Reproducing butterflies do not increase intake of antioxidants when they could benefit from them

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The significance of dietary antioxidants may be limited by the ability of animals to exploit them. However, past studies have focused on the effects of dietary antioxidants after ‘antioxidant forced-feeding’, and have overlooked spontaneous antioxidant intake. Here, we found that reproducing female Bicyclus anynana butterflies had higher antioxidant defences and enhanced fecundity when forced to consume antioxidants (polyphenols). Interestingly, these positive effects were not constant across the oviposition period. When given the choice between food resources with and without antioxidants, reproducing butterflies did not target antioxidants when they could have benefited the most from them. Moreover, they did not consume more antioxidants than non-reproducing butterflies. These results emphasize that, despite potential positive effects of dietary antioxidants, the ability of animals to exploit them is likely to restrict their ecological significance.

1. Introduction

Because dietary antioxidants can affect important life-history traits, such as fertility or ageing, individuals able to optimize antioxidant intake to maximize fitness should present an adaptive advantage. Examining how animals select food in relation to its antioxidant content is therefore likely to provide important insights into the actual significance of dietary antioxidants [1].

Animals may consume antioxidant resources if they (i) can find them in their habitat, (ii) recognize them as food resources, and (iii) are morphologically and physiologically equipped to consume them. If the above prerequisites hold true, animals are expected to exploit antioxidant resources when their need for antioxidants increases, and if the associated benefits surpass potential costs. For instance, consuming antioxidant resources may prevent animals from consuming energy resources, and may also result in pro-oxidant effects under particular conditions [1].

In mammals, oxidative damage increases across gestation, suggesting that reproduction progressively hinders an appropriate endogenous antioxidant response by females [2]. Similarly, egg-laying reduces antioxidant defences in female insects [3]. Reproducing females would therefore be expected to increase antioxidant intake during periods of high reproductive activity to enhance antioxidant defences. However, studies examining antioxidant intake across reproduction are currently lacking [4].

In female butterflies, reproductive investment changes over time, with oviposition typically peaking just a few days after mating [5]. This peak is thus expected to be associated with highest need for antioxidants. Because, in the wild, butterflies feed on nectar or fruit containing variable antioxidant concentrations, they have the opportunity to increase antioxidant intake when needed.

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Accordingly, virgin *Bicyclus anynana* butterflies strongly increase antioxidant intake under hot conditions [6]. However, whether butterflies regulate antioxidant intake when reproducing is unknown.

Here, we followed a three-step approach to assess the importance of dietary antioxidants (polyphenols) for female *B. anynana* across the oviposition period: we (i) assessed the effects of polyphenol supplementation on fitness components, (ii) assessed its effects on oxidative status, and (iii) examined whether butterflies targeted polyphenols when they could benefit the most from them.

2. Methods

(a) Animals

We used 389 2-day-old females in three experiments: (i) 121 to examine fitness components, (ii) 172 to measure oxidative status, and (iii) 96 to monitor feeding behaviour. For fitness components, all females were necessarily mated, whereas for oxidative status and feeding behaviour, only half of them were mated (virgin females served as controls). After copulation, females were randomly assigned to a given experimental treatment. The study was conducted at 27°C, 70% humidity with a photoperiod of L12 : D12.

(b) Fitness components

Mated females were placed individually into 1 l containers with a maize leaf to stimulate egg-laying. They each received a 20 ml glass vial containing cotton soaked with a sugar or a sugar–polyphenol solution, both solutions containing 10% sugar. We used *Aronia melanocarpa* extract (Artemis International, Fort Wayne, IN) as the source of polyphenols in the sugar–polyphenol solution, which contained 0.5% of this extract. We used this ‘polyphenol cocktail’ [7] to better reflect the antioxidant content of fruit that butterflies may naturally consume. Because of the large number of females to monitor simultaneously in our study, only mortality was monitored daily. Eggs were counted on days 1, 3, 5, 7, 9 and 10 after mating, as most eggs are laid during this period [5].

(c) Oxidative status

Mated and virgin females were placed individually into 1 l containers (with a maize leaf for mated females), and received a 20 ml glass vial containing the sugar or the sugar–polyphenol solution described above. They experienced these conditions for 1, 5 or 10 days, after which they were frozen and stored at −80°C. We then measured their total antioxidant capacity (TAC; expressed in mmol 2 HOCl neutralized) using the OXY-adsorbent test (Diacron International, Grosseto, Italy), and assessed oxidative damage by measuring hydroperoxide concentrations (expressed in mg dl–1 H2O2 equivalent) with the d-ROM test (Diacron International). For both tests, we followed protocols described in [6].

(d) Feeding behaviour

Butterflies were transferred to 16 8 l cages 1 day before monitoring. Eight cages contained six mated females, and eight cages contained six virgin females. Cameras were placed above cages to focus on four 20 ml glass vials: two contained cotton soaked with a sugar solution, and two contained cotton soaked with a polyphenol solution (0.5% *Aronia* extract). To dissociate food resources that butterflies could use to cover energy versus antioxidant requirements [1], sugar concentration was 10% in the sugar solution but only 1% in the polyphenol solution.

We monitored feeding behaviour during the first 3 h of each day (when food consumption is maximal [6]) until day 10. A feeding event started when a butterfly unrolled its proboscis to feed, and ended when it retracted it. For further analyses, we summed the duration of all feeding events (in seconds) per cage, solution and day.

(e) Statistics

We used Student’s *t*-test to examine whether food affected the number of eggs laid over the oviposition period, and within each time window when eggs were counted. We did not directly examine how fecundity varied over time, as the duration of the time windows when eggs were counted varied (24 or 48 h).

To determine whether polyphenol intake affected survival, we implemented Kaplan–Meier survival analyses with *p*-values calculated with the Breslow and log-rank tests, which, respectively, better detect early and late differences between treatments.

To examine oxidative status, we used a general linear model for TAC values (log-transformed) and a generalized linear model with a gamma distribution for oxidative damage. Day, food and reproductive status were used as fixed factors. When the effect of
Figure 2. Oxidative status of female *B. anynana* over time. Butterflies are pooled across (a,b) or separated by reproductive status (virgin: c,d; mated: e,f). They fed on a sugar or a sugar–polyphenol solution. Two indices of oxidative status were measured: total antioxidant capacity (TAC) and hydroperoxide concentration to evaluate oxidative damage.

3. Results

(a) Fitness components

Overall, butterflies feeding on the sugar–polyphenol solution laid 20% more eggs than butterflies feeding on the sugar solution (153 ± 8 versus 127 ± 10 eggs; \( t_{1,119} = -1.983, p = 0.050 \)). These effects were also found between days 2 and 3 \( (t_{1,119} = -2.208; p = 0.029) \), and between days 8 and 9 of oviposition \( (t_{1,119} = -2.362; p = 0.020) \), but were not found in other time windows (all \( p > 0.114; \) figure 1a).

Polyphenols also tended to improve the survival of butterflies during oviposition, but these effects were not statistically significant (Breslow test: \( \chi^2 = 2.894, p = 0.089 \); log-rank test: \( \chi^2 = 2.234, p = 0.127 \); figure 1b).

(b) Oxidative status

TAC values and oxidative damage were higher (i) in butterflies feeding on the sugar–polyphenol solution, (ii) in virgin butterflies and (iii) in the middle of oviposition \( (p < 0.001 \) for comparisons between day 5 and days 1 and 10) (figure 2 and table 1). A significant interaction between food and day indicated that polyphenol supplementation affected TAC values at the onset (day 1: \( p < 0.001 \)) and in the middle of oviposition (day 5: \( p = 0.017 \)) but not afterwards (day 10: \( p = 0.959 \); figure 2a).

(c) Feeding behaviour

Butterflies fed longer at the onset of oviposition, and more than twice as long on the sugar solution as on the polyphenol solution \( (2446 ± 105 \text{ s} \) versus \( 955 ± 165 \text{ s} \)). Finally, mated females fed less than virgin females \( (1520 ± 142 \text{ s} \) versus \( 1882 ± 178 \text{ s} \)), and this difference tended to be higher in the first days of oviposition (figure 1c,d and table 1).

4. Discussion

For the first time, to the best of our knowledge, we tested whether reproducing animals regulate antioxidant intake when given the choice between food resources with variable antioxidant contents. Towards this end, we used a frugivorous butterfly known to increase antioxidant intake under hot conditions [6]. Despite significant effects of antioxidant supplementation on fecundity, reproducing butterflies did not target antioxidant resources correspondingly. Feeding behaviour therefore appears to be the main limitation constraining the significance of dietary antioxidants in this species.

The positive effects of polyphenol intake on fecundity were accompanied by both pro- and antioxidant effects. Interestingly, the balance between these contrasting effects varied across the oviposition period. While pro- and antioxidant effects were of similar amplitude at the onset and at the end of oviposition, antioxidant effects were preponderant over pro-oxidant effects in the middle of oviposition (figure 2a,b). This suggests that the cost/benefit ratio of polyphenol intake depended on time and tipped in favour of antioxidant defences only in the middle of oviposition.
Butterflies fed the most on polyphenols at the onset of oviposition, as was the case for sugar intake. Such behaviour may appear paradoxical, as the highest benefits of polyphenol intake on fecundity and antioxidant status occurred later. It may therefore be hypothesized that butterflies anticipated their needs for antioxidants and energy in view of the peak of oviposition to come. However, this hypothesis is unlikely, as (i) hydrophilic polyphenols are rapidly eliminated from the organism [1] and (ii) mated females fed less than virgin ones, especially at the onset of oviposition. This suggests that egg-laying directly competed with feeding, and that reproducing females favoured reproduction (egg-laying) over self-maintenance (feeding). The lower intake of polyphenols by mated females coupled with their utilization for reproduction (which enhanced fecundity) may therefore explain why the effects of polyphenols on their oxidative status were weaker than in virgin females, and why polyphenol supplementation did not significantly improve survival during oviposition. Finally, as food intake and oxidative status varied in a similar manner over time in virgin and mated females, it is likely that age rather than reproduction was responsible for these temporal changes.

The fact that mated butterflies did not increase polyphenol intake does not mean that butterflies avoided polyphenols or did not detect them. Indeed, changes in polyphenol intake paralleled changes in sugar intake over time, suggesting that butterflies did not progressively avoid the polyphenol solution because of its low energy content. Moreover, mated and virgin butterflies fed only twice as long on the sugar solution as on the polyphenol solution, whereas its sugar concentration was 10 times higher. This suggests that polyphenols were attractive for butterflies and altered their feeding selection. Accordingly, we previously found that virgin female B. anynana increased polyphenol intake under hot conditions [6]. However, in this study, butterflies did not increase polyphenol intake because of reproduction. These incongruent results suggest that (i) reproduction prevents animals from modulating their feeding behaviour, because reproduction competes with feeding activities, and (ii) animals do not simply rely on their physiological state but additionally need external cues (e.g. temperature) to increase antioxidant intake. Finally and most importantly, these results further emphasize that, even though dietary antioxidants can improve fitness after ‘antioxidant forced-feeding’, their ecological importance is restricted to situations where animals are able to find, recognize and exploit them.

**Table 1.** Results of general and generalized linear models examining the effects of time (T), food (F) and reproductive status (R) on antioxidant capacity (TAC, n = 172), oxidative damage (n = 172) and feeding behaviour (n = 16) in female B. anynana. The effect of time$^2$ ($T^2$) was also examined for feeding behaviour. Italicized values indicate $p < 0.05$. For the directions of effects, see figures.

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**Ethics.** All experiments complied with German laws on animal experimentation.

**Data accessibility.** The datasets supporting this article have been uploaded as electronic supplementary material.

**Authors’ contributions.** M.B. and K.F. designed this study, M.B. and I.L. collected fitness data and conducted laboratory analyses. I.B. and L.S. conducted behavioural analyses. All authors contributed to the writing of the article, gave final approval for publication, and agree to be accountable for all aspects of the work performed.

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