

## RESEARCH ARTICLE

# Feeding on ripening and over-ripening fruit: interactions between sugar, ethanol and polyphenol contents in a tropical butterfly

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## ABSTRACT

In ripe fruit, energy mostly derives from sugar, while in over-ripe fruit, it also comes from ethanol. Such ripeness differences may alter the fitness benefits associated with frugivory if animals are unable to degrade ethanol when consuming over-ripe fruit. In the tropical butterfly *Bicyclus anynana*, we found that females consuming isocaloric solutions mimicking ripe (20% sucrose) and over-ripe fruit (10% sucrose, 7% ethanol) of the palm *Astrocaryum standleyanum* exhibited higher fecundity than females consuming a solution mimicking unripe fruit (10% sucrose). Moreover, relative to butterflies consuming a solution mimicking unripe fruit, survival was enhanced when butterflies consumed a solution mimicking either ripe fruit supplemented with polyphenols (fruit antioxidant compounds) or over-ripe fruit devoid of polyphenols. This suggests that (1) butterflies have evolved tolerance mechanisms to derive the same reproductive benefits from ethanol and sugar, and (2) polyphenols may regulate the allocation of sugar and ethanol to maintenance mechanisms. However, variation in fitness owing to the composition of feeding solutions was not paralleled by corresponding physiological changes (alcohol dehydrogenase activity, oxidative status) in butterflies. The fitness proxies and physiological parameters that we measured therefore appear to reflect distinct biological pathways. Overall, our results highlight that the energy content of fruit primarily affects the fecundity of *B. anynana* butterflies, while the effects of fruit consumption on survival are more complex and vary depending on ripening stage and polyphenol presence. The actual underlying physiological mechanisms linking fruit ripeness and fitness components remain to be clarified.

**KEY WORDS:** Diet, Frugivory, Insect, Nutrient interactions, Tropics

## INTRODUCTION

In their natural habitat, frugivorous animals are exposed to a variety of fruit, which differs in ripeness (Corlett, 2011). They typically consume ripe fruit but may also feed on unripe or over-ripe fruit depending on abundance and detectability (Foster, 1977; Lasa et al., 2017). Consuming fruit of different ripeness is likely to affect the fitness of frugivores, as fruit chemical composition differs between ripening stages, particularly regarding sugar and ethanol concentrations (Dudley, 2004). In ripe fruit, the accumulation of soluble sugars results from the hydrolysis of starch during fruit formation or from the importation of sugar from the plant (Seymour et al., 1993). Ripe fruit can thus contain more than twice as much

sugar as unripe fruit, thereby constituting an important energy resource for frugivorous animals (Dudley, 2004). If ripe fruits are not promptly consumed, they undergo an over-ripening process during which sugar can be converted into ethanol. This process occurs when fruit becomes infected by yeasts capable of producing ethanol via fermentation under anaerobic conditions (Levey, 2004). Ethanol production by yeasts increases their competitiveness, as ethanol may deter frugivorous competitors (Dudley, 2004; Janzen, 1977; Ruxton et al., 2014). However, the negative relationship between sugar and ethanol content in fruit is quite weak, presumably because over-ripe or rotting fruit constitutes a heterogeneous milieu for micro-organisms. Indeed, fruit becomes contaminated by yeasts present in the environment (e.g. soil, insects) through damage because of abrasion, falling or partial consumption and inoculation by animals (Becher et al., 2012; Carrigan et al., 2015; Dudley, 2004; Janzen, 1977). Ethanol is therefore common in over-ripe fruit but typically occurs heterogeneously and at low concentrations (Dudley, 2004; Levey, 2004). Precise data on ethanol concentration are, however, available only for some fruit species consumed by animals (Dominy, 2015).

Exposure to ethanol may differ for frugivorous animals depending on their latitudinal distribution. Indeed, fruit fermentation is likely to occur more frequently in the tropics, where environmental conditions are more favourable for microbial growth and ethanol production than in temperate regions (Levey, 2004). Moreover, frugivory appears to be more widespread in tropical regions, presumably because fruit diversity is high and fruit abundance is not as seasonal (i.e. the fruiting season is longer) as in other regions (Hanya et al., 2013; Kissling et al., 2009). Paradoxically, Afrotropical *Drosophila melanogaster* flies appear more sensitive to ethanol than European ones, as they die more rapidly when consuming a sucrose solution supplemented with 5–15% ethanol (Chakir et al., 1993; Fry, 2014). Therefore, should this pattern apply to other species, tropical frugivorous animals may be more likely to become intoxicated by ethanol than temperate ones.

Survival differences between Afrotropical and European flies appear to be due to higher levels of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in European flies (Chakir et al., 1993; Fry, 2014). ADH initiates ethanol detoxification by degrading ethanol into acetaldehyde, which is then converted into acetate by ADH and ALDH. Acetate is, in turn, used to produce lipids and generate energy. Consequently, animals able to degrade ethanol should target food containing ethanol, as it represents an important source of energy. In contrast, animals unable to degrade ethanol should avoid such food, as they are likely to experience detrimental side effects rather than energetic advantages. This is exemplified by studies on *D. melanogaster* flies able to degrade ethanol and which target sucrose solutions supplemented with 5–15% ethanol primarily for their energy content (e.g. Pohl et al., 2012). However, an impairment of their capacity to produce

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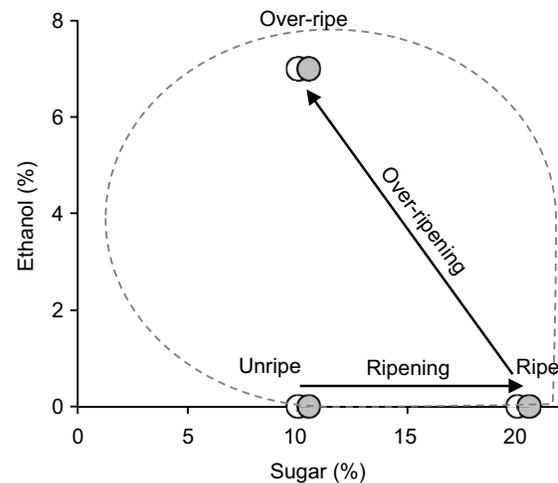
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ADH reduces this intrinsic preference (Ogueta et al., 2010). Consequently, the propensity of animals to feed on over-ripe fruit depends on their capacity to degrade ethanol, which determines the balance between the energetic benefits and the detrimental side effects associated with ethanol consumption. Some animals discriminate against over-ripe fruit containing ethanol (Levey, 2004; Sánchez et al., 2004), suggesting that they have low thresholds of ethanol tolerance, and that detrimental side effects rapidly overrule energetic advantages. This suggests that these animals have a narrow hormetic zone in response to ethanol concentration (i.e. inverted U-shape response in relation to ethanol concentration with maximal positive effects for intermediate concentrations; Costantini et al., 2010; Dudley, 2004). However, other animals do not discriminate against food containing alcohol (Mazeh et al., 2008; Zungu and Downs, 2017), and some even actively seek such food (Gochman et al., 2016; Ogueta et al., 2010; Wiens et al., 2008), suggesting that energetic advantages may also overrule side effects.

Variation in oxidative status owing to ethanol consumption may underlie the detrimental side effects on fitness following the consumption of over-ripe fruit. Indeed, ethanol consumption has been described as altering the oxidative balance of organisms by increasing the production of reactive oxygen species (ROS) and decreasing endogenous antioxidant defences, thereby increasing oxidative damage on biomolecules (Albano, 2006; Comporti et al., 2010). This variation in oxidative status can, in turn, negatively affect the fitness of animals by decreasing reproductive performance and survival probability (Monaghan et al., 2009). Such adverse effects may, however, be reduced if frugivorous animals are able to benefit from antioxidant compounds present in fruit if ingested along with ethanol (Beaulieu and Schaefer, 2013). Indeed, several studies have shown that the pro-oxidant effects of ethanol disappear when it is consumed together with antioxidant compounds such as polyphenols (Devipriya et al., 2007, 2008; Faremi et al., 2008; Kasdallah-Grissa et al., 2007). Moreover, the solubility and the absorption of polyphenols can be enhanced when consumed in combination with ethanol (Manach et al., 2004; Scholz and Williamson, 2007). Consequently, the ethanol and polyphenol contents of fruit are likely to interact, such that the presence of polyphenols in fruit may allow frugivorous animals to more readily exploit over-ripe fruit despite its ethanol content. Importantly, the polyphenol content of fruit does not necessarily vary in a constant manner during the ripening and over-ripening process (Ayala-Zavala et al., 2004; Castrejón et al., 2008; Castro-Concha et al., 2014; Eid et al., 2013; Erkan et al., 2008; Kalt et al., 1999; Miletic et al., 2012). Consequently, frugivorous animals have the choice to feed on fruit of variable ripening stages, with differing polyphenol levels.

Here, we examine the fitness, physiological and behavioural consequences associated with the chronic consumption of feeding solutions mimicking unripe, ripe and over-ripe fruit in a tropical frugivorous butterfly. Towards this end, we consider three aspects of the ripening process: sucrose, ethanol and polyphenol contents (Fig. 1). Because of the high sugar and energy content of ripe fruit, we predict that the consumption of a feeding solution mimicking this ripening stage should enhance fitness proxies such as fecundity and survival probability (hypothesis 1). Moreover, we predict that the consumption of an isocaloric solution mimicking over-ripe fruit (containing sugar and ethanol) should increase fitness if butterflies are able to degrade ethanol (hypothesis 2). If unable to do so, butterflies may still be able to benefit from the energy content of over-ripe fruit if it contains high amounts of polyphenols, as these



**Fig. 1. Relationship between sugar and ethanol concentration in fruit of variable ripening stage.** The graphic illustrates in a simplified way the relationship between sugar and ethanol in the fruit of the Neotropical palm *Astrocaryum standleyanum* as given by Dudley (2004). The dashed line delimits the approximate space in which sugar and ethanol contents have both been measured in the same fruits. The circles represent the treatments used in our experiment (white circles, without polyphenols; grey circles, with polyphenols): unripe fruit contains low levels of sugar (10%) and does not contain ethanol (0%), ripe fruit contains high levels of sugar (20%) and no ethanol (0%), and over-ripe fruit contains low levels of sugar (10%) and high levels of ethanol (7%).

antioxidant compounds may help them to maintain their oxidative balance and fitness (hypothesis 3). In that case, butterflies should avoid feeding on solutions with ethanol and devoid of polyphenols, but target solutions with ethanol supplemented with polyphenols (hypothesis 4).

## MATERIALS AND METHODS

### Biological model

The subtropical and tropical *Bicyclus anynana* (Butler 1879) butterfly originates from woodland habitats in Eastern Africa. Adult butterflies typically fly close to the ground and feed on fallen fruit (Brakefield et al., 2009a). The butterflies used in our study came from our stock population established at the University of Greifswald (Germany) in 2008. Our captive population derives from the population established at the University of Bayreuth (Germany) in 2003, which itself derives from a stock population established at the University of Leiden (The Netherlands) in 1988 using 80 gravid females captured in the wild in Malawi. To maintain high levels of heterozygosity within captive populations (Van't Hof et al., 2005), several hundred individuals are reared together in each generation, and the vast majority of individuals per generation reproduce. Across generations, adult butterflies were fed exclusively with banana slices, while larvae were raised on maize sprouts (*Zea mays*) (Brakefield et al., 2009a,b).

In our experiment, animals were kept in climate chambers set at 27°C, 70% humidity and a photoperiod of 12 h:12 h light:dark. Such conditions were expected to mimic environmental conditions during the tropical wet season, when reproduction occurs. Larvae were first raised on maize sprouts and were collected once they had pupated. At that stage, they were transferred into hanging cages, from which eclosed individuals were later collected on a daily basis. Females and males were kept in separate cages, where they could feed on banana slices covered with soaked cotton wool and laid on the top of the cage. Once butterflies were 2 days old, mating sessions

were conducted by introducing males into the cage of females. Copulating pairs were isolated and transferred individually into 1-litre cylindrical plastic containers (height: 15 cm, upper opening: 85 cm<sup>2</sup>) covered with a gauze lid. Males were removed from the plastic containers immediately after copulation. For the purpose of our study, we used a total of 273 females: 177 for the measurements of fitness and physiological parameters, and 96 for the measurement of behavioural parameters (see below).

### Fitness and physiological parameters

Females were randomly assigned to six feeding treatments characterized by variable sugar, ethanol and polyphenol concentrations (Fig. 1). Each female was maintained in a 1-litre plastic container (as described above) with a 20 ml glass vial (height: 4.7 cm, upper opening: 2.8 cm<sup>2</sup>) positioned in its centre and containing cotton wool soaked with one of these six feeding solutions.

To our knowledge, there are no data about the sugar, ethanol and polyphenol contents of fruit that *B. anynana* butterflies actually consume in their natural habitat. For this reason, we had to use sugar and ethanol concentrations known from other tropical fruit. Specifically, the concentrations that we used reflected the natural concentrations found in the fruit of the Neotropical palm *Astrocaryum standleyanum* depending on its ripening stage (Dudley, 2004). However, in this species, ripening stages partially overlap in terms of sugar and ethanol concentrations. Therefore, to better characterize the effects of the consumption of unripe, ripe and over-ripe fruit, we chose sugar and ethanol concentrations that were specific to a given ripening stage: the feeding solution mimicking unripe fruit contained 10% w/v sucrose and 0% v/v ethanol, the feeding solution mimicking ripe fruit contained 20% w/v sucrose and 0% v/v ethanol, and the feeding solution mimicking over-ripe fruit contained 10% w/v sucrose and 7% v/v ethanol (Fig. 1). Feeding solutions were either not supplemented or supplemented with a polyphenol extract (0.5% w/v *Aronia melanocarpa* extract; Artemis International Inc., Fort Wayne, IN, USA; see Kim et al., 2013 for a precise description of the polyphenol composition of this extract), thereby resulting in six feeding treatments. The polyphenol concentration used matches the natural content of fruit rich in polyphenols. Indeed, the dry extract that we used contains 1530 mg polyphenols 100 g<sup>-1</sup> (Artemis International), thus resulting in a final concentration of 76.5 mg polyphenols l<sup>-1</sup> (Denev et al., 2012; Manach et al., 2004). The energy content of the solutions mimicking unripe fruit (1600 kJ l<sup>-1</sup>) was reduced to 50% as compared with the solutions mimicking ripe and over-ripe fruit (3200 kJ l<sup>-1</sup> for both). The viscosity of all solutions was comparable, as their sucrose concentrations were below the threshold (30–40% sucrose) beyond which viscosity increases and the imbibition rate of the proboscis decreases (Krenn, 2010; Nardone et al., 2013). All feeding solutions were topped up every day, and entirely renewed every other day across the oviposition period.

To stimulate oviposition, a maize leaf was placed into each plastic container and renewed daily. Eggs were counted daily over the first 10 days following copulation, as most eggs are laid during this period (Fischer, 2007; Fischer et al., 2004). Some individuals died during this period, which allowed us to analyze whether feeding treatment affected survival probability in addition to fecundity over the oviposition period.

After the 10 days of oviposition, all females that were still alive ( $N=138$ ) were quickly frozen in powdered dry ice and stored at  $-80^{\circ}\text{C}$ . We then measured ADH activity and the oxidative status of butterflies by following protocols described in Fischer et al. (2014) and Beaulieu et al. (2015). Briefly, the frozen body (thorax and

abdomen) of each butterfly was crushed in 200  $\mu\text{l}$  of phosphate-buffered saline (100 mmol l<sup>-1</sup>, pH 7.4) with one tungsten carbide bead (3 mm) at high speed shaking (30 times s<sup>-1</sup>, 1 min). After centrifugation (twice at 15,000 g, 10 min, 4°C), the supernatant was collected. For the measurement of ADH activity, 10  $\mu\text{l}$  of supernatant was added to 190  $\mu\text{l}$  of a reaction solution (0.15 mol l<sup>-1</sup> Tris-HCl, 30 mmol l<sup>-1</sup> isopropanol, 3 mmol l<sup>-1</sup> NAD<sup>+</sup>, pH 8.5). The increase in absorbance was measured at 340 nm every 15 s for 10 min. To calculate ADH activity, the slope of the reaction between the second and sixth minute was used, and the blank value was subtracted from each measurement. The oxidative status of the supernatant was also measured using (1) the d-ROM test (Diacron International, Grosseto, Italy), which measures the concentration of hydroperoxide, a reactive oxygen metabolite resulting from the attack of ROS on organic substrates (expressed in mg dl<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> equivalent), and (2) the OXY-adsorbent test (Diacron International), which measures the total antioxidant capacity of samples (expressed in mmol<sup>-1</sup> HOCl neutralized). Spectrophotometric measurements were conducted at 490 nm. We measured the total protein content of samples with the Bradford protein assay to express physiological markers per milligram of proteins.

### Feeding behaviour

We examined the feeding behaviour of butterflies in relation to the ethanol and polyphenol content of the feeding solutions. Towards this end, each female butterfly was placed in the morning (i.e. after fasting at night) into a 1-litre transparent plastic container with a single 20 ml glass vial positioned in its centre (as described above). The glass vials contained cotton wool soaked with a sugar solution (10% w/v sucrose) with or without ethanol (7 or 0% v/v) and polyphenols (0.5 or 0.0% w/v of *Aronia* extract). These four feeding solutions therefore correspond to the aforementioned feeding solutions mimicking unripe and over-ripe fruit, with or without polyphenols. Plastic containers were then placed into two climate cabinets (Sanyo MIR 553, Osaka, Japan) with the same environmental settings as those described above. All treatments were balanced between climate cabinets. Each cabinet was equipped with eight digital cameras (Samsung SEB-1005R, Gyeonggi-do, South Korea) connected to a digital video recorder (Samsung SDE-5001N) and a monitor. The feeding behaviour of each butterfly was observed with the cameras from the side of the plastic containers (several butterflies could be observed with the same camera), which enabled us to quantify the time when butterflies started to feed (feeding latency) and for how long they did so (feeding duration). Videos were recorded for 8 h. For further analyses, we only considered individuals who showed an interest in feeding and consumed the feeding solution at least once ( $n=53$ ). The proportion of feeding individuals did not differ between treatments (Fisher's exact tests: all  $P>0.380$ ).

### Statistical analyses

To analyse whether feeding treatments affected the survival of butterflies over the oviposition period, we used a generalized linear model (GzLM; binomial distribution) with survival (1: individual survived, 0: individual died) as the dependent variable and ripening stage (unripe, ripe, over-ripe), polyphenol concentrations (0.0 or 0.5%) and the interaction between both terms as independent factors. To analyse the effects of food on fecundity, we used a general linear model (GLM) with egg number as the dependent variable and the same fixed factors as above. We also examined how food affected the physiology of butterflies by using GzLMs (gamma distribution and log-link function; data distributions were skewed to the right) with oxidative damage, antioxidant capacity and ADH

**Table 1. Results of generalized and general linear models examining the effects of feeding solutions (mimicking different ripening stages and with or without supplemented polyphenols) on fitness proxies (survival and fecundity) and physiological parameters [alcohol dehydrogenase (ADH) activity, oxidative damage, antioxidant capacity] in female *Bicyclus anynana* butterflies**

	Survival	Fecundity	ADH	Oxidative damage	Antioxidant capacity
Ripeness	$\chi^2_2=1.41$ , $P=0.493$	$F_{1,171}=4.68$ , $P=0.011$	$\chi^2_2=33.00$ , $P<0.001$	$\chi^2_2=11.16$ , $P=0.004$	$\chi^2_2=12.10$ , $P=0.002$
Polyphenols	$\chi^2_1=4.58$ , $P=0.032$	$F_{1,171}=1.81$ , $P=0.180$	$\chi^2_1=0.49$ , $P=0.485$	$\chi^2_1=1.37$ , $P=0.243$	$\chi^2_1=6.32$ , $P=0.012$
Ripeness × polyphenols	$\chi^2_2=12.23$ , $P=0.002$	$F_{1,171}=1.71$ , $P=0.185$	$\chi^2_2=7.39$ , $P=0.025$	$\chi^2_2=4.82$ , $P=0.090$	$\chi^2_2=2.63$ , $P=0.268$

Bold text indicates significant results.

activity as dependent variables, and the same fixed factors as above. Finally, using a GLM, we analysed whether the feeding behaviour of butterflies depended on the presence of ethanol and polyphenols in their feeding solution. This GLM included feeding latency or feeding duration (log-transformed) as dependent variables, and ethanol (0 or 7%) and polyphenol (0.0 or 0.5%) concentrations and their interactions as independent factors. The normality of residuals was assessed through the Shapiro–Wilk test, and multiple comparisons were examined with Benjamini–Hochberg adjusted  $P$ -values (with a false discovery rate set at 0.25). All analyses were performed using SPSS 22.00 (IBM, Armonk, NY, USA).

Raw data are available in Tables S1 and S2.

## RESULTS

### Fitness parameters

Regarding statistical main effects, survival was not significantly affected by ripening stage but was significantly enhanced when food was supplemented with polyphenols (Table 1, Fig. 2A). However, the significant interaction between ripening stage and polyphenols revealed that the latter effect was restricted to the feeding solution mimicking ripe fruit (although this effect also tended to occur for the feeding solution mimicking unripe fruit;  $P=0.076$ ). Therefore, in the presence of polyphenols, survival was highest in butterflies feeding on the solution mimicking ripe fruit. In contrast, in the absence of polyphenols, survival was highest in butterflies feeding on the solution mimicking over-ripe fruit (Table 1, Fig. 2A).

Fecundity was only significantly affected by the factor ripening stage, indicating that butterflies fed with solutions mimicking ripe and over-ripe fruit laid more eggs than those fed with the solution mimicking unripe fruit, irrespective of the presence of polyphenols (Table 1, Fig. 2B).

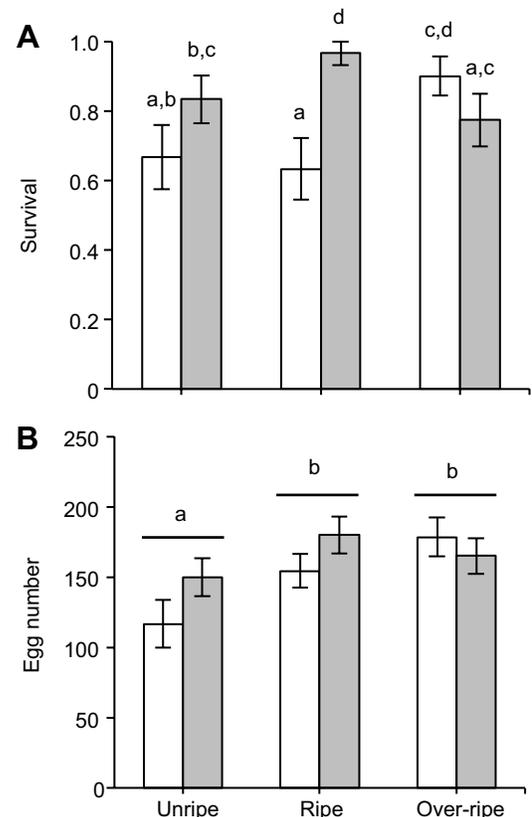
### Physiological parameters

All three physiological parameters were significantly affected by the factor ripening stage (Table 1). This was because butterflies feeding on the solution mimicking ripe fruit showed lower ADH activity, higher oxidative damage and lower antioxidant defences than butterflies feeding on the solutions mimicking unripe and over-ripe fruit (Fig. 3). Moreover, the interaction between ripening stage and polyphenols was significant for ADH activity, because ADH activity decreased only in the presence of polyphenols in butterflies feeding on the solution mimicking over-ripe fruit (Table 1, Fig. 3A). Ripening stage and polyphenols also tended to interact for oxidative damage (Table 1), presumably because polyphenol consumption tended to decrease oxidative damage only in butterflies feeding on the solution mimicking over-ripe fruit (Fig. 3B). In contrast, polyphenol consumption did not interact with ripening stage for antioxidant defences, even though it generally decreased them (Table 1, Fig. 3C).

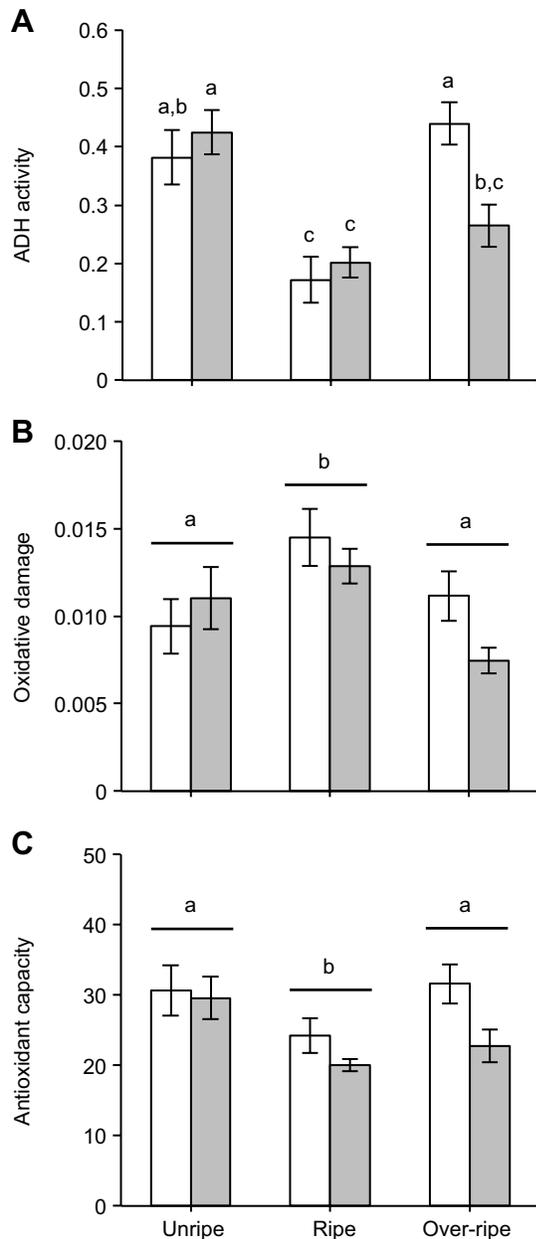
### Feeding behaviour

Butterflies did not feed more rapidly on the solutions supplemented with ethanol than on the solutions devoid of ethanol ( $F_{1,49}=0.877$ ,  $P=0.354$ ). Similarly, feeding latency did not depend on the presence of polyphenols ( $F_{1,49}=2.339$ ,  $P=0.133$ ). Finally, the interaction between ethanol and polyphenols was not significant for feeding latency ( $F_{1,49}=1.352$ ,  $P=0.251$ ; Fig. 4A).

Feeding duration did not depend on the presence of polyphenols in the feeding solution ( $F_{1,49}=0.156$ ,  $P=0.694$ ). In contrast, butterflies spent less time feeding on the solutions supplemented

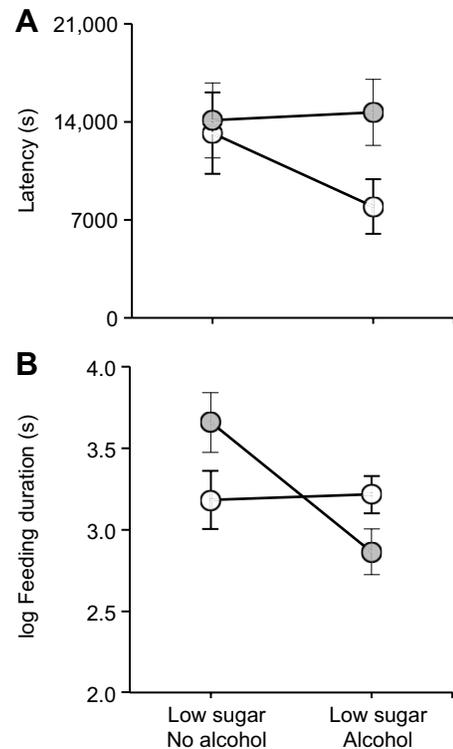


**Fig. 2. Fitness parameters in female *Bicyclus anynana* butterflies feeding on solutions mimicking variable fruit ripening stages.** (A) Survival and (B) fecundity were measured during the 10 days of oviposition. White bars represent butterflies feeding on solutions without polyphenols (unripe fruit:  $N=27$ , ripe fruit:  $N=30$ , over-ripe fruit:  $N=30$  butterflies) and grey bars represent butterflies feeding on solutions supplemented with polyphenols (unripe fruit:  $N=30$ , ripe fruit:  $N=29$ , over-ripe fruit:  $N=31$  butterflies). Different letters above bars indicate significant differences between groups ( $P<0.05$ ). Results are presented as means $\pm$ s.e.m.



**Fig. 3. Physiological parameters in female *B. anynana* butterflies feeding on solutions mimicking variable fruit ripening stages.** (A) Alcohol dehydrogenase (ADH) activity, (B) oxidative damage and (C) antioxidant capacity were measured after the 10 days of oviposition. White bars represent butterflies feeding on solutions without polyphenols (unripe fruit:  $N=18$ , ripe fruit:  $N=19$ , over-ripe fruit:  $N=26$  butterflies) and grey bars represent butterflies feeding on solutions supplemented with polyphenols (unripe fruit:  $N=23$ , ripe fruit:  $N=28$ , over-ripe fruit:  $N=24$  butterflies). ADH activity is expressed in optical density units, oxidative damage was assessed with the d-ROM test, which measures hydroperoxide concentration (expressed in milligrams per decilitre of  $H_2O_2$  equivalent), and antioxidant capacity was measured with the OXY-adsorbent, which that measures total antioxidant capacity (expressed per millimole of HOCl neutralized). All results are expressed per milligram of proteins, and are presented as means $\pm$ s.e.m. Different letters above bars indicate significant differences between groups ( $P<0.05$ ).

with ethanol than on the solutions devoid of ethanol ( $F_{1,49}=6.014$ ,  $P=0.018$ ). This effect, however, was restricted to butterflies exposed to solutions containing polyphenols (interaction ethanol $\times$  polyphenols;  $F_{1,49}=7.088$ ,  $P=0.010$ ; Fig. 4B).



**Fig. 4. Feeding parameters in female *B. anynana* butterflies.** (A) Latency to feed and (B) feeding duration were measured at the beginning of the oviposition period. The solution with low sugar concentration and no ethanol mimics unripe fruit, and the solution with low sugar concentration and ethanol mimics over-ripe fruit. White circles represent butterflies feeding on the solutions without polyphenols (unripe fruit:  $N=12$ , over-ripe fruit:  $N=13$  butterflies) and grey circles represent butterflies feeding on the solutions supplemented with polyphenols (unripe fruit:  $N=12$ , over-ripe fruit:  $N=16$  butterflies). The interaction between sugar concentration and polyphenols is only significant for feeding duration ( $P<0.05$ ). Results are presented as means $\pm$ s.e.m.

## DISCUSSION

### Effects of fruit ripening stage in the absence of polyphenols

In agreement with our first hypothesis, we found that, relative to individuals feeding on a solution mimicking unripe fruit (with low sugar concentration and without ethanol), the consumption of a feeding solution mimicking ripe fruit (with high sugar concentration and without ethanol) enhanced the fecundity of female *B. anynana* butterflies. Moreover, we found that the consumption of a feeding solution mimicking over-ripe fruit (with low sugar concentration but with ethanol) also enhanced fecundity. The high energy content of these two feeding solutions likely underlies these positive effects. Moreover, in contrast to the solution mimicking ripe fruit, the consumption of the solution mimicking over-ripe fruit also increased the survival probability of butterflies, suggesting that butterflies could derive benefits from ethanol not only for reproduction but also for self-maintenance.

The positive effects of ethanol ingestion that we observed were unlikely to be due to the microbicidal properties of ethanol in the gut of butterflies. Indeed, the butterflies that we used in our experiment have been kept under laboratory conditions and have not been exposed to pathogens for generations. Consequently, if ethanol had microbicidal effects in butterflies, its effects would more likely occur on their gut microbiota rather than on potential pathogens, which in turn might reduce fitness components (Engel and Moran, 2013). Instead, the fact that

butterflies benefited from food containing ethanol suggests that they were able to degrade ethanol and take advantage of its energy content (hypothesis 2).

Despite the fitness benefits associated with ethanol intake in *B. anynana*, effects on ADH activity were not conclusive. While ADH activity was higher in butterflies feeding on the solution mimicking over-ripe fruit (containing ethanol) than in those feeding on the solution mimicking ripe fruit as would be expected, it was equally high in butterflies feeding on solutions mimicking unripe and over-ripe fruit. It is therefore possible that butterflies do not possess a type of ADH that can be induced by ethanol, as observed in adult *D. melanogaster* fruit flies (Geer et al., 1988; Hernández-Tobías et al., 2011). Butterflies lacking this physiological protective response would be expected to experience the detrimental effects of ethanol intake such as its pro-oxidant effects (Albano, 2006; Comporti et al., 2010). However, we did not detect any pro-oxidant effects following ethanol consumption, suggesting that butterflies were efficiently protected against ethanol exposure. Moreover, the fact that ethanol consumption even increased the survival probability of butterflies suggests that self-maintenance mechanisms were not compromised by ethanol consumption. *Bicyclus anynana* butterflies therefore appear to exhibit constitutively high ADH levels independently of ethanol exposure, which may allow them to avoid activating genes repeatedly as well as the latency related to gene expression when feeding on over-ripe fruit. Alternatively, the fact that ethanol consumption enhanced fitness proxies in *B. anynana* without altering physiological parameters may also suggest that ethanol detoxification and caloric utilization reflect distinct biological pathways, as suggested in *D. melanogaster* (Devineni and Heberlein, 2013).

Because of the benefits associated with ethanol intake, *B. anynana* butterflies were expected to be more inclined to feed on a solution supplemented with ethanol than on a solution devoid of ethanol. Accordingly, previous studies have shown that their feeding response (i.e. the uncoiling of the proboscis after placing the tarsi of butterflies in direct contact with feeding solutions for 30 s) increased with ethanol concentration (range: 0.01–10%) (Dierks and Fischer, 2008; Kehl and Fischer, 2012). Following these results, it was hypothesized that the presence of ethanol in fruit may help butterflies to locate food, as suggested for other insects (Hoffmann and Parsons, 1984; Utrio and Eriksson, 1977) and primates (Dudley, 2004; Levey, 2004). However, in the present study, where butterflies were free to feed by themselves over several hours, we did not detect positive effects of ethanol on feeding latency and duration. Experimental biases, such as the very high ethanol concentration and the absence of yeasts in the feeding solution mimicking over-ripe fruit, may explain this lack of preference (Becher et al., 2012; Mazeh et al., 2008; Xu et al., 2012). However, previous studies examining the feeding response of *B. anynana* butterflies in relation to ethanol concentration found that the strongest response occurred when butterflies were exposed to even higher ethanol concentrations (10%) than in the present study, and that butterflies do not alter their feeding response in relation to the presence of yeasts in their food (Dierks and Fischer, 2008; Kehl and Fischer, 2012). It might also be argued that the high ethanol concentration that we used slightly sedated butterflies, thereby reducing feeding preferences (Devineni and Heberlein, 2013). However, even before consuming ethanol for the first time, butterflies did not appear to show any preference for the feeding solution containing ethanol, as feeding latency was not affected by ethanol. It might also be argued that the fact that we examined the feeding behaviour of butterflies naive to ethanol explains their lack

of preference for ethanol (Devineni and Heberlein, 2013; Lee et al., 2009; Peris et al., 2015). A final explanation for our results is that the presence of ethanol in over-ripe fruit does actually not facilitate fruit localization and consumption in *B. anynana*, as observed in other frugivorous species (Mazeh et al., 2008). Ethanol may therefore elicit feeding only when butterflies are in direct contact by chance with fruit containing ethanol (Dierks and Fischer, 2008; Kehl and Fischer, 2012). Given the favourable conditions for fruit to ferment and produce ethanol in the tropics (Levey, 2004), this scenario is likely to be highly probable for butterflies in their natural habitat.

In our study, the solutions mimicking ripe and over-ripe fruit were isocaloric. Accordingly, their consumption similarly increased the fecundity of butterflies. However, only the consumption of the solution mimicking over-ripe fruit increased their survival. The fact that the survival of butterflies was not enhanced when consuming the feeding solution mimicking ripe fruit may be due to the high sucrose concentration that we used in this solution. Indeed, previous studies have shown that some insects are unable to convert excess sugar into lipid stores that are subsequently used to enhance fitness components (Arrese and Soulages, 2010; Visser and Ellers, 2008). Even though the mechanisms underlying this phenomenon remain unclear, this suggests that the consumption of large sugar quantities entails some physiological costs that can impact on fitness components. Accordingly, we found that high sugar consumption was related to lower ADH activity, higher oxidative damage and lower antioxidant capacity in butterflies. A previous study also found that ADH activity decreased with sucrose concentration in *D. melanogaster* larvae (McKechnie and Geer, 1984). Moreover, sucrose consumption has also been found to increase oxidative damage and decrease antioxidant defences in rats, presumably because of their higher mitochondrial metabolism, ROS production and utilization of antioxidant defences (Busserolles et al., 2002; Chaudhary et al., 2007).

#### Effects of fruit ripening stage in the presence of polyphenols

In a previous study, we found that the consumption of polyphenols increased fitness, oxidative damage and antioxidant capacity in *B. anynana* butterflies (Beaulieu et al., 2016). In the presence of high sugar concentrations, we also found here that the consumption of polyphenols could increase fitness proxies, as it significantly increased survival probability in butterflies feeding on the solution mimicking ripe fruit (and it tended to increase it in butterflies feeding on the solution mimicking unripe fruit). As the survival of butterflies feeding on the solution mimicking ripe fruit devoid of polyphenols was not enhanced, this result suggests that polyphenols can regulate the allocation of sugar to maintenance mechanisms. However, the effects of polyphenol consumption on oxidative markers strongly differed from those that we previously found. Indeed, polyphenol intake had no significant effects on oxidative damage and significantly decreased antioxidant defences. Lower oxidative damage as a result of polyphenol consumption has been reported by studies examining the oxidative status of animals consuming ethanol (Devipriya et al., 2007, 2008; Faremi et al., 2008; Kasdallah-Grissa et al., 2007). In contrast to these studies, ethanol consumption per se was not associated with pro-oxidant effects in our study. Therefore, polyphenols were not used to re-optimize the oxidative status of butterflies consuming ethanol, which may explain why we only found a weak tendency for polyphenols to decrease oxidative damage in butterflies consuming ethanol. We also found that the combination of ethanol and polyphenols in the feeding solution mimicking over-ripe fruit resulted in significantly lower ADH activity. This may be due to

ADH interacting with the hydroxyl group of polyphenols, which may ultimately reduce its availability for ethanol. Butterflies feeding on the solution mimicking over-ripe fruit supplemented with polyphenols may therefore be less efficient at detoxifying ethanol than butterflies consuming ethanol alone. This may explain why their survival was not enhanced relative to butterflies feeding on solutions mimicking unripe or ripe fruit. Consequently, in contrast to our fourth hypothesis, butterflies should not necessarily target fruit containing both ethanol and polyphenols. Accordingly, we found that butterflies did not feed for as long on a solution combining ethanol and polyphenols as on a solution supplemented with ethanol alone. Similar to *D. melanogaster* flies (Ogueta et al., 2010), the impaired ADH activity resulting from the simultaneous consumption of ethanol and polyphenols may underlie the reluctance of butterflies to feed on the ethanol/polyphenol cocktail.

### Conclusions

Overall, our study shows that tropical *B. anynana* butterflies can increase their fitness by feeding on ripe and over-ripe fruit. However, in terms of survival, the benefits associated with the consumption of fruit depend on ripening stage and polyphenol content: ripe fruit are more beneficial when containing polyphenols, while over-ripe fruit are more valuable when devoid of polyphenols. Interestingly, *B. anynana* butterflies appear to be able to adapt their feeding behaviour accordingly. Even though these results shed some light on the interactions between fruit components during ripening and their effects on the fitness and feeding behaviour of frugivorous animals, this study represents only a first step. Indeed, even though *B. anynana* butterflies have remained genetically diverse across generations in captivity (Van't Hof et al., 2005), it is currently unknown whether stable (e.g. temperature, humidity, light) and artificial (e.g. diet, lack of pathogens, competitors, predators) conditions in captivity have affected their physiology and behaviour (as observed in other species; Griffith et al., 2017). Therefore, the results of the present study may not be entirely comparable to those obtained in wild animals subject to natural conditions, and have to be considered with caution. Moreover, fruit ripening is more complex than just changes in sucrose, ethanol and polyphenol contents, as it can alter fruit characteristics in many other dimensions (e.g. acidity decline, fruit softening, production of flavour volatiles, production of carotenoids, skin coloration). To further examine how fruit ripening affects frugivorous animals, future studies should therefore consider as many ripeness dimensions as possible, but most importantly, also the interactions between these dimensions.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: M.B., K. Franke; Methodology: M.B., K. Franke; Writing - original draft: M.B., K. Franke, K. Fischer; Writing - review & editing: M.B., K. Fischer; Supervision: K. Fischer.

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