restricted to the egg stage, but were also present in hatchlings resulting from these eggs (though amounts of glycogen and free carbohydrates were not significantly higher in hatchlings originating from eggs produced at 20℃, but note their low overall quantities). Note that the amount of protein was not constant during development from egg to hatchling, indicating an incomplete transformation into structural components due to metabolic losses.

As expected, egg development time was strongly affected by temperature, being longer at the colder than at the warmer temperature (Jarošík et al., 2004; Liu et al., 1995; Wagner et al., 1984). The effect of oviposition temperature (and the interaction with developmental temperature) is probably an experimental artefact, caused by the fact that eggs were collected and divided once per day, meaning that part of the egg development took place at the oviposition rather than at the developmental temperature. Embryonic development at the higher temperature resulted in smaller hatchlings regardless of oviposition temperature and therefore egg size (thus following the temperature-size rule; Angilletta and Dunham, 2003; Atkinson, 1994; Atkinson et al., 2006; Walters and Hassall, 2006), with lower amounts of protein and glycogen. The lower amount of protein found in hatchlings having developed at 27℃ indicates a relatively lower conversion of egg proteins into hatchling structural elements at the higher temperature (Chapman, 1998; Gillot, 2005; Van Handel, 1993). Thus, proteins at the warmer temperature may have served to a larger extent

as energetic resource for somatic maintenance and growth due to higher metabolic rates, which may also account for glycogen as a pure energetic resource (Jarošík et al., 2004).

Both components showing variation across developmental temperatures (proteins, glycogen) were involved in interactions with oviposition temperature, with the effect of developmental temperature being more pronounced for hatchlings originating from eggs produced at 20°C (see further below). For hatchling water content there was also a significant interaction between oviposition and developmental temperature, with the amount of water being highest in hatchlings developing at the temperature they were produced at. Thus, water loss was higher when eggs were transferred to a new temperature for embryonic development. As water content is considered important for fitness (with higher amounts decreasing desiccation risk; Fischer et al., 2003a, 2006), these findings indicate that mothers tuned their offspring's phenotype for the environment experienced during oviposition, suggesting beneficial acclimation (Wilson and Franklin, 2002; Woods and Harrison, 2002).

Relative composition of eggs and hatchlings (per 1 mg dry mass)

In relative terms, eggs produced at 20°C and 27°C showed a fairly similar composition, suggesting that the larger size of the eggs produced at the lower temperature is achieved by a considerably higher energetic investment per offspring (see also below). This is particular true for the major egg components, lipids and protein, which showed no variation across oviposition temperatures. Relative water and free carbohydrate content was higher while glycogen content was lower in eggs produced at the higher temperature. Note though that the latter two compounds were generally present in fairly small amounts in eggs, and that variation in egg water content was overall small (reduction by 1% only). The relatively reduced amount of glycogen in eggs produced at 27°C is probably related to the higher realized fecundity at the higher temperature. In *B. anynana* egg glycogen content is known to decrease throughout the oviposition period (Karl et al., 2007). The increased concentration of free carbohydrates found in eggs produced at 27°C, in contrast, probably reflects a higher metabolic activity at the higher temperature, causing enhanced glycogenolysis or lipid degradation (Jarošík et al., 2004; Lorenz, 2003; Thompson et al., 2003).

Generally, lipids and proteins were present in higher concentrations in hatchlings compared to eggs, while concentrations of free carbohydrates and especially glycogen was much lower in hatchlings. Although glycogen is a poorer energy source compared to lipids, this suggests that it represents an important energy sources for the developing embryo (e.g. Guisande and Harris, 1995; Van Handel, 1993). The differences found in relative egg composition (see above) are only partly echoed in the resulting hatchlings. Hatchlings originating from eggs produced at 27°C also showed higher relative water and free carbohydrate contents, but glycogen content was increased rather than decreased, and additionally protein content was reduced. The relatively higher water amount in hatchlings from eggs produced at 27°C may be related to a higher desiccation risk at the higher temperature. The higher amounts of glycogen in hatchlings from 27°C eggs despite a lower initial concentration in these eggs indicates a (for unknown reasons) relatively reduced consumption of this compound during embryonic development in eggs produced at 27°C compared to 20°C. In contrast, the conversion rate of protein from egg to hatchling was lower for eggs produced at 27°C.

In agreement with results on absolute egg composition, development at 27°C caused reductions in relative protein and glycogen amounts (see above). Additionally though, developing at the higher temperature resulted in increased water, lipid and free carbohydrate concentrations. The higher relative water content for hatchlings having developed at 27°C might be causally related to shorter development times (~3.5 days shorter), reducing water loss (Fischer et al., 2003b; Jarošík et al., 2004). The opposing patterns for protein and glycogen on the one hand and lipids and free carbohydrates on the other suggest a differential use of these compounds across developmental temperatures, with a relatively higher consumption/loss of proteins and glycogen at the higher temperature, but a relatively higher consumption/loss of lipids and free carbohydrates at the lower temperature. A striking finding in this context is that this effect was much more pronounced for eggs produced at the lower compared to the higher temperature.

We presume that those patterns might be related to the different survival probabilities during the wet (warm) and the dry (cold) season in *B. anynana's* natural environment.

Under wet season conditions, survival probability of eggs and hatchlings is generally high, while it is much lower under dry season conditions (Brakefield, 1997; Fischer et al., 2003a, b). As egg/hatchling protein content is at least often closely related to fitness (Diss et al., 1996), an increased preservation of proteins (through an increased consumption of other compounds, mainly lipids) should be more beneficial under more stressful conditions (i.e. low ambient temperatures). Under warm wet season conditions, in contrast, survival probability is high regardless of egg quality (Fischer et al., 2003a, b), such that an enhanced preservation of protein should be less important. Under this adaptive scenario, effects would be expected to be more pronounced when females had the chance to tune their offspring's development through maternal effects, which indeed seems to be the case: effects are much more pronounced for eggs produced at 20°C compared to 27°C. Such maternal effects may evolve for cross-generational phenotypic plasticity with mothers passing on their experience to the offspring to increase offspring fitness in predictable environments (Mousseau and Dingle, 1991; Mousseau and Fox, 1998a; Rossiter, 1996).

Energy content and turnover

Although egg size may be uncorrelated with energy content (Azevedo et al., 1997; Baur and Baur, 1997; Giron and Casas, 2003; Guisande and Harris, 1995; Jaeckle, 1995; Karl et al., 2007; McIntyre and Gooding, 2000), in *B. anynana* larger eggs and hatchlings did contain more energy throughout. Size differences were closely related to differences in energy content. Relative energy content, which increased from eggs to hatchlings, was higher for hatchlings having developed at 27°C (with a respective tendency occurring in hatchlings originating from eggs produced at 27°C), but did not differ across eggs produced at 20 or 27°C. Thus, as expected, patterns in relative energy content closely resembled those found for relative lipid contents.

Loss in dry mass and energy during embryonic development was higher for hatchlings originating from eggs produced at 20°C compared to 27°C, and was higher during development at 27°C compared to 20°C, again reflecting changes in relative lipid content. Concomitantly, energetic efficiency was higher for eggs produced at 27°C, and (by 10.2%) higher when developing at 20°C compared to 27°C. The higher efficiency during embryonic development at lower temperatures may have important implications for the above-mentioned temperature-size rule,

providing a possible mechanism how larger sizes at lower temperatures are achieved.

Conclusions

As expected, *B. anynana* females ovipositing at a lower temperature produced larger eggs and vice versa. Larger egg size was achieved by provisioning those eggs with larger quantities of all compounds investigated, whilst variation in relative egg composition was small. Consequently, egg size was rather strongly related to energy content. Very similar patterns were found for hatchlings, although variation in relative hatchling composition was more pronounced indicating differences in the use and turn-over of specific compounds across eggs produced at different temperatures. Glycogen was used to a large extent for embryonic development, while much larger parts of lipids and proteins were transferred to hatchlings. The temperature experienced during embryonic development had considerable effects on hatchling size and composition. According to the temperature-size rule development at a higher temperature resulted in smaller hatchlings (with lower amounts of protein and glycogen). Turnover rates of protein/glycogen and lipids/free carbohydrates differed across developmental temperatures, suggesting an emphasis on hatchling quality (i.e. protein content) at the more stressful low temperature, and therefore the presence of adaptive maternal effects. Overall, growth was much less efficient at the higher developmental temperature, which my have important bearings on the patterns of covariation between body size and temperature. Our study exemplifies the complex interactions between temperature environments and biochemical egg and offspring composition, allowing conclusions to be drawn about energy budgets and turn-over rates. As those aspects of temperature-mediated plasticity are generally under-explored, we suggest that there is much to gain from carrying out more such detailed analyses.

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Manuscript 4

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Adult nutrition and butterfly fitness: effects of diet quality on reproductive output, egg composition, and egg hatching success

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Abstract

In the Lepidoptera it was historically believed that adult butterflies rely primarily on larval-derived nutrients for reproduction and somatic maintenance. However, recent studies highlight the complex interactions between storage reserves and adult income, and that the latter may contribute significantly to reproduction. Effects of adult diet were commonly assessed by determining the number and/or size of the eggs produced, whilst its consequences for egg composition and offspring viability were largely neglected (as is generally true for insects). We here specifically focus on these latter issues by using the fruit-feeding tropical butterfly *B. anynana*, which is highly dependent on adult-derived carbohydrates for reproduction. Adult diet of females had pronounced effects on fecundity, egg composition and egg hatching success, with butterflies feeding on the complex nutrition of banana fruit performing best. Adding vitamins and minerals to a sucrose-based diet increased fecundity, but not offspring viability. All other groups (plain sucrose solution, sucrose solution enriched with lipids or yeast) had a substantially lower fecundity and egg hatching success compared to the banana group. Differences were particularly pronounced later in life, presumably indicating the depletion of essential nutrients in sucrose-fed females. Effects of adult diet on egg composition were not straightforward, indicating complex interactions among specific compounds. There was some evidence that total egg energy and water content were related to hatching success, while egg protein, lipid, glycogen and free carbohydrate content did not seem to limit successful development. The patterns shown here exemplify the complexity of reproductive resource allocation in insects, and the need to consider egg composition and offspring viability when trying to estimate the effects of adult nutrition on insect fitness.

Keywords

Bicyclus anynana, income breeding, reproductive resource allocation, insect

Introduction

Availability of adequate nutrition is of crucial importance for successful reproduction. A multitude of studies documented pronounced effects of diet quality and quantity on female reproductive output and thereby fitness (e.g. Braby and Jones, 1995; Leather, 1995; Willers et al., 1987). Fitness, however, is composed of various components, such that determining individual fitness is a challenging enterprise. Although frequently used, simply counting offspring (egg) numbers or using proxies only vaguely related to survival (such as egg size) might be misleading (Bernardo, 1996; McIntyre and Gooding, 2000). In order to gain a more integrated understanding of reproductive resource allocation, we need to shed more light on the interplay between reserves originating from storage versus income, between diet quality / quantity and egg composition, and the associated consequences for offspring viability.

Holometabolous insects are interesting models for studying reproductive resource allocation, because diets and energetic needs change dramatically between life stages, warranting integrated strategies for timed nutrient accumulation, storage and release (e.g. Boggs, 1981; Braby and Jones, 1995; Pan and Telfer, 2001; Stearns, 1992; Wheeler, 1996). This seems particularly important for female insects, since nutrient investment into eggs constitutes a major expenditure of energy (Boggs, 1981; Braby and Jones, 1995; Parker and Begon, 1986). The Lepidoptera, feeding as larvae on protein-rich plant foliage, were historically believed to primarily rely on larval-derived nutrients for reproduction and somatic maintenance (Jervis and Boggs, 2005; Leather, 1995; Mevi-Schütz and Erhardt, 2005; O'Brien et al., 2002; Telang et al., 2001). However, recent studies highlight the complex interactions between storage reserves and adult income, and that the adult diet may contribute significantly to reproductive output (e.g. Fischer et al., 2004; Hill, 1989; Jervis et al., 2005; Leather, 1995; Mevi-Schütz and Erhardt, 2005; O'Brien et al., 2004). Even other substrates ranging from pollen to mud, dung or carrion may comprise important sources of scarce nutrients (e.g. Beck et al., 1999; Boggs and Jackson, 1991; Gilbert, 1972; Smedley and Eisner, 1995). While substantial progress was made in some of these areas in recent years (especially in relation to use of income versus storage; Fischer et al., 2002, 2004; Jervis and Boggs, 2005; Mevi-Schütz and Erhardt, 2005;

O'Brien et al., 2002, 2004), others remain poorly understood. This is particularly true for the effects of adult nutrition on egg composition and in turn on offspring fitness (Casas et al., 2005; Giron and Casas, 2003; Jann and Ward, 1999; Karl et al., 2007; Kyneb and Toft, 2006).

We here draw on these under-explored areas of insect reproduction by using the tropical, fruit-feeding butterfly *Bicyclus anynana* as model organism. In this species adult-derived carbohydrates are essential for egg production, without which no eggs will be produced ("income breeder"; Bauerfeind and Fischer, 2005a; Fischer et al., 2004). Consequently, even the first eggs laid contain considerable amounts of adult-derived nutrients, followed by a quick shift towards even higher ratios (Fischer et al., 2004). Note in this context that fruit is not necessarily a richer source of nutrients than nectar, but is generally rather similar in terms of carbohydrate and nitrogen content (Bosque and Pacheco, 2000; Levey et al., 2000; Levey and Del Rio, 2001; Ômura and Honda, 2003). However, fruit may contain considerable amounts of lipids and a variety of micronutrients that may also benefit reproduction (University of Hohenheim, 1996). Recent studies using this species showed that females have a significantly higher reproductive output and longer life-spans when fed with banana as compared to sugar-based diets (Bauerfeind and Fischer, 2005a; Bauerfeind et al., 2007). As various compounds such as lipids or amino acids had no positive effects on the above traits, it was concluded that the beneficial effects of fruit are caused by resource congruence (i.e. the use of nutrient types in a specified ratio; Bazzaz, 1996) rather than any specific compound (Bauerfeind and Fischer, 2005a; Bauerfeind et al., 2007). In addition to reproductive output, we here investigate the effects of adult diet on egg composition and egg hatching success. Specifically we examine whether adult diet has a direct impact on egg composition (such that e.g. a lipid-rich diet causes an increased lipid content of eggs), and whether any potential variation in egg content is related to offspring viability.

Material and Methods

Study organism and experimental population

For this study the tropical butterfly *Bicyclus anynana* Butler, 1879 (Lepidoptera, Nymphalidae, Satyrinae) was used. *B. anynana* is a fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen, 1991). 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 over 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof et al., 2005). Animals from the Bayreuth stock were used for this study.

Experimental design

To investigate the effects of adult nutrition on reproductive output, egg composition and egg hatching success all *B. anynana* individuals were reared within the same environmental cabinet at a constant temperature of 27℃, high relative humidity (70%) and a photoperiod of L12:D12. Larvae were reared on young maize plants in population cages (50 x 50 x 80 cm), with plants being regularly replaced. The resulting pupae were collected from the plants and transferred to cylindrical hanging cages (30 x 38 cm). Following adult eclosion, female butterflies were randomly divided among five nutritional treatments: 1) moist banana [hereafter ‘banana’, for a general composititon af banana see University of Hohenheim (1996)], 2) sucrose solution (20 Vol%; ‘sugar’), 3) sucrose solution (20 Vol%) enriched with lipids (1 Vol% olive oil [Roth, Karlsruhe, Germany], containing ~14 % 16:0 palmitic acid, ~3% 16:1 palmitoleic acid, ~3% 18:0 stearic acid, ~70 % 18:1 oleic acid, ~12 % 18:2 linoleic acid and 18:3, 20:0, 20:1, 22:0, 22:1 acids < 0.1 Vol%; ‘lipid’), 4) sucrose solution (20 Vol%) enriched with a combination of minerals and vitamins readily found in banana (cf. Bauerfeind and Fischer, 2005a; Table 1; 'MV') and 5) a sucrose solution (20 Vol%) enriched with baker's yeast (*Saccharomyces cerevisiae*; ~0.5 Vol%; ‘yeast’). Sucrose solution enriched with lipids was prepared by using ultrasound with the resulting suspension being clearly stable for the day after which diets were generally renewed. Commercial baker’s yeast was solved each day in the appropriate amounts

in sugar solution before daily exchange. As all butterflies were facing no-choice situations, it is highly unlikely that effects are based on different rates of intake. Furthermore, sucrose solution was found not to influence longevity of butteflies (Bauerfeind and Fischer, 2005a). On the day after eclosion, all females were set up for mating with random males for two days. All male butterflies were fed moist banana, as their contribution to reproduction (e.g. transfer of sodium via spermatophores) is considered to be negligible (Ferkau and Fischer, 2006; Fischer et al., 2003b). Following the mating period, females were marked individually and placed into translucent plastic pots (1 L, covered with gauze), containing a fresh cutting of maize for egg-laying. Females were provided with their respective nutritional treatments throughout their lives.

Table 1: Amounts of minerals and vitamins per 1 L 20 Vol% sucrose solution for the feeding treatment 'sucrose solution enriched with minerals and vitamins' (MV).

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		

As a proxy for early and late fecundity the eggs laid on days 3 and 4 and 16 and 17 of adult life were counted. The eggs laid on the first day of oviposition (day 3) were discarded, as they contain relatively high amounts of larval resources (see Fischer et al. 2004), while the focus of the present study is on adult-derived resources. Days 3 and 4 represent the beginning of oviposition in *B. anynana* with a maximum of daily egg numbers, followed by a continuous decrease with female age. Under experimental conditions at 27℃ maximum life-spans prolong until ~30 days with half of the butterfly population still alive at the later chosen collection days (Bauerfeind and Fischer, 2005a). Per female, 20-40 eggs starting from day 4 (early) and day 16 (late) were collected and each day randomly divided into two groups: one half was

used for recording egg hatching success, whereas the other half was stored at -20℃ for later analysis of egg composition. For the early collection period 1.9 ± 0.08 additional days were necessary to collect enough eggs for further experiments, while for the later +3.9 ± 0.12 days were needed. Butterfly age for the early period is therefore defined days 4-6 and the late days 16-20. The two time points were chosen as differences in adult diet may take some time to take effect, i.e. any potential differences might be hidden at the beginning of the oviposition period due to overriding effects of larval storage reserves.

Egg hatching success

Eggs were, separated by female, transferred to Petri dishes and reared at the conditions described above. Hatchlings were counted and removed daily until no more larvae had hatched for at least two days. Finally, remaining eggs were counted and the proportion of eggs hatched was calculated. As successful developed hatchlings are generally able to feed by themselves, egg composition provided by resources of mothers is especially of importance for embryonic development, making egg hatching success an adequate parameter for testing effects of maternal diet on progeny fitness.

Egg composition

Only females having laid ≥ 20 eggs during both collecting periods were used for egg content analyses. If more eggs were available, a maximum of 20 randomly chosen eggs were used. To rule out confounding effects of (potentially occurring) egg shell contamination with dietary fluids, eggs were washed prior to analyses as follows: egg samples were put into 250 µl H_2O, washed for 10 sec in an ultrasonic bath, transferred to 500 µl $CHCl_3$:MeOH (1:1), and washed again for 10 sec. in an ultrasonic bath. Then, the solvent was removed using a borosilicate filter membrane on a vacuum pump, and the samples were rinsed with 1 ml H_2O. The latter procedure was repeated once. Before washing, egg fresh mass was determined, and after the washing procedure eggs were dried for 24h at 70℃ and weighed again. Egg water content was estimated as mass difference between egg fresh and dry mass. The extraction and separation of egg lipid, protein, glycogen, and free carbohydrate from the same samples was carried out as described by Lorenz (2003). Colorimetric

determination of total lipid, glycogen, and free carbohydrate was performed using modified sulphophosphovanillin and anthrone methods. Protein was measured with an EL 808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, Vermont, USA) using the RotiQuant Universal assay (Roth, Karlsruhe, Germany) and bovine serum albumin as a standard (Karl et al., 2007; Lorenz, 2003). Throughout, data were corrected using measured recovery rates (free carbohydrate: 96.5 ± 2.4%; lipid: 91.1 ± 2.1 %; protein: 72.7 ± 2.6 %; glycogen: 83.4 ± 1.9 %; Lorenz 2003). To correct for different recovery rates between treatments due to different days of analysis and sample handling, data were standardized to 100 % dry mass. From the resulting values for egg components, energy investment per mg egg dry mass was calculated using average caloric values of 17.2 kJ g^{-1} for free carbohydrates, proteins and glycogen and 39.0 kJ g^{-1} for lipids (Ganong, 1974; Silbernagel and Despopolos, 1991).

Statistical analyses

Data were analyzed using repeated measures ANOVAs with the nutritional treatments as categorical factor and both collecting periods as repeated measures. In case of a global significance, differences between treatment groups were localized using Tukey's HSD. Pearson correlations were used to analyze the relationships between different egg components. All statistical tests were performed using STATISTICA 6.1. Throughout the text, means are given ± 1 SE.

Results

Fecundity, egg size and hatching success

Adult diet significantly affected fecundity of *B. anynana* females ($F_{4,250}$ = 6.1, P < 0.001). The banana group achieved the highest egg numbers (86.2 ± 4.5) followed by the MV group (71.6 ± 4.1), while the remaining treatment groups laid significantly fewer eggs and were statistically indistinguishable (63.8 ± 2.3; Tukey HSD after ANOVA; Fig. 1A). While fecundity at days 3-4 was fairly similar across treatment groups (except from slightly higher values for the banana group), variation in later fecundity was higher with the banana and MV groups showing substantially higher values than all other groups (significant treatment x time interaction; $F_{4,250}$ = 2.5, P = 0.041). Generally, females produced much more eggs early compared to late in life ($F_{1,250}$= 256.3, P < 0.001). Egg dry mass did not differ significantly across treatment groups ($F_{4,129}$ = 0.260, P = 0.903), but was overall significantly lower (reduction by ca. 11 %) later than earlier in life ($F_{1,129}$ = 23.8, P < 0.001; treatment x time interaction: $F_{4,129}$ = 0.66, P = 0.624; Fig. 1B).

Egg hatching success was overall significantly higher in the banana compared to all other groups, with the latter four groups being statistically indistinguishable (Tukey HSD; $F_{4,213}$ = 10.8, P < 0.001; Fig. 1C). The overall better performance of the banana group resulted mainly from a constantly high hatching success (early life: 82.4 ± 3.6 %, late life: 71.9 ± 4.6%), whilst in the other treatment groups hatching success dropped markedly from ~70 % (early) to ~30% (late; time: $F_{1,213}$ = 177.3, P < 0.001; treatment x time: $F_{4,213}$ = 6.2, P < 0.001; Fig. 1C).

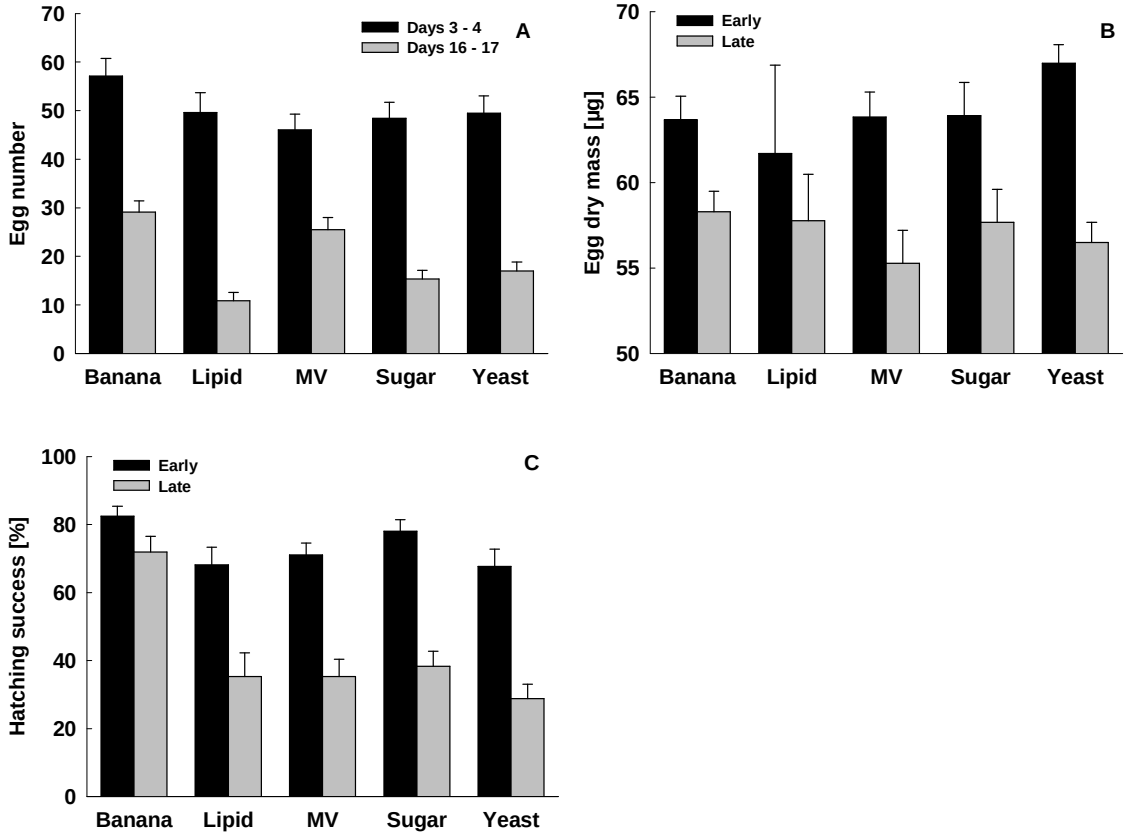

Fig. 1: Mean egg number (A), egg dry mass (B), and egg hatching success (C) for female *Bicyclus anynana* in relation to adult feeding and female age. Early: Eggs from days 4-6 of adult life; late: eggs from days 16-20. Banana: moist banana; Lipid: 20 Vol% sucrose plus 1 Vol% olive oil; MV: 20 Vol% sucrose plus minerals and vitamins; Sugar: 20 Vol% sucrose; Yeast: 20 Vol% sucrose plus baker's yeast.

Egg composition

Across treatments and sampling periods, eggs consisted predominantly of water (84.1 ± 0.24%), followed by lipids (6.8 ± 0.11%), proteins (6.5 ± 0.19%), glycogen (2.1 ± 0.05%) and free carbohydrates (0.4 ± 0.02%). Egg water content was significantly higher in the banana than in the yeast group, while all other pairwise comparisons were not significant (Tukey HSD after ANOVA; $F_{4,129} = 2.5$, $P = 0.049$; Fig. 2A). Overall, water content was significantly lower in later than in earlier eggs ($F_{1,129} = 5.1$, $P = 0.026$; treatment x time interaction: $F_{1,129} = 0.6$, $P = 0.677$). Adult diet further significantly influenced egg lipid ($F_{4,131} = 13.4$, $P < 0.001$; Fig. 2B), protein ($F_{1,129} = 10.7$, $P < 0.001$; Fig. 2C), glycogen ($F_{4,131} = 4.1$, $P < 0.001$; Fig. 2D) and free carbohydrate content ($F_{4,130} = 10.1$, $P < 0.001$; Fig. 2E).

Lipid content was higher in the banana and MV groups compared to all other treatments. Further, lipid content increased with female age ($F_{4,130}$ = 62.0, P < 0.001), particularly in the banana and MV groups (treatment x time interaction: $F_{4,130}$= 17.7, P < 0.001). Protein content, in contrast, was significantly lower in the banana and MV than in the other groups. While protein content decreased with female age in those two treatment groups, it increased in the remaining three groups (treatment x time interaction: $F_{4,130}$ = 19.0, P < 0.001). Accordingly, no overall time effect for protein content was observed ($F_{2,130}$ = 2.5, P = 0.129).

Glycogen content was significantly reduced in the yeast compared to all other groups, and was also in tendency reduced in late compared to early eggs ($F_{4,131}$ = 365.7, P < 0.001; treatment x time interaction: $F_{4,131}$ = 2.2, P = 0.071). Free carbohydrate content was significantly higher in the sugar and yeast treatments. While free carbohydrate content was generally rather constant over time ($F_{4,131}$ = 3.1, P = 0.082), it strongly increased with female age in the sugar group, but slightly decreased in the yeast group (treatment x time interaction: $F_{4,131}$ = 5.3, P < 0.001).

Across both sampling periods and all treatments egg lipid content was rather strongly negatively correlated to protein content (i.e. the higher the lipid, the lower the protein content and vice versa; Table 2). Further, glycogen tended to be negatively related to protein content (significant in 8 out of 10 cases). Correlations between lipid and glycogen content as well as free carbohydrates to the other three compounds revealed no conclusive pattern.

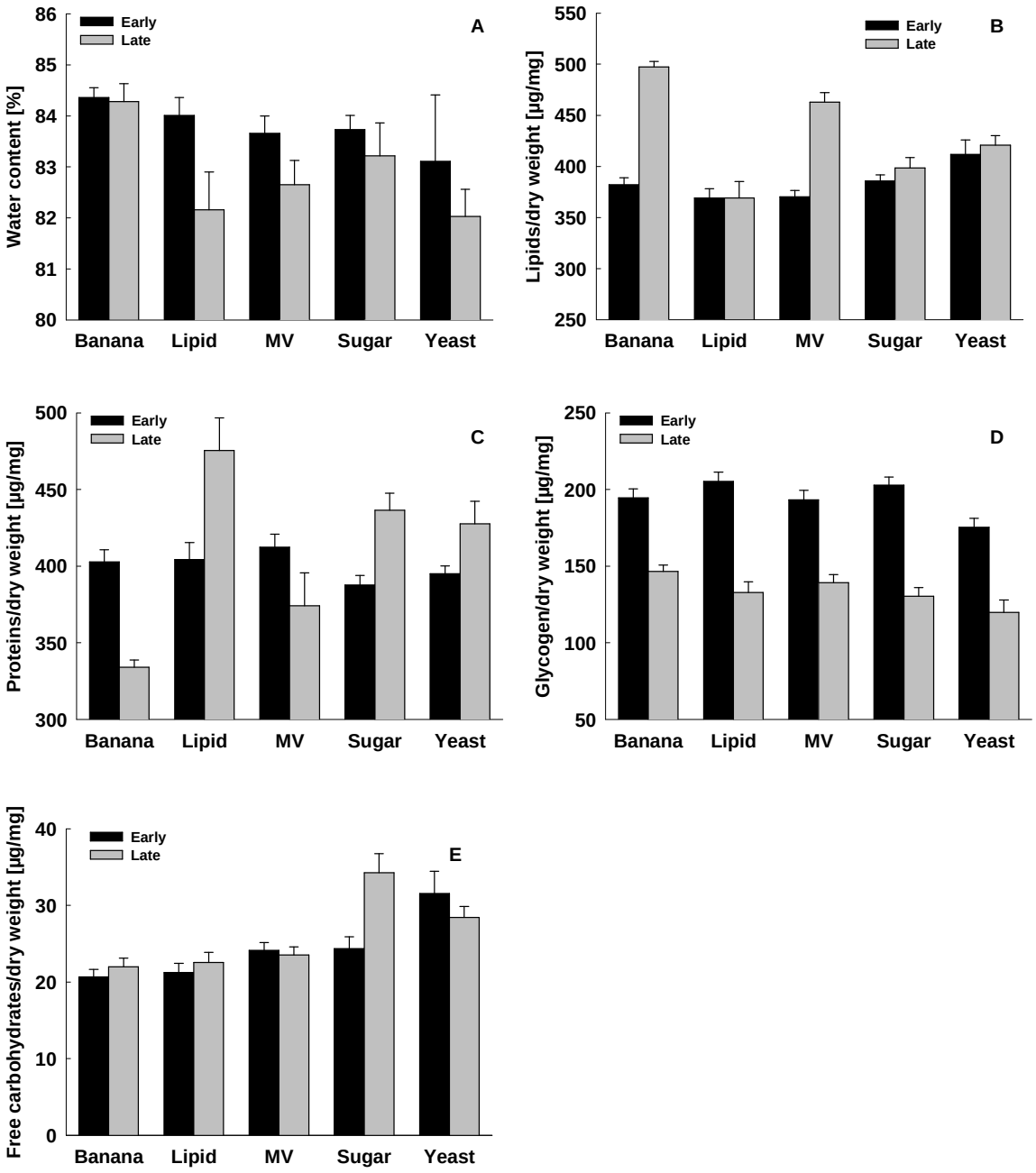

Fig. 2: Relative egg water (A), lipid (B), protein (C), glycogen (D) and free carbohydrate content (E; means ± 1 SE) for female *Bicyclus anynana* in relation to adult feeding and female age. Early: Eggs from day 4-6 of adult life; late: eggs from days 16-20. For explanations of dietary treatments see Fig. 1.

Table 2: Inter-correlations between *Bicyclus anynana* egg compounds (Carb. = Free carbohydrates) per 1 mg dry mass in relation to adult feeding treatment and time. Early: Eggs from days 4-6 of adult life; late: eggs from days 16-20. Given are correlation coefficients. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

		Early			**Late**		
		Lipid	Glycogen	Lipid	Lipid	Glycogen	Lipid
	N	Protein	Protein	Glycogen	Protein	Protein	Glycogen
Banana	29	***** - 0.69**	***** - 0.60**	- 0.15	***** - 0.62**	- 0.35	***** - 0.47**
Lipid	18	***** - 0.82**	*** - 0.55**	-0.01	***** - 0.96**	***** - 0.76**	*** + 0.56**
MV	30	***** - 0.60**	***** - 0.64**	- 0.20	***** - 0.87**	*** - 0.57**	-0.12
Sugar	30	***** - 0.64**	**** - 0.56**	- 0.23	***** - 0.84**	***** - 0.63**	+ 0.14
Yeast	29	***** - 0.56**	- 0.33	***** - 0.53**	***** - 0.80**	***** - 0.76**	+ 0.23
		Carb.	Carb.	Carb.	Carb.	Carb.	Carb.
		Protein	Lipid	Glycogen	Protein	Lipid	Glycogen
Banana	29	+ 0.04	- 0.32	+ 0.13	+ 0.01	*** - 0.37**	+ 0.21
Lipid	18	- 0.35	+ 0.27	+ 0.04	*** - 0.47**	+ 0.43	+ 0.26
MV	30	**** - 0.53**	+ 0.19	+ 0.35	+ 0.08	- 0.23	+ 0.05
Sugar	30	+ 0.21	- 0.35	- 0.18	+ 0.31	*** *- 0.57**	- 0.04
Yeast	29	*** - 0.45**	- 0.04	+ 0.12	***** - 0.60**	+ 0.26	***** + 0.61**

Energy investment

Energy investment per 1 mg egg dry mass was significantly higher in banana and MV females compared to other treatments (Tukey HSD after ANOVA; $F_{4,128} = 15.4$, $P < 0.001$), and was higher in eggs laid later in life than in those laid earlier ($F_{1,128} = 83.4$, $P < 0.001$; Fig. 3A). The latter effect was particularly pronounced in the banana and MV treatments (treatment x time interaction; $F_{4,128} = 22.7$, $P < 0.001$). Energy investment per egg, in contrast, did not differ significantly between treatments ($F_{4,128} = 1.2$, $P = 0.337$), but was significantly lower (by about 6%) in eggs oviposited later in life ($F_{1,128} = 6.9$, $P < 0.001$; treatment x time interaction: $F_{1,128} = 1.7$, $P = 0.156$; Fig. 3B). Across treatment groups, egg dry mass was significantly positively related to egg energy content (early: $r = 0.98$, $P < 0.001$, $N = 135$; late: $r = 0.93$, $P < 0.001$, $N = 136$).

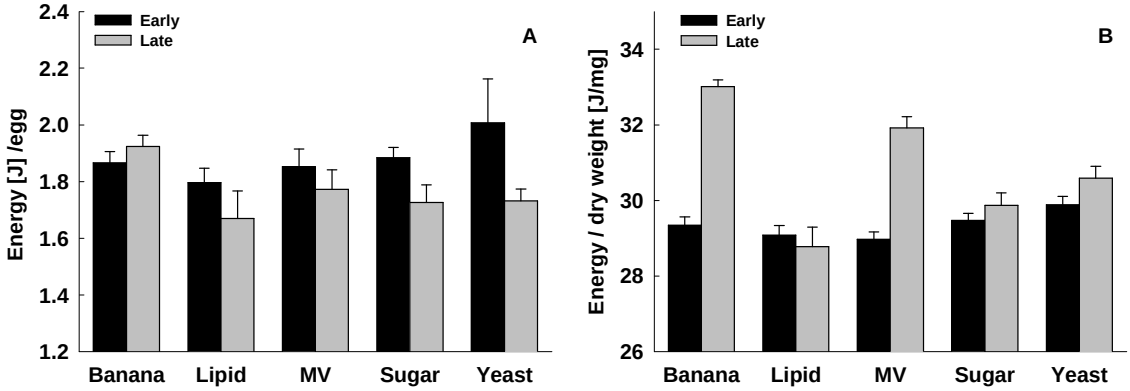

Fig. 3: Egg energy content per 1 mg dry mass (A) and per egg (B) (means ± 1 SE) for female *Bicyclus anynana* in relation to adult feeding and female age. Early: Eggs from days 4-6 of adult life; late: eggs from days 16-20. For explanations of dietary treatments see Fig. 1.

Discussion

Fecundity, egg size and hatching success

Female *B. anynana* butterflies feeding on the complex nutritional diet of banana showed highest fecundity levels, followed by the group fed a sugar solution supplemented with minerals and vitamins (MV). The higher average egg numbers in these groups mainly reflect a much less pronounced reduction in daily fecundity with female age compared to the other groups. The general decline in egg numbers with female age in turn probably reflects the depletion of essential (larval-derived) resources (Giron and Casas, 2003; Karlsson and Wiklund, 1984). Thus, these results show, in line with previous findings, that banana is the superior food in terms of egg numbers, with only the solution containing vitamins and minerals rising fecundity above the level of a plain sugar solution (Bauerfeind and Fischer, 2005a; Bauerfeind et al., 2007).

As insect eggs are primarily composed of protein and lipid (Engelmann, 1999; Karl et al., 2007; Kawooya and Law, 1988; Lorenz, 2003; Ziegler and Van Antwerpen, 2006), ovipositing females should be in high demand for such compounds, and yeast is known to be an excellent source of protein to insect frugivores (Good and Tatar, 2001). Regarding lipids, insects are known to be able to produce the triacylglycerol needed for egg production from the free fatty acids provided by our lipid treatment, and also to transport those into developing oocytes (Ferenz, 1985; Lubzens et al., 1981; Ziegler and Van Antwerpen, 2006). As nevertheless neither yeast nor lipid affected *B. anynana* reproductive output it can be concluded that female reproduction is usually not constrained by adult-derived lipid or protein (Bauerfeind and Fischer, 2005a; Bauerfeind et al., 2007; Jervis and Boggs, 2005; Mevi-Schütz and Erhardt, 2005).

On the other hand, the evidence suggests that females may eventually run out of vitamins or minerals carried over from the larval stage during oviposition. Interestingly, adding either minerals or vitamins to the adult diet had no effect on *B. anynana* fecundity, but only the combination of both (Bauerfeind and Fischer, 2005a). Positive effects of additionally providing minerals or vitamins, though not necessarily in combination with each other, on fecundity have been reported also for some other

insects (Engelmann, 1999; Pappas and Fraenkel, 1977). Generally, however, the role of those compounds in insect reproduction is only poorly understood, while their importance for insect energy metabolism, growth, development or detoxication is rather well established (e.g. Barbehenn et al., 2001; Lindroth et al., 1991; Vanderzant et al., 1962).

In contrast to fecundity, egg size was not affected by nutritional treatment, although former studies reported slightly larger eggs laid by females fed banana compared to other diet groups (Bauerfeind and Fischer, 2005a; Bauerfeind and Fischer, 2007). Anyway, when manipulating diet quality or other factors, variation in egg size was generally found to be much less pronounced as compared to fecundity (Bauerfeind and Fischer, 2005a, 2005b; Bauerfeind et al., 2007; Steigenga et al., 2005). The decrease in egg size with female age is a common feature repeatedly documented for a variety of butterfly species (e.g. Braby and Jones, 1995; Hill and Pierce, 1989; Jones et al., 1982; Karlsson and Wiklund, 1984).

In addition to producing most eggs, banana-fed females also had the highest egg hatching success, again documenting the high quality of this adult diet. While early in life hatching success was very similar across feeding treatments, it dropped dramatically later in life except for the banana group. These findings suggest the depletion of one or more crucial substances in the females fed sucrose-based diets, and that the respective compounds can obviously be drawn from the fruit diet. We do not know the compounds in question, but can largely rule out proteins, lipids, and the minerals and vitamins tested. Most likely this pattern is caused by resource congruence rather than by any specific compound (note that hatching success dropped substantially even in the MV treatment), as has been implicated before with regard to reproductive output (Bauerfeind and Fischer, 2005a; Bauerfeind et al., 2007). While a substantial number of studies examined the effects of larval and adult nutrition on reproductive output, very little is known on the effects of adult nutrition on offspring fitness (Jann and Ward, 1999; Kyneb and Toft, 2006). In the rove beetle *Tachyporus hypnorum*, for instance, maternal diet affected larval development and offspring survival (Kyneb and Toft, 2006), while in the dung fly *Scathophaga stercoraria* low adult food quality had no effect on egg hatching success (Jann and Ward, 1999). We will below discuss the associations between offspring fitness and

egg composition which might causally underlie the variation in hatching success found.

Egg composition

Generally, egg size and composition are not necessarily tightly correlated, and variation in egg composition can be ecologically and evolutionarily more important than variation in egg size (Azevedo et al., 1997; Fox and Czesak, 2000; Giron and Casas, 2003; McIntyre and Gooding, 2000). In line with other data on insects (Van Handel, 1993; Ziegler and Van Antwerpen, 2006), *B. anynana* eggs consist mainly of water, proteins and lipids. An egg water content of about 84 % is typical for satyrine butterflies (García-Barros, 2006). Highest values were found in the banana, lowest in the yeast group. The latter may reflect dehydration associated with the production of ethanol by yeast (Cowmeadow et al., 2005). Egg water content was generally lower in late than in early eggs, except for the banana group (but note that the interaction is not significant; see also Karl et al., 2007). Although water content variation seems to be small by percentage, such differences represent a large part eggs, especially as egg dry weight compounds only reflect about 16% of it. As the banana group is the only one maintaining a high egg hatching success later in the oviposition period, egg water content might have a significant impact on egg hatching success (compare Fig. 1C and 2A; see below).

Compared to all other treatments, the eggs produced by banana and MV females showed higher amounts of lipids, but lower amounts of proteins. In both groups this pattern is primarily caused by variation in the later eggs. While relative lipid content remained largely similar over time in the sugar, yeast and lipid group, it increased dramatically in late compared to early eggs in the banana and MV group. Similar results were obtained from lines selected for differences in egg size (Karl et al., 2007). Regarding relative protein content, it decreased with female age in the banana and MV group, while it increased in the remaining groups. The similarity of patterns across both groups is striking and may suggest that the increased fecundity of the banana and MV groups caused a gradual depletion of nitrogenous compounds, compensated by an increased investment of lipids. Likewise, a reduction in protein investment with female age was found in *B. anynana* females having been exposed to larval food stress (Karl et al., 2007). It is generally assumed that the vast majority

of nitrogenous compounds used for reproduction is accumulated and stored during the larval phase (e.g. Jervis and Boggs, 2005; O'Brien et al., 2002; Wheeler, 1996), though adult diet may in some cases compensate larval deficiencies (Mevi-Schütz and Erhardt, 2005).

The general importance of lipids and proteins for embryonic and larval development in insects is well established (Beenakkers et al., 1985; Diss et al., 1996; Van Handel, 1993). Lipids are considered to cover the energetic demands of the developing embryo, while proteins are mainly structural components, but may additionally serve as energetic resource (Beenakkers et al., 1985). Furthermore, yolk protein has been previously shown to be a good predictor for neonate fitness (Diss et al., 1996). Nevertheless, neither compound had a detectable influence on egg hatching success in *B. anynana*. Despite a strong reduction of relative protein content in late eggs, banana-fed females were able to maintain egg hatching success on a high level, while the increased investment of protein in other groups could not prevent a reduction in egg viability. Likewise hatching success of later eggs in the MV group was as low as for the other groups (except banana), despite a clearly increased relative lipid content.

Glycogen content was similar across treatments except for the yeast group, but was in tendency lower in late compared to early eggs (see also Karl et al., 2007), and may reflect the beginning use up of stored glucose for reproduction or in the build up of it in eggs itself. Free carbohydrate levels in the hemolymph are known to increase with carbohydrate-rich diet (Thompson, 1998; Thompson et al., 2003). Here, free carbohydrate content in the eggs was higher in the sugar and yeast groups, although all treatments should have provided sufficient amounts of free carbohydrates. In all other treatments free carbohydrates were similar in early and late eggs, at it has been reported before (Karl et al., 2007). Neither glycogen nor free carbohydrates showed any obvious association with fecundity or hatching success.

The negative association between lipid and protein across adult diet groups described above is also found within groups: throughout, strong negative correlations between both compounds were found. Further, glycogen tended to be negatively

related to protein content. These findings may suggest a trade-off between the investment into structural elements and storage reserves.

Energy investment

Estimates of reproductive investment often exclusively rely on egg numbers and/or egg size (Azevedo et al., 1997; Bernardo, 1996; McIntyre and Gooding, 2000). This approach is problematic as egg size can be uncorrelated with energy content (Jaeckle, 1995), as individual egg compounds may vary independently of egg size (Baur and Baur, 1997), and as energetic investment may decrease with female age (Giron and Casas, 2003; Mousseau and Dingle, 1991). Nevertheless studies on insects taking the actual energetic investment into account (by means of biochemical analyses) are scarce (Casas et al., 2005; Diss et al., 1996; Giron and Casas, 2003; Quickenden and Roemhild, 1969; Van Handel, 1993). In *B. anynana* egg dry mass correlated strongly with energy content, but nevertheless individual compounds showed substantial variation, suggesting that allocation strategies may differ in spite of similar egg size and energy content.

Investment per 1 mg dry mass followed, as expected, patterns of variation in lipid content. Consequently eggs from the banana and MV groups showed an overall higher relative energy investment, especially so in late eggs. It is noteworthy that relative investment was not reduced in later eggs in any group. In contrast, absolute investment per egg was similar across treatment groups, but was ca. 6% lower in later as compared to earlier eggs (except for the banana group). This can be largely attributed to variation in egg dry mass, declining by ca. 11%. The association between a high energetic investment even in late eggs and high hatching success found in the banana group suggests that egg energy content might be crucial for egg viability (see below).

While the general decline in egg provisioning with female age (egg size, absolute energy content) is in agreement with other studies (e.g. Braby and Jones, 1995; Giron and Casas, 2003; Karlsson and Wiklund, 1984; McIntyre and Gooding, 2000; Mousseau and Dingle, 1991), relative investment (per mg dry mass) as well as absolute investment in animals fed with a high quality food remained unaffected (see also Karl et al., 2007). The latter was achieved, despite a substantial decline in egg

size, by increasing relative energy investment (i.e. provisioning with lipids). Thus, caution is needed when trying to draw general conclusions, and studies on reproductive resource allocation should take into account variation in egg quality (McIntyre and Gooding 2000).

Conclusions

Individual fitness is a complex trait that is difficult to measure. A commonly used method for estimating the fitness effects of dietary treatments (and other factors) is determining the number (and sometimes size) of eggs produced (see Azevedo et al., 1997; Bernardo, 1996). Studies directly measuring offspring viability, in contrast, are much less frequent (Capinera et al., 1977; Diss et al., 1996; McIntyre and Gooding, 2000; Quickenden and Roemhild, 1969). Not accounting for differences in offspring viability is obviously problematic, and very little is known to date on the interplay between diet quality, age, egg content, and offspring viability (Casas et al., 2005; Karl et al., 2007; McIntyre and Gooding, 2000). Our study provides some insights regarding the latter. Clearly, the complex nutritional composition of banana fruit was superior compared to alternative diets, not only increasing reproductive output compared to sugar-based diets (Bauerfeind and Fischer, 2005a; Bauerfeind et al., 2007), but also positively affecting egg hatching success. As has been argued before (Bauerfeind and Fischer, 2005a; Bauerfeind and Fischer, 2007) we believe the increased reproductive output to be a consequence of resource congruence, as sugar enriched with minerals and vitamins increased fecundity (though not reaching the levels of the banana group), while either adding protein or lipids had no detectable effect.

Adult diet had striking effects on egg composition, namely on water, protein, lipid and carbohydrate content. The effects, however, were not straightforward (e.g. lipid content was not increased in the lipid group, and protein content was not increased in the yeast group), indicating complex interactions among specific compounds. Protein content declined with female age, but only in the groups exhibiting highest fecundity (banana, MV), suggesting that *B. anynana* reproduction strongly depends on nitrogenous resources accumulated during the larval phase (see also Jervis and Boggs, 2005; O'Brien et al., 2002; Wheeler, 1996). This development was counter-balanced by an increased investment of lipids into later eggs, presumably

synthesised from the adult diet (Bauerfeind and Fischer, 2005b; Fischer et al., 2004). Despite the overall pronounced variation in egg composition, no single compound showed any clear association with egg hatching success, and neither did egg size (note the high hatching success for the relatively small eggs produced late by banana-fed females).

Only two traits showed at least some evidence for an association with offspring viability: absolute energy content and egg water content. Both compounds were present in largely similar quantities in early and late eggs from the banana group (being associated with a high hatching success throughout), while they clearly decreased with female age in all other groups, followed by a decrease in hatching success. We tentatively conclude that egg protein content is not limiting in *B. anynana*, possibly based on a minimum threshold beyond which no successful development is possible. This threshold may not have been touched here. Note that protein content in *B. anynana* was remarkably stable across eggs artificially selected for large and small size (Karl et al., 2007). Rather, the amount of energy available for embryonic development and egg water content may determine hatching success. There is some evidence already that a high water content, presumably reducing desiccation risk, may be important for successful egg development in *B. anynana* (Fischer et al., 2003a; Fischer et al., 2006). Consequently, water should not be exclusively considered a cheap filler, especially since probably some energy is needed to incorporate water into eggs. The importance of adult diet for different components of *B. anynana* fitness exemplifies the complexity of reproductive resource allocation in insects, which were formerly assumed to rely primarily on larval stores (Leather, 1995; Telang et al., 2001). Clearly, more such efforts are needed before general conclusions on the effects of adult diet on egg composition and the role of specific compounds for egg hatching success can be drawn.

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List of publications

Geister T. L, Lorenz, M.W., Hoffmann K.H. and Fischer, K. (2008) Adult nutrition and butterfly fitness: effects of diet quality on reproductive output, egg composition, and egg hatching success *Frontiers in Zoology* **submitted**

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe. Ferner erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Bayreuth, der 09.05.2008 - Thorin Lukas Geister