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PLANT-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Enhancing offspring quality or quantity? Different ways for using nectar amino acids in female butterflies

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Abstract Butterfly-pollinated flowers offer nectar with higher amino acid concentrations than most flowers pollinated by other animals, and female butterflies of some species prefer to consume amino acid-rich nectar. However, for over 30 years, there has been an ongoing discussion about whether nectar amino acids benefit butterfly fitness. A clear positive effect was only shown for the nectar-feeding *Araschnia levana*, and females of the fruit-feeding *Bicyclus anynana* also increased offspring quality when they were fed amino acids as adults. Thus, severe doubts remain about the general significance of these single positive results. We therefore tested a further species from a phylogenetically different butterfly subfamily, the small heath (*Coenonympha pamphilus* L., Satyrinae), taking into account feeding conditions over the whole life cycle of this species. *C. pamphilus* females receiving nectar amino acids as adults, irrespective of larval food quality, produced heavier larvae and also increased the hatching success of their eggs over the oviposition period. Furthermore, females raised under nitrogen-poor larval conditions tended to use nectar amino acids to increase the number of eggs laid. Thus, *C. pamphilus* females used nectar amino acids primarily to increase their offspring quality, and secondly tended to increase offspring quantity, if larval resources were scarce, showing a resource allocation pattern differing from both *B. anynana* and *A. levana*. Our study supports the old postulate that nectar amino acids generally enhance butterfly fitness.

Keywords Butterfly reproduction · *Coenonympha pamphilus* · Larval feeding · Lepidoptera pollination

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Introduction

Approximately two-thirds of all flowering plants are pollinated by insects (Schoonhoven et al. 2006). Apart from olfactory and visible attractants, flowers lure potential pollinators with rewards such as nectar, pollen, or oil. Nectar is composed mainly of water and sugars, but can also contain significant amounts of amino acids (Ziegler 1956; Lüttge 1961; Baker and Baker 1973, 1986; Baker 1975). Amino acid concentration in floral nectar is relatively constant within species and can typify certain plant families and genera (Baker and Baker 1986). Moreover, the amino acid concentration in floral nectar correlates with specific pollinator types, and nectar of flowers adapted to pollination by butterflies contains higher levels of amino acids than most flowers pollinated by other animal types (Baker and Baker 1975, 1986). As a consequence of this finding, there has been an ongoing discussion for over 30 years as to whether amino acids obtained from nectar in the adult diet enhance butterfly fitness.

Insect eggs consist primarily of protein (Engleman 1984), and the amount of acquired nitrogen is a key factor for fitness and reproduction in insects, but larval host plants often do not provide optimal amounts of nitrogen (Schoonhoven et al. 2006). Hence, the limiting resource for reproduction seems to be nitrogenous compounds (Mattson 1980; Boggs 1981), and insects are dependent on dietary

sources for 10 out of 20 amino acids (Dadd 1973). Thereby, resource allocation differs under unconstrained, benign conditions and stressful, resource-poor environments (Boggs 2009). However, butterflies can also synthesise non-essential amino acids from carbohydrates in adult nectar diet (O'Brien et al. 2002).

Depending on an animal's stage of development and current environmental circumstances, nutrients are required at optimal levels to maximize fitness (Simpson and Raubenheimer 1993). Furthermore, allocation patterns of nutrients at each developmental stage are not independent of each other and also interact with the nutritional environment (Boggs 2009). Thus, in holometabolous insects, larvae and adults can feed on different food sources and both stages can contribute nutrients to reproduction. In butterflies, nitrogen used in egg production is mainly derived from stored larval reserves (Boggs 1981, 1997a; O'Brien et al. 2002), but the importance of larval reserves declines with increasing quality of adult nutrition (Boggs 2009). Nitrogen sources in the adult stage are adult diet (Boggs 1997a; O'Brien et al. 2002) and 'nuptial gifts' that females receive in the form of spermatophores from males at mating (Boggs and Gilbert 1979; Boggs 1981). Hence, female butterfly fecundity can be increased by the availability of amino acid-rich nectar in the adult diet (Mevi-Schütz and Erhardt 2005), by a plain sugar solution enriched with amino acids and salts (Bauerfeind and Fischer 2009), by the utilization of nitrogen-rich pollen (O'Brien et al. 2003), and by nitrogen-rich nuptial gifts received during mating (Wiklund et al. 1993; Karlsson 1998; Arnqvist and Nilsson 2000). Furthermore, female butterflies of some species prefer to consume amino acid-rich nectar (Alm et al. 1990; Erhardt and Rusterholz 1998; Mevi-Schütz and Erhardt 2002), suggesting that amino acids derived from adult diet are generally important for female butterfly reproduction. For instance, female butterflies of *Araschnia levana* and *Coenonympha pamphilus* L. (Lepidoptera: Satyrinae) raised on nitrogen-poor larval host plants showed an increased preference for amino acid-rich nectar (Mevi-Schütz and Erhardt 2003a; Mevi-Schütz et al. 2003).

Another important factor affecting resource allocation patterns is aging. Food intake for any given life stage does not necessarily match life-history trait requirements for that particular life stage (Boggs 2009). For example, nectar intake varies with age (Boggs and Ross 1993; Boggs 1997b), and the source (larval or adult income) of egg nutrients varies over the female oviposition period (Boggs 1997a, b), thereby also affecting fecundity (Boggs and Freeman 2005).

Nevertheless, the role of amino acids in nectar for butterfly reproduction remains controversial. For instance, females of the tropical papilionids *Battus philenor* and *Ornithoptera priamus* (Erhardt 1991, 1992) and male butterflies in general did not show a preference for amino acid-rich nectar (e.g., Mevi-Schütz and Erhardt 2003a; Mevi-Schütz et al. 2003). Furthermore, female fecundity of several butterfly species was not influenced by nectar amino acids (Moore and Singer 1987; Hill 1989; Mevi-Schütz and Erhardt 2003b), leaving some ambiguity about the general significance of nectar amino acids for butterfly reproduction. To date, only two studies have shown conclusive evidence of a beneficial effect of amino acids from adult diet on butterfly fecundity (Mevi-Schütz and Erhardt 2005; Bauerfeind and Fischer 2009). However, in the study of Mevi-Schütz and Erhardt (2005), positive fitness effects of nectar amino acids in the adult diet of *A. levana* females only became apparent when larvae were raised under nitrogen-poor dietary conditions. In contrast, increased egg size resulting from an amino acid-rich adult diet in the fruit-feeding *Bicyclus anynana* was unaffected by larval food conditions (Bauerfeind and Fischer 2009). Previous studies which found no effect of amino acids in the adult diet on butterfly reproduction neglected nutrients acquired during the larval phase. As, in these studies, larvae were raised under benign conditions, positive effects of nectar amino acids from adult feeding might have been masked (Moore and Singer 1987; Hill 1989; Mevi-Schütz and Erhardt 2003b). Hence, further manipulative studies of resource acquisition and utilization in phylogenetically diverse butterflies are needed to test the hypothesized universality of the relationship between nectar amino acids, reproduction, and larval food stress among butterflies, as stated by Jervis and Boggs (2005).

The objective of the present study was therefore to clarify this issue by investigating the small heath (*Coenonympha pamphilus* L., Satyrinae), an additional butterfly species from a different subfamily than the previously tested nectar-feeding *A. levana* and fruit-feeding *B. anynana*, taking into account the food quality of the juveniles as well as of the adults. We measured not only quantitative reproductive parameters (i.e. egg number) but also qualitative, fitness-relevant traits such as hatching success of eggs and progeny's larval hatching mass, and refer to these traits as offspring quality in this paper. As nutrients from adult feeding can be stored and can be used only later during the oviposition period after resources from the larval phase are depleted (Boggs 1997a), we also analysed time patterns of reproductive parameters over the whole female oviposition period.

Materials and methods

Study species

Coenonympha pamphilus, the small heath, is a common butterfly in Eurasia and is found primarily on unfertilized

meadows. It generally has three generations per year and overwinters as half-grown larva (Ebert and Rennwald 1991). Larvae of this species feed on a variety of grasses (*Cynosurus*, *Poa*, and *Anthoxanthum* spp.; Koch 1991), but *Festuca rubra* is favored (Goverde and Erhardt 2003). Eighteen *C. pamphilus* females were collected from an unfertilized meadow in the northern Jura mountains (Liesberg BL, Switzerland) and placed in cages containing pots of *F. rubra* for oviposition. The eggs were carefully collected (the butterflies laid their eggs directly on the cage netting so it was necessary to cut the netting to collect the eggs) and placed in Petri dishes.

Plant material

Larval food plants of *F. rubra* were grown in 750-ml plastic pots filled with untreated calcareous soil from a nutrient-poor meadow near Liesberg BL in Switzerland. Each pot was planted with 450 seeds (UFA Samen, Basel, Switzerland). Plants were grown in a greenhouse at the University of Basel, with ambient sun light in summer and supplement light (1,000 W broad spectrum, light period from 0600 to 2000 hours) during cloudy weather conditions and a day/night cycle of 25/19°C. All pots were watered when necessary. High-quality larval food plants were obtained by fertilizing half the pots once a week with 50 ml Algoflash (Laboratoire Algochemie Z. I. Nord, Chateau-Renault, France; N:P:K = 1:1:1). The low-quality larval food plants received only water. Prior to introducing the larvae, the following plant quality parameters were analyzed from 8-week-old grass samples: Leaf water content was calculated as the difference between dry and fresh leaf mass (drying by 80°C for 48 h), dry leaves were ground for leaf nitrogen (N) and carbon (C) analysis using a CHN analyzer (LECO Instruments, model 1932; St. Joseph, MI, USA).

Larval diet

L1 larvae from the 18 ovipositing females were randomly assigned to either the high- or low-quality larval food plants and kept in groups of 10. All lineages were included in the analysis.

Larvae were raised in the same greenhouse under the same climatic conditions as larval food plants. Larvae from each female were reared separately in order to later trace back each butterfly to the ovipositing female. After 10 days, larvae were separated and kept individually in Petri dishes enclosed with nylon mesh. About a third of all larvae entered diapause and had therefore to be excluded from the experiment. There was no effect of larval food quality on the proportion of larvae entering diapause ($\chi^2 = 2.46$, $df = 1$, $P = 0.12$). The larvae continued to receive their assigned larval food quality diet. To achieve a difference in nitrogen reserves from the larval phase, larvae raised on high-quality larval food plants received an abundant supply of fertilized *F. rubra* ad libitum, whereas larvae from the low-quality treatment received a quantitatively limited amount of unfertilized larval host plants during the last larval instar (ca. 50% of the amount of the high-quality larvae). Considering not only food quality but also the amount of available food is important in order to evaluate effects of food quality on performance in feeding experiments due to potential compensatory feeding (Carvalho et al. 2005; Simpson and Raubenheimer 2007). Pupae were collected and placed in individual compartments until emergence.

Butterfly diet

Females were weighed within 24 h after emergence (Mettler Toledo Ab 204-S; Mettler Instruments, Switzerland), placed in individual nylon mesh cages (20 cm × 20 cm × 40 cm), and allowed to mate once with an unrelated male. All butterflies that did not mate within the first 2 days after emergence were excluded from the analysis. Female butterflies from the high- and low-quality larval food treatments were randomly assigned to a nectar diet treatment consisting either nectar mimic with amino acids (AA) or without amino acids (NAA). Four treatment groups resulted: high-quality larval food and adult diet with amino acids (high/AA, $n = 13$), high-quality larval food and adult diet without amino acids (high/NAA, $n = 16$), low-quality larval food and adult diet with amino acids (low/AA, $n = 11$) and low-quality larval food and adult diet without amino acids (low/NAA, $n = 13$).

A nectar mimic of the plant *Lantana camara* was used in this experiment. Although this plant does not occur naturally in the habitat of *C. pamphilus*, nectar mimics of *L. camara* have been used in former experiments with *C. pamphilus* (Mevi-Schütz and Erhardt 2003a) as well as with a number of other butterfly species (Alm et al. 1990; Erhardt and Rusterholz 1998; Mevi-Schütz et al. 2003; Mevi-Schütz and Erhardt 2005) and have become established as a reference for comparing studies of different butterfly species. The nectar mimic of the group fed without amino acids contained only sucrose, glucose, and fructose, whereas the diet of the amino acid-fed group corresponded to the complete nutrient spectrum of *L. camara* nectar, additionally containing nonessential and essential amino acids (for exact composition, see Alm et al. 1990). The test solutions were made with sodium-free substances.

Preliminary experiments showed that *C. pamphilus* butterflies rejected a daily feeding (Cahenzli and Erhardt, unpublished data). In contrast to the varying availability of floral nectar quality and quantity in nature (Rusterholz and Erhardt 1998a, b), nectar supply at the artificial feeding

station was not limited. In the present experiment, the butterflies consumed obviously enough nutrients by feeding only every second day. Furthermore, the butterflies were kept in cages, reducing their mobility and diminishing their energy consumption. We therefore fed the butterflies their respective nectar diet every second day and allowed them to consume nectar until they voluntarily left the feeding station. *C. pamphilus* butterflies did not recognize the artificial feeding station as a natural nectar source, so we placed the butterflies beside the nectar-filled tube and dipped the rolled-out proboscis with the help of a needle into the nectar mimic to initiate feeding. To measure the amount of nectar consumed, we used a 100-µl Hamilton syringe.

Reproductive parameters

Larval host plants for oviposition were offered, but in our experiment *C. pamphilus* females laid their eggs mainly on the cage netting. We recorded the longevity of each butterfly and collected all eggs laid by cutting them out of the cage netting.

Egg duration (number of days from when the egg was laid to when larva hatched), the egg hatching success (number of eggs hatched per female), and larval hatching mass (mg) of progeny were recorded for all eggs collected from each butterfly. Previous studies with other butterfly species showed that egg mass and larval hatching mass are tightly correlated (Karlsson and Wiklund 1984; Nakasuji and Kimura 1984) and larger eggs produce larger hatchlings (Fischer et al. 2002). Thus, the measured larval hatching mass in this study is a good substitute for egg mass. To measure larval hatching mass instead of egg mass was necessary because *C. pamphilus* females frequently lay their eggs on the cage netting, which made weighing eggs without destroying them impossible. Eggs laid on the same day by the same butterfly were placed together in Petri dishes. Freshly hatched larvae were weighed within 24 h (Mettler M3; Mettler Instruments) before they were fed. Furthermore, we characterized relationships between butterfly mass and reproduction, between butterfly age and number of eggs, and between larval hatching success and larval hatching mass.

Statistical analysis

Plant quality parameters (leaf water concentration, leaf nitrogen, and C/N ratio) of fertilized and unfertilized larval food plants were compared using a one-way MANOVA.

The larval quality traits $\log_n$ transformed larval duration, pupal duration and adult emergence mass, the reproductive traits $\log_n$-transformed longevity of female butterflies, the $\log_n$-transformed average amount of nectar mimic consumed by females, total number of eggs laid, hatching success of eggs, and progeny's egg duration and larval hatching mass were analyzed with mixed-effects models. Because related individuals (individuals from the same maternal lineage) are not independent of each other, effects and interactions were tested against the random factor lineage. Larval quality traits were tested against the factors larval food quality (low, high) and sex. The reproductive traits were tested against the factors nectar amino acid diet (AA vs. NAA) and larval food quality (high vs. low), and the covariates female emergence mass and the average amount of nectar consumed per feeding. The average amount of nectar mimic consumed was tested against the factor nectar diet and the covariate female emergence mass. A stepwise model reduction of these models was employed, with the least significant interactions always removed first (Crawley 2007). Tukey–Kramer's HSD comparisons ($P < 0.05$) were performed between the levels of significant factors.

Mixed-effects models with temporal pseudoreplication (repeated measures on the same females) were used to test whether day of oviposition, adult diet, individual, and the interaction between day of oviposition and adult diet affected time patterns of reproductive traits. Number of eggs and larval hatching success of eggs over time were analyzed with generalized linear mixed models with temporal pseudoreplication due to non-normal data structure (Crawley 2007). Furthermore, quadratic regression was used to characterize trends in the time pattern of hatching success of eggs over the oviposition period from females fed with nectar without amino acids, whereas the time pattern of hatching success of eggs from females fed with amino acid-rich nectar was analyzed with linear regression (Crawley 2007). The time pattern of number of eggs laid over the oviposition period of females fed with nectar lacking amino acids was characterized with exponential regressions, whereas the corresponding time pattern of females fed with amino acid-rich nectar was analyzed with quadratic regression (Crawley 2007). Time patterns of progeny's larval hatching mass were analyzed using quadratic regressions (Crawley 2007).

Correlation analysis was used to examine if female emergence mass affected total number of eggs laid positively or negatively. We also used correlation analysis to detect a possible trade-off between total number of eggs laid and progeny's larval hatching mass, as it was not clear whether number of eggs or larval hatching mass is the dependent variable.

To maintain consistency in the investigation of the effect of nectar amino acids on female butterfly reproduction, we used analogous statistical analyses as in the experiment with *A. levana* (Mevi-Schütz and Erhardt 2005). All statistical analyses were calculated with R Statistical Software (v.2.9.1; R Development Core Team 2009). All treatment means are indicated with standard errors.

Table 1 Treatment means for *Coenonympha pamphilus* butterflies

	Low/NAA	Low/AA	High/NAA	High/AA
Total no. eggs laid	84.15 ± 4.71 a	98.64 ± 8.06 ab	132.75 ± 5.73 c	121.15 ± 7.37 bc
Egg duration (day)	7.74 ± 0.06	7.82 ± 0.12	7.77 ± 0.06	7.62 ± 0.08
Hatching success	0.87 ± 0.02	0.85 ± 0.04	0.82 ± 0.05	0.88 ± 0.03
Hatching mass (mg)	0.163 ± 0.005 bc	0.185 ± 0.006 a	0.159 ± 0.004 c	0.178 ± 0.005 ab
Nectar/feeding (µl)	9.35 ± 0.56	9.58 ± 0.72	9.46 ± 0.35	10.01 ± 0.47
Female Longevity (day)	22.77 ± 11.04	24.18 ± 10.34	24.07 ± 11.27	26.46 ± 10.56

Butterflies were raised on a low- or high-quality larval diet and fed a nectar mimic with (*AA*) or without (*NAA*) amino acids (means ± SE)
Different letters indicate significant differences among treatment groups (Tukey–Kramer HSD, $P < 0.05$)

Results

Laval host plant quality

Fertilized *F. rubra* had a significantly higher plant quality (leaf nitrogen, water content, and C/N ratio) than unfertilized larval food plants ($F_{1,26} = 79.88$, $P < 0.001$). As expected, fertilized *F. rubra* had significantly higher leaf nitrogen (low-quality: 2.85 ± 0.08 g N/100 g dry weight; high-quality: 4.16 ± 0.07 g N/g dry weight; $P < 0.001$), a higher water content (low-quality: 0.08 ± 0.01 g H_2O/100 g dry weight; high-quality: 0.12 ± 0.01 g H_2O/100g dry weight; $P = 0.006$), and a lower C/N ratio (low-quality: 16.23 ± 0.53; high-quality: 10.37 ± 0.25; $P < 0.001$) than unfertilized larval food plants.

Effects of larval diet

Larval duration (high-quality: females 24.69 ± 0.54 days; males 22.25 ± 0.31 days; low-quality females 26.48 ± 0.62 days; males 24.47 ± 0.53 days) was significantly affected by larval diet quality ($F_{1,49} = 27.31$, $P < 0.001$), sex ($F_{1,49} = 23.63$, $P < 0.001$), and marginally by lineage ($F_{1,49} = 3.51$, $P = 0.076$). Additionally, there was a marginal interaction between sex and larval diet quality ($F_{1,49} = 3.54$, $P = 0.06$), since in males the difference of larval duration between low- and high-quality larval food treatment groups was more pronounced than in females.

Pupal duration (high-quality: females 7.52 ± 0.12 days; males 8.36 ± 0.74 days; low-quality: females 7.52 ± 0.10 days; males 7.67 ± 0.10 days) was not affected by larval diet quality ($F_{1,50} = 0.28$, $P = 0.60$), by sex ($F_{1,50} = 2.42$, $P = 0.12$), or by lineage ($F_{1,50} = 0.10$, $P = 0.75$).

Emergence mass (high-quality: females 31.55 ± 1.04 mg; males 21.45 ± 0.52 mg; low-quality: females 26.18 ± 0.77 mg; males 18.73 ± 0.91 mg) was significantly affected by larval diet quality ($F_{1,49} = 56.84$, $P < 0.001$) and sex ($F_{1,49} = 157.80$, $P < 0.001$), whereas lineage had no significant effect ($F_{1,49} = 1.60$, $P = 0.22$). Additionally, there was a significant interaction between sex and larval diet quality ($F_{1,49} = 4.25$, $P = 0.042$), since in males the difference of emergence mass between low- and high-quality larval food treatment groups was more pronounced than in females.

Effects of nectar amino acids on female reproduction

The reproductive traits total number of eggs laid, progeny's larval hatching mass, egg duration, hatching success of eggs, female longevity, and the amount of consumed nectar were affected differently by the measured parameters.

The total number of eggs laid by females differed significantly among the four treatment groups ($F_{3,49} = 12.1$, $P < 0.001$; Table 1). Total number of eggs laid was significantly affected by larval food quality, whereas nectar diet, average amount of nectar consumed, emergence mass, and lineage had no significant effects (Table 2). Additionally, there was a significant interaction between larval and adult diet quality (Table 2).

Progeny's larval hatching mass differed significantly among the four treatment groups ($F_{3,49} = 5.71$, $P = 0.003$; Fig. 1; Table 1). Larval food quality, emergence mass and lineage did not affect progeny's mean larval hatching mass, whereas nectar diet had a significant effect, and the average amount of consumed nectar only a marginal effect (Table 2). There was no trade-off between number of eggs and progeny's larval hatching mass in females fed nectar without amino acids ($r = -0.10$, $df = 27$, $P = 0.61$) or in females fed nectar containing amino acids ($r = 0.28$, $df = 22$, $P = 0.18$).

Egg duration ($F_{3,49} = 0.73$, $P = 0.6$; Table 1) and hatching success of eggs ($F_{3,49} = 0.45$, $P = 0.8$; Table 1) did not differ significantly among the four treatment groups and were not influenced by larval food quality, female emergence mass, average amount of nectar mimic consumed, adult nectar diet, or lineage (Table 2).

Female longevity did not differ among the four treatment groups ($F_{3,49} = 0.34$, $P = 0.8$; Table 1) and was not influenced by larval food quality, average amount of nectar mimic consumed, adult nectar diet, or lineage (Table 2). Emergence mass had a marginal effect on longevity (Table 2).

Table 2 Effects of larval food quality, emergence mass, adult nectar diet, daily amount of nectar consumed and lineage on reproduction of *Coenonympha pamphilus* butterflies, showing *F* values, *P* values, and the effect size (*R*) of the ANCOVA

	Number of eggs ($df = 1, 30$)			Egg duration ($df = 1, 31$)			Egg hatching success ($df = 1, 31$)			Larval hatching mass ($df = 1, 31$)			Longevity ($df = 1, 31$)		
	F	*P*	*R*	*F*	*P*	*R*	*F*	*P*	*R*	*F*	*P*	*R*	*F*	*P*	*R*
Nectar quality	<0.01	0.98	0.00	0.19	0.66	0.08	0.42	0.52	0.11	17.11	**<0.001**	0.60	0.28	0.60	0.09
Larval food quality	33.75	**<0.001**	0.73	0.69	0.41	0.15	1.75	0.20	0.23	0.90	0.35	0.17	0.84	0.37	0.16
Emergence mass	1.14	0.29	0.19	2.09	0.16	0.25	3.07	0.09	0.30	0.08	0.78	0.05	1.78	0.19	0.23
Amount of nectar	2.71	0.11	0.29	0.64	0.43	0.14	0.62	0.44	0.14	4.07	0.053	0.34	1.38	0.17	0.21
Lineage	<0.001	1.00	0.00	1.78	0.19	0.23	0.002	0.97	0.01	1.06	0.32	0.25	2.02	0.17	0.33
Larval food quality × nectar quality	4.50	**0.042**	0.36	–	n.s	–	–	n.s.	–	–	n.s.	–	–	n.s.	–

Emergence mass is shown as an expression of larval food quality. Adult nectar diet is shown as AA versus NAA

$R = \sqrt{(F)}\,(\sqrt{(F+df)})^{-1}$, *df* in lineage = 16

Significant values ($P < 0.05$) shown in bold

Table 3 Effects of day of oviposition, adult nectar diet and individual on time patterns of reproductive parameters of *Coenonympha pamphilus* butterflies, showing *Z* or *t* values, *P* values, degrees of freedom (*df*), and the effect size (*R*) of the ANCOVA

	Egg number				Larval hatching mass				Egg hatching success			
	z	*P*	*R*	*df*	*t*	*P*	*R*	*df*	*z*	*P*	*R*	*df*
Day of oviposition	−15.05	**<0.001**	0.43	1,994	21.58	**<0.001**	0.95	1,754	5.78	**<0.001**	0.21	1,754
Individual	3.92	**<0.001**	0.49	1,50	0.41	0.65	0.03	1,50	1.41	0.16	0.20	1,50
Adult diet	−1.97	**0.049**	0.27	1,994	4.32	**<0.001**	0.52	1,754	3.79	**<0.001**	0.47	1,754
Adult diet × day of ovipostion	–	n.s.	–	–	–	n.s.	–	–	−4.32	**<0.001**	0.16	1,754

Adult nectar diet is shown as AA versus NAA

$R = t(\sqrt{(t^2+df)})^{-1}$ or $z(\sqrt{(z^2+df)})^{-1}$

Significant values ($P < 0.05$) shown in bold; *n.s.* not significant

Adult treatment groups fed with the nectar mimic containing amino acids (high/AA and low/AA) did not consume more nectar than those fed with the nectar mimic without amino acids (high/NAA vs. low/NAA; $F_{3,49} = 0.4$, $P = 0.8$; Table 1). Larval food quality ($F_{1,32} = 0.47$, $P = 0.50$), female emergence mass ($F_{1,49} < 0.01$, $P = 0.95$), nectar diet quality ($F_{1,32} = 0.55$, $P = 0.46$), and lineage ($F_{1,49} = 0.78$, $P = 0.39$) had no significant effects on nectar consumption.

Female emergence mass was positively correlated with the number of eggs laid ($r = 0.40$, $n = 53$, $P = 0.003$), since heavier females laid more eggs.

Time effects over oviposition period

The reproductive traits number of eggs laid, progeny's larval hatching mass, and hatching success of eggs were affected differently by the measured parameters over the female oviposition period. Furthermore, regression analyses revealed how time patterns changed over the female oviposition period:

Generalized linear mixed model analysis revealed that day of oviposition, individual, and adult diet had a significant effect on the time pattern of number of eggs laid (Table 3). Furthermore, exponential regression analysis showed that the number of eggs laid of females that received no amino acids as adults decreased over the female oviposition period (high/NAA and low/NAA; $R^2 = 0.79$, $n = 29$, $P < 0.001$; Fig. 2). Quadratic regression analysis also showed that the number of eggs laid of the females fed amino acid-rich nectar decreased over the female oviposition period (high/AA and low/AA; $R^2 = 0.85$, $n = 30$, $P < 0.001$).

Generalized linear mixed model analysis showed significant effects of adult diet and day of oviposition on time patterns of progeny's larval hatching mass (Table 3). Quadratic regression analyses revealed that progeny's larval hatching mass decreased over the oviposition period in both adult feeding groups (high/AA and low/AA: $R^2 = 0.82$, $n = 28$, $P < 0.001$; high/NAA and low/NAA: $R^2 = 0.85$, $n = 29$, $P < 0.001$).

Day of oviposition, adult nectar diet, and the interaction between day and adult diet significantly affected the time

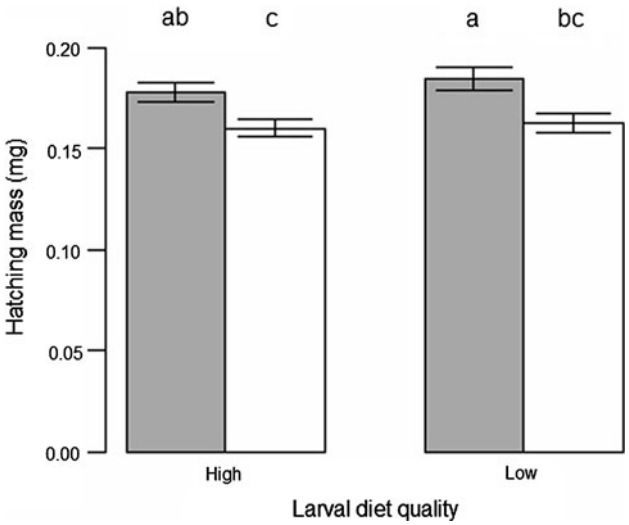

Fig. 1 Hatching mass of larvae laid by *Coenonympha pamphilus* butterflies raised on low-quality (*low*) or high-quality (*high*) *Festuca rubra* and fed nectar mimics with amino acids (*AA*; *dark bars*) or without amino acids (*NAA*; *bright bars*). Means ± SE. Different *letters* indicate significant differences for larval hatching mass (high/NAA-high/AA: $P = 0.047$; low/AA-high/AA: $P = 0.8$; low/NAA-high/AA: $P = 0.2$; low/AA-high/NAA: $P = 0.005$; low/NAA-high/NAA: $P = 0.1$; low/NAA-low/AA: $P = 0.03$)

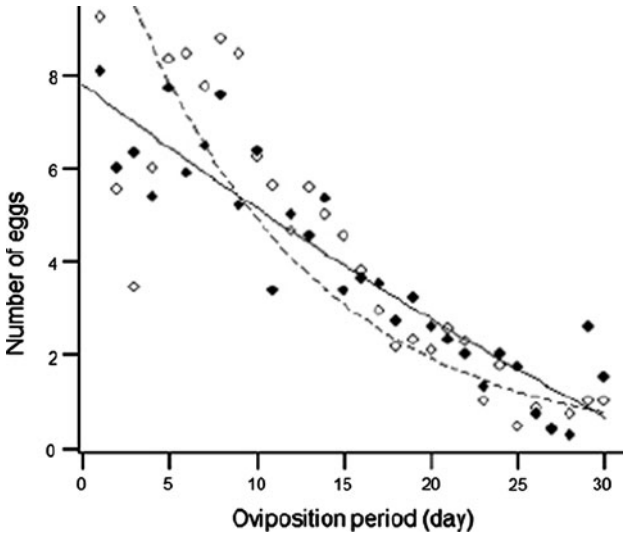

Fig. 2 Regression analyses show that the number of eggs laid from *Coenonympha pamphilus* females fed with amino acid-rich nectar (*filled dots, solid lines*; $R^2 = 0.85$, $n = 30$, $P < 0.001$) versus females receiving no amino acids (*empty dots*, *dashed lines*; $R^2 = 0.79$, $n = 29$, $P < 0.001$) both decrease over the oviposition period. There was no difference between high- and low-quality larval food groups

pattern of hatching success of eggs, whereas individual had no significant effect (Table 3). Quadratic regression analysis showed that hatching success of eggs increased over the oviposition period for AA females ($R^2 = 0.74$, $n = 24$, $P < 0.001$, Fig. 3). For NAA females, linear regression

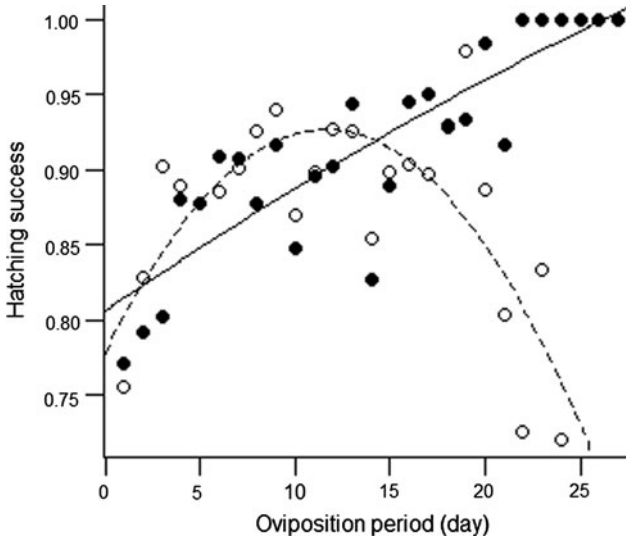

Fig. 3 Regression analyses show that hatching success of eggs from *Coenonympha pamphilus* females fed with amino acid-rich nectar (*filled dots, solid lines*; $R^2 = 0.74$, $n = 24$, $P < 0.001$) increased significantly, whereas females receiving no amino acids (*empty dots*, *dashed lines*; $R^2 = 0.57$, $n = 27$, $P < 0.001$) showed first an increase and afterwards a decrease. There was no difference between high- and low-quality larval food groups

analysis revealed that hatching success of eggs increased up to day 12 and decreased afterwards ($R^2 = 0.57$, $n = 27$, $P < 0.001$; Fig. 3).

Discussion

Effects of nectar amino acids on butterfly reproduction

This study clearly shows that floral nectar amino acids can influence reproduction in female *Coenonympha pamphilus* butterflies. Similar to the fruit-feeding *B. anynana* females (Bauerfeind and Fischer 2009), *C. pamphilus* females receiving amino acids in their adult diet produced significantly heavier offspring (Fig. 1; Tables 1, 2). Several previous studies with other butterfly species showed benefits of increased egg and larval size (Murphy et al. 1983; Braby 1994; Fischer et al. 2003; Seko and Nakasuji 2004; Fischer et al. 2006; but see Wiklund and Persson 1983; Karlsson and Wiklund 1984; Wiklund and Karlsson 1984). Furthermore, *C. pamphilus* lay their eggs not only on benign larval food plants but also on dead plant material, from which freshly hatched larvae must find new host plants (Wiklund 1984). In general, larger hatchling larvae can travel longer distances, thereby increasing the likelihood to find fresh larval food plants, as stated by Murphy et al. (1983). However, further work is required to determine the advantage of heavier larval masses in *C. pamphilus*. Furthermore, a disadvantage regarding sibling competition was found for

species of Hesperioidea with bigger eggs and longer egg duration (Garcia-Barros 2000). However, this was not apparent in our study, since egg duration was the same for all treatment groups (Table 1).

In addition, we did not observe a trade-off between progeny's larval hatching mass (as a substitute for egg mass) and number of eggs in our study, an analogous result as in *A. levana* (Mevi-Schütz and Erhardt 2005). Furthermore, females raised on high-quality larval food plants laid similar numbers of eggs in the present study as did *C. pamphilus* females in another experiment (Karlsson and Wiklund 2005).

Nitrogen-rich larval food significantly affected emergence mass and the number of eggs laid (Tables 1, 2). Furthermore, emergence mass and the number of eggs laid were positively correlated. The achieved adult mass of females raised under variable larval food conditions also correlated strongly with their reproductive success in other butterfly species (Mevi-Schütz and Erhardt 2005; Bauerfeind and Fischer 2009). Low/AA females used nectar amino acids primarily to increase larval hatching mass and egg hatching success over time (Tables 1, 3; Figs. 1, 3), but obviously also invested additional nitrogen from adult feeding to slightly increase the number of eggs, as indicated by the significant interaction between larval and adult diet quality on egg number.

The findings of the present study support the idea that reproduction can be increased by amino acids obtained from adult diet, and this may even be a general feature in nectar feeding butterflies. It also demonstrates that allocation patterns of amino acids acquired from adult diet differ between different butterfly subfamilies, possibly reflecting different life history traits and strategies.

Time effects over oviposition period

In our study, the number of eggs laid over the female oviposition period decreased in all four treatment groups. Females fed with nectar lacking amino acids tended to lay more eggs early in their life, whereas females fed amino acid-rich nectar tended to extended the number of eggs over the whole oviposition period (Fig. 2), possibly relying on the better adult food quality (Boggs 1997b). A corresponding result has been found in the fruit-feeding *B. anynana* (Bauerfeind and Fischer 2009).

The progeny's hatching mass of larvae decreased steadily with increasing female age in the present study. This result is in accordance with the decreasing egg mass produced by *C. pamphilus* females towards the end of their life (Wickman and Karlsson 1987) and the decreasing egg mass with increasing age of several other butterfly species (Wiklund and Persson 1983; Karlsson and Wiklund 1984; Mevi-Schütz and Erhardt 2003a, 2005; Bauerfeind and Fischer 2009; but see Bauerfeind and Fischer 2007).

However, patterns of hatching success of eggs differed considerably between females fed with or without amino acid-rich nectar (Fig. 3). Hatching success of eggs steadily increased with female age for butterflies fed with amino acid-rich nectar, whereas females that received no amino acids as adults reached a peak hatching success of eggs before it decreased again. Similarly, differences in hatching success of eggs were more pronounced in later phases of the oviposition period in differently fed females of the fruit-feeding butterfly *B. anynana* (Geister et al. 2008). Nutrient types that are scarce or non-existent in adult food indeed seem to limit reproduction, causing it to decline to zero as juvenile stores are depleted (Boggs 1997b). For instance, nitrogen-rich pollen in the adult diet significantly prolonged lifetime fecundity and oviposition rate in female *Heliconius charitonia* butterflies (Dunlap-Pianka et al. 1977). However, the differing time patterns of the hatching success over a female's oviposition period were not reflected in the quantitative analysis between treatment groups in *C. pamphilus* (Tables 1, 2). This seemingly contradictory finding could be caused by the fact that the potential to realize positive effects of nectar amino acids on female fecundity is truncated by the relatively short life span of *C. pamphilus*. This in turn may represent a life history strategy adapted to temperate, nutrient-poor habitats. It is, however, remarkable that *C. pamphilus* females show a resource allocation pattern which is more pronounced and more successful in longer lived butterflies living under more benign environmental conditions, such as *Charaxes fulvescens* (Molleman et al. 2008) or the tropical *Heliconius* butterflies (Dunlap-Pianka et al. 1977).

Regarding allocation patterns of larval versus adult nutrients for reproduction, *C. pamphilus* is a nectar-feeding species with some mature eggs at emergence and therefore an intermediate type between 'capital' and 'income' breeder (Boggs 1997a, b; Casas et al. 2005). Our study reveals a remarkably differentiated resource use strategy of *C. pamphilus.* Egg number depended mainly on larval resources (Table 2), but the significant effect of nectar diet quality on the number of eggs laid over female oviposition period (Table 3), and the significant interaction between adult and larval diet on the total number of eggs laid, also reveal slight effects of nectar amino acids. In contrast, only adequate adult resources (i. e. amino acid-rich nectar) increased progeny's larval hatching mass (Tables 1, 2; Fig. 1) and could compensate for a decline in hatching success of eggs in *C. pamphilus* (Fig. 3). These results support the findings of Casas et al. (2005) in the ectoparasitoid *Eupelmus vuilletti*, indicating that a strict classification of breeding types, based on resource allocation of larval reserves versus adult incoming nutrient use, may be difficult, as different reproduction parameters and nutrient use are highly variable within a single species.

Conclusions

This study shows that the uptake of amino acids in floral nectar can enhance reproduction in female *C. pamphilus* butterflies. However, resource allocation of amino acids from adult diet in combination with nitrogen gained from the larval phase may affect female reproduction in different butterfly species in different ways. Thus, further work is required to clarify if and how amino acids gained from floral nectar and adult diet in general affect butterfly reproduction. Nevertheless, the results of the present study support previous findings suggesting a coevolutionary process between butterflies and flowers dependent on butterfly pollination.

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