Mating frequency influences nectar amino acid preference of *Pieris napi*

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It is generally assumed that butterflies, as is the case with many holometabolous insects, rely primarily on nutrients gathered by larval feeding for somatic maintenance and fecundity. These reserves can be supplemented by adult feeding and in some cases by nuptial gifts passed from the males to the females during mating. Recent findings indicate that female butterflies detect and prefer nectar with high levels of amino acids, thus calling new attention to this nutritive source. Polyandrous species can further supplement their larval stores with additional nuptial gifts. This study examined how mating frequency of the polyandrous butterfly *Pieris napi* affects the female's preference for nectar amino acids. Females of this species generally detect and prefer nectar mimics containing amino acids. However, nectar amino acid preference is significantly lower in mated females. Furthermore, nectar amino acid preference increases when females are not allowed to remate, whereas the preference of twice-mated females remains constant at a lower level. These results indicate a versatile response of females to nectar amino acids, depending on their nutritional status; they may even switch their source of amino acids between adult feeding and nuptial gifts.

Keywords: fecundity; nectar amino acids; nuptial gifts; polyandry

1. INTRODUCTION

Reproductive output in butterflies depends on nutrients gathered by larval feeding, adult uptake and, in some cases, 'nuptial gifts' received by the female from the male at mating (Boggs 1995). Resources acquired and stored during the larval stage are generally believed to be the primary nutrients used for reproduction. However, this rather narrow view neglects the importance of adult-derived nutrients. Adult diet can supplement larval reserves and, to date, nectar sugars (Stern & Smith 1960; O'Brien *et al.* 2000), pollen (Gilbert 1972; Dunlap-Pianka *et al.* 1977), rotting fruit (Braby & Jones 1995) and mud puddles (Pivnick & McNeil 1987) have been shown to increase reproductive output. In addition, nuptial gifts are known to increase female fecundity and longevity significantly in some species (Rutowski *et al.* 1987; Karlsson 1998) and multiple matings can further enhance female reproductive success (Wiklund *et al.* 1993; Boggs 1995; Stjernholm & Karlsson 2000). The use and importance of these three sources for reproductive success are complex; an understanding of the allocation and possible interactions of these three nutritional sources gives further insight into factors affecting fecundity.

Larval and adult diets are nutritionally distinct, hence adult butterflies use other nitrogen sources (i.e. nectar, puddles, carrion) and when necessary compensate for inadequate larval reserves. The use of nectar as an alternative and beneficial source of nitrogen is controversial. Butterfly-visited flowers have higher concentrations of nectar amino acids than do flowers visited by other pollinators (Baker & Baker 1973, 1975) and female butterflies prefer nectar sources containing amino acids in the

majority of species tested (Alm *et al.* 1990; Erhardt & Rusterholz 1998; Rühle 2000; J. Mevi-Schütz, personal observation). Poor larval food quality increases (Mevi-Schütz & Erhardt 2003) whereas fertilization of the larval food plant decreases nectar amino acid preference (Mevi-Schütz *et al.* 2003). Furthermore, ageing butterflies lose their nectar amino acid preference in conjunction with decreased oviposition rates (J. Mevi-Schütz and A. Erhardt, unpublished data). All of these results implicate a link between nectar amino acids and butterfly fitness, and suggest that butterflies benefit from nectar amino acids. However, research on nectar amino acids has shown little or no effect on reproductive output or longevity in butterflies (Murphy *et al.* 1983; Moore & Singer 1987; Hill 1989; Hill & Pierce 1989, O'Brien *et al.* 2000).

Spermatophores contain, aside from sperm and hormones, nutrients such as amino acids, protein, lipids, hydrocarbons, salts and water (Marshall 1982; Bissoondath & Wiklund 1995). Radiotracer studies on several species have shown that some of these nutrients are incorporated into female soma and eggs (Boggs & Gilbert 1979). The amount of nutrients that females receive from males depends on several factors: the mating system (monandry or polyandry) and the mating status of the male (virgin or previously mated), coupled with the degree to which females absorb these nutrients (Boggs 1981; Svard & Wiklund 1989; Kaitala & Wiklund 1994; Oberhauser 1997). In the polyandrous green-veined white butterfly *Pieris napi*, ejaculates average 15% of the male body mass (Forsberg & Wiklund 1989) and virgin males transfer nitrogen equivalent to that in ca. 70 eggs (Karlsson 1998). Multiply mated females are known to have a higher lifetime fecundity, lay larger eggs, use more of their stored resources and live longer than once-mated females (Wiklund *et al.* 1993; Karlsson 1998). Furthermore, these females actively forage for matings to acquire additional

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nutrients (Kaitala & Wiklund 1994), because *P. napi* females receive enough sperm from the first mating to fertilize their egg load (Wiklund *et al.* 1993). Apparently, *P. napi* females greatly benefit from male-derived nutrients, so we expect a reduced reliance on adult uptake, in particular a reduction in nectar amino acid preference, with increased mating frequency.

In this study, the effect of mating on the nectar amino acid preference of female *P. napi* was tested to determine whether mating frequency and the male-derived nutrients influence the butterflies' preference.

2. MATERIAL AND METHODS

(a) *Study organism*

Pieris napi L. 1758, the green-veined white butterfly, is a common butterfly found in pristine meadows, urban areas and intensively used agricultural land. This species is polyvoltine, overwinters as pupa and is known to be polyandrous (Ebert & Rennwald 1991). The primary larval host is *Cardamine pratensis* L., but a wide range of other Brassicaceae, including cultural cabbage forms, are used. The adults forage for nectar on a variety of flowers including *C. pratensis*, *Lythrum salicaria* and *Geranium robertianum* (Ebert & Rennwald 1991).

Pieris napi females were collected in July 2000 from four different field sites (three on the Dinkelberg, Germany and one by Möhlin, Switzerland). The butterflies were placed in cages (40 cm × 20 cm × 20 cm) containing pots of juvenile cabbage (*Brassica capitata* L. f. *alba*) for oviposition, and fed a balanced 20% sugar solution (sucrose : fructose : glucose, 1 : 1 : 1) in a greenhouse at the Botanical Institute of the University of Basel. The greenhouse was maintained at a day temperature of 26 °C and a night temperature of 16 °C and artificial lighting was set at a 14 L : 10 D cycle throughout the experiment. The eggs and hatching larvae remained on the juvenile plants until the L2 stage, at which time the larvae were transferred to individual plastic rearing cages and provided with fresh cabbage leaves *ad libitum*. Upon emergence the butterflies were weighed, sexed and transferred to individual flight cages (40 cm × 20 cm × 20 cm).

(b) *Mating treatment and nectar amino acid preference*

Females ($n = 50$) were randomly assigned to a mating regime: unmated ($n = 16$); once-mated ($n = 18$); or twice-mated ($n = 16$). On day 1, a virgin male was placed in each cage containing a female of the once- or twice-mated groups. The butterflies were allowed to mate once and then the males were removed. *Pieris napi* generally remates after 3–5 days (Wiklund *et al.* 1993), so on day 5, a virgin male was placed in each cage of the twice-mated group and removed after mating. To avoid effects of cage location, cages were rotated on a daily basis.

A feeding station, consisting of two artificial purple silk flowers attached to the open ends of 240 µl glass culture tubes protruding out of green florist moss, was placed in each cage. In preliminary trials the butterflies were found to prefer these purple artificial flowers to other offered colours and paper flowers. The culture tubes were filled with artificial nectar mimicking *Lantana camara* nectar, chosen because this flower is often visited by butterflies, has a high amino acid concentration and has been used in several previous studies of amino acid preference. Each feeding station offered one flower with a nectar mimic containing amino acids (termed AA) and one flower with a nectar mimic without amino acids (termed NAA). Both nectar mimics

contained 0.547 M of sucrose, 0.282 M of glucose and 0.316 M of fructose per litre of distilled water. The nectar mimic AA contained additionally non-essential amino acids (0.718 mM of alanine, 0.421 mM of asparagine, 0.326 mM of glutamic acid, 0.931 mM of glutamine, 2.371 mM of glycine, 2.23 mM of proline and 1.37 mM of serine) and essential amino acids (0.201 mM of arginine, 0.672 mM of threonine, 0.221 mM of tyrosine and 0.137 mM of valine). The test solutions were made with sodium-free substances.

The feeding station was removed each day and the volume of nectar mimic consumed was determined by refilling the culture tubes with the corresponding nectar (AA or NAA) mimic using a Hamilton syringe (100 µl, gas tight, no. 710). The degree of preference was based on the amount of nectar mimic containing amino acids consumed in relation to the total amount consumed from both artificial flowers. The butterflies preferred the nectar mimic with amino acids, accepted both mimics equally (indifferent response) or preferred the nectar mimic without amino acids. Evaporation rates of control feeding stations showed that evaporative losses did not differ between the two nectar mimics ($p = \text{n.s.}$; nectar mimic with amino acids ($\pm \text{s.d.}$): 11.6 ± 0.09 µl; without amino acids: 12.0 ± 0.05 µl). The glass culture tubes were replaced daily and the feeding stations were randomly located in the cages to prevent position effects.

Each day fresh *B. capitata* leaves were provided for oviposition, the eggs were collected and the hatching success was documented. Any female that failed to lay viable eggs (for the mated groups), failed to mate a second time (for the twice-mated group) or died prior to day 5 was excluded from the analysis. Longevity was recorded for each female. In total 34 females fulfilled the criteria: ten unmated, 15 once-mated and nine twice-mated.

(c) *Statistical analysis*

Analysis of nectar amino acid preference was carried out using the data from days 1–10 because less than half of the butterflies remained after this date. Emergence weight did not differ significantly between unmated, once-mated and twice-mated females ($F_{2,31} = 1.763$, $p = 0.1883$, $n = 34$) so that the potential effect of female emergence weight on nectar amino acid preference was not taken into account. A χ^2 -test was used to analyse overall preference for the nectar mimic with amino acids.

(d) *Analyses between mating groups*

Data from the once-mated and twice-mated groups were combined during the first 5 days of the experiment, because at this time both groups had been allowed to mate only once and analysis with a Tukey–Kramer test showed no significant difference between these two groups ($p > 0.05$). The effects of mating treatment (days 1–5, unmated versus once-mated; days 6–10, unmated versus once-mated versus twice-mated) and experimental day on the nectar amino acid preference were tested with ANOVA. Data on nectar amino acid preference were arcsine transformed and distribution of residuals was normal. The average longevities in the different mating treatments were compared using ANOVA after the data were log transformed (to improve normality). Differences between the treatments were determined using Tukey–Kramer *post hoc* comparisons ($p < 0.05$).

To examine changes in nectar amino acid preference over the duration of the experiment (days 1–10) an ANCOVA with mating treatment and experimental day (and their interaction) as factors and individual butterfly as a category variable was used. A jackknife analysis (excluding each individual once) was carried

out to test the influence of individual butterfly (this takes the individual longevity into account) on the significant outcome of mating treatment.

(c) Analyses within mating groups

To compare the nectar amino acid preference between days 1–5 and days 6–10 within each mating-treatment group, nectar amino acid preferences for each butterfly were pooled for day 1 through to day 5 and for day 6 through to day 10. These data were used in Student's *t*-tests. Regression analyses using the amino acid preference data for each day were performed to examine changes in nectar amino acid preference within the 5 day periods for each group. Means are presented $\pm$ s.e. All statistical analyses were performed using JMP v. 3.1 (SAS 1995).

3. RESULTS

(a) Overall nectar amino acid preference

Pieris napi females preferred the *L. camara* nectar mimic containing amino acids over the nectar without amino acids ($\chi^2 = 22.7$, $n = 34$, $p < 0.01$). Twenty-six females showed a clear preference, whereas 8 individuals were indifferent.

(b) Nectar amino acid preference between mating treatments

Nectar amino acid preference differed significantly between the unmated and the once-mated females ($F_{1,29} = 30.47$, $p = 0.0053$) from day 1 through to day 5 (figure 1a), but experimental day had no effect ($F_{4,29} = 2.28$, $p = 0.222$). Mating treatment had a marginally significant effect on nectar amino acid preference ($F_{2,28} = 4.33$, $p = 0.041$) from day 6 through to day 10 and experimental day did not affect preference ($F_{4,28} = 3.05$, $p = 0.109$). Comparisons of nectar amino acid preference for all pairs using Tukey–Kramer honestly significant difference tests showed a significant difference between unmated and twice-mated females ($p < 0.05$), but no significant difference was found between unmated and once-mated, or between once-mated and twice-mated, butterflies (figure 1b).

Both mating treatment ($F_{2,31} = 4.95$, $p = 0.0078$) and experimental day ($F_{1,31} = 6.28$, $p = 0.0128$) affected nectar amino acid preference over the duration of the entire experiment (days 1–10), but the interaction was not significant ($F_{2,31} = 1.40$, $p = 0.248$). The jackknife statistic confirmed that the observed changes in nectar amino acid preference were determined by the whole dataset and not by individual females ($F_{\text{mean}} = 4.85 \pm 0.09$, $t_s = 3.611$ d.f. = 2, $p < 0.001$), so that individual variation and individual longevity did not affect the outcome.

(c) Difference in nectar amino acid preference within mating treatment

Unmated females maintained the same preference for nectar amino acids throughout the experiment: preference did not differ between days 1–5 (0.52 ± 0.005) and days 6–10 (0.51 ± 0.006 ; $t = 1.324$, $p = 0.2041$, $n = 10$). However, regression analysis showed that preference increased during the first 5 days ($r^2 = 0.16$, $p = 0.0041$) and levelled off after day 6 ($r^2 = 0.053$, $p = 0.291$). Once-mated females increased their preference for nectar amino acids: preference was significantly higher for days 6–10

Figure 1. (a) Nectar amino acid preference from day 1 to day 5 of unmated ($n = 10$) and once-mated ($n = 24$, see § 2b) *Pieris napi* females. (b) Nectar amino acid preference from day 6 to day 10 of unmated ($n = 10$), once-mated ($n = 15$) and twice-mated ($n = 9$) *P. napi* females. Error bars indicate standard errors. Different letters indicate significant differences between mating treatments (Tukey–Kramer honestly significant difference tests, $p < 0.05$).

(0.51 ± 0.004 ; $t = 2.925$, $p = 0.0072$, $n = 15$) than for days 1–5 (0.49 ± 0.003). However, there was no increase in preference during the first 5 days ($r^2 = 0.005$, $p = 0.553$) or during day 6 through to day 10 ($r^2 = 0.05$, $p = 0.109$). Twice-mated females showed no change in nectar amino acid preference between days 1–5 (0.506 ± 0.008) and days 6–10 (0.508 ± 0.008 ; $t = 0.168$, $p = 0.869$, $n = 9$). Preference was fairly constant during days 1–5 ($r^2 = 0.012$, $p = 0.472$) and days 6–10 ($r^2 = 0.004$, $p = 0.688$). Figure 2 summarizes the relative change in nectar amino acid preference in each of the mating groups over the duration of the experiment.

(d) Longevity

Mating treatment significantly affected longevity ($F_{2,31} = 8.089$, $p = 0.0015$). The longevity of unmated (8.5 ± 0.8 days) and once-mated (9.7 ± 0.6 days) groups did not differ, but these two groups differed significantly from the twice-mated (12.9 ± 0.8 days) females (figure 3).

Figure 2. Within-treatment-group comparison of nectar amino acid preference over time for: (a) unmated; (b) once-mated; and (c) twice-mated *Pieris napi* females. Preference increased significantly after day 5 in the once-mated group, whereas no significant difference in preference was found in the other two groups (see data in § 3c). Arrows show the time of mating. Preference for nectar with amino acids is shown above the 0.5 level. Error bars indicate standard errors.

4. DISCUSSION

(a) *Mating frequency and nectar amino acid preference*

Mating and mating frequency clearly affected the nectar amino acid preference of *P. napi* females. The nectar

Figure 3. Longevity of the *Pieris napi* females is influenced by the number of matings. Error bars indicate standard errors. Different letters indicate significant differences between mating treatments (Tukey–Kramer honestly significant difference tests, $p < 0.05$).

amino acid preference during the first 5 days was highest in unmated females, which had not received a nutritious spermatophore. By contrast, once-mated females showed only a slight preference for nectar amino acids during the initial 5 days. During mating males transfer substances that are incorporated into female soma and reproductive tissues and the quantity of material passed by the males to the females during mating influences reproductive output in some species (Rutowski *et al.* 1987). When females fail to receive a spermatophore, mature eggs can be reabsorbed and used for somatic maintenance (Watanabe 1988). Females may, in the absence of a spermatophore, rely more on adult uptake. The difference in nectar amino acid preference between unmated and mated females during the first 5 days of this experiment suggests that butterflies attempt to compensate for lack of nutrients by selectively choosing nectar rich in amino acids. The preference difference between the mating groups appears rather slight, but our experimental conditions (same flower colour and shape for both nectar mimics) excluded a learning effect, so that the response was conservative. Under natural conditions, floral variation (and differences in nectar amino acid concentration) could result in a more pronounced effect. A rough estimate of the actual importance of nectar amino acids in egg provisioning can be made using egg nitrogen information from Karlsson's (1998) work on the same species. He found that one egg contains 0.003 mg of nitrogen and a spermatophore from a virgin male contains nitrogen equivalent to 71 eggs. Our butterflies consumed on average 0.0119 mg of nitrogen in the nectar mimic daily, which represents *ca.* four eggs per day and, if the butterflies live an average of 10 days, could be equivalent to 40 eggs. Recent work on larval and adult dietary resources suggests that lepidopterans use different sources of essential and non-essential amino acids for egg provisioning (O'Brien *et al.* 2002). The nectar mimic of *L. camara* used in our study contains primarily non-essential

amino acids (see § 2b). However, if the egg amino acids are drawn from a pool corresponding to the free amino acids in haemolymph (O'Brien *et al.* 2002), then the allocation of non-essential amino acids taken directly from nectar versus the synthesis of non-essential amino acids from adult dietary sugars would be comparable.

In the second half of the experiment (days 6–10), the nectar amino acid preference of unmated females remained high. The once-mated females, presumably having used up their spermatophoral reserves, showed an increased preference for nectar amino acids. By contrast, the twice-mated females, which were able to replenish their nutritive stores via a second spermatophore, showed a constant level of preference for the nectar amino acids. Polyandrous *P. napi* females have been shown to have a higher lifetime fecundity, maintain egg weight at a higher level and live longer than once-mated females (Wiklund *et al.* 1993; Kaitala & Wiklund 1994). Males of polyandrous species have been shown to produce larger and protein-rich spermatophores than males of monandrous species, with *P. napi* investing the most per body weight (Bischoodath & Wiklund 1995). Furthermore, *P. napi* females appear to need at least two full-sized spermatophores to realize their potential fecundity (Karlsson & Wickman 1989). The transfer of a second nutritious nuptial gift in the twice-mated group obviously curtailed the need for alternative nitrogen sources and therefore the nectar amino acid preference did not change.

(b) Alterations of nectar amino acid preference

Nectar amino acid preference increased significantly in the once-mated females from day 5 (figure 2). This corresponds to studies that show that female *P. napi* mated to virgin males actively forage for rematings 4–5 days after their initial mating (Kaitala & Wiklund 1994). When no male is available, females are forced to seek alternative food sources, in this case nectar amino acids. Furthermore, females from species that mate less frequently are more likely to visit mud puddles than females from species that multiply mate (Sculley & Boggs 1996), indicating that monandrous females need to replenish stores. Boggs (1990) introduced a model that describes the material going into egg production as a combination of larval reserves, adult diet and male-derived nutrients. The butterflies in the present study emerged with similar weights from controlled larval conditions, so that larval reserves were the same. Although it is generally accepted that amino acids are primarily derived from the larval diet, and the benefits from adult diet and nuptial gifts are limited, these sources still could affect fecundity. Variations in mating frequency, in this case unmated versus once-mated versus twice-mated, obviously affect adult uptake by altering nectar amino acid preference. Even though little evidence has linked nectar amino acid uptake to improved fecundity (Murphy *et al.* 1983; Moore & Singer 1987; Hill 1989; Hill & Pierce 1989; O'Brien *et al.* 2000), we may expect a heavier reliance on adult uptake and the use of nectar amino acids under poor larval conditions or lower mating frequency.

Variations in the quality of the nuptial gifts may also affect nectar amino acid preference. Apparently, *P. napi* females are unable to assess the quality of the spermatophore (virgin male versus previously mated male) prior to

mating (Wiklund *et al.* 1993). However, females delay remating for longer after receiving a large spermatophore than after receiving a small one (Oberhauser 1989; Wiklund & Kaitala 1993). The reliance of females on alternative amino acid sources (nectar, puddles, etc.) may increase when small spermatophores are passed at mating. Recent work has shown that male *P. napi* transfer an anti-aphrodisiac to the female at mating, and this curtails remating until the first spermatophore is digested (Forsberg & Wiklund 1989; Andersson *et al.* 2000). However, very little is known about mechanisms that allow females to assess their nutritional level. The stretch receptors in the bursa copulatrix can assess the mechanical load (Sugawara 1979), but it is not presently clear whether this mechanical stimulus affects nectar amino acid preference nor how nutrient-deficient females learn to forage actively for amino acid rich nectar.

(c) Longevity

Male-donated material has been shown to increase longevity in several species (Wiklund *et al.* 1993; Boggs 1995; Karlsson 1998; Kawagoe *et al.* 2001). Our study supports these previous findings. The twice-mated females lived longer than the once-mated individuals, which outlived the unmated females. Longevity is perhaps the most important aspect in butterfly fitness: longer life means higher fecundity (Leather 1988). Further studies of the effect of nectar amino acid uptake on longevity under varying mating frequencies may improve our understanding of compensatory feeding.

(d) Conclusion

The preference for and probable use of nectar amino acids by the female butterfly to compensate for insufficient spermatophoral input (either too few or too small) and/or inadequate larval reserves demonstrates the nutritional plasticity available to holometabolous insects. This change in preference and uptake of nectar amino acids may facilitate fecundity when nutritional reserves and nuptial gifts are lacking. Additional studies are needed to test the actual fecundity of once-mated females fed on an amino acid rich diet versus multiply mated females.

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