ORIGINAL ARTICLE

Transgenerational inheritance of learned preferences for novel host plant odors in *Bicyclus anynana* butterflies

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Many phytophagous insects have strong preferences for their host plants, which they recognize via odors, making it unclear how novel host preferences develop in the course of insect diversification. Insects may learn to prefer new host plants via exposure to their odors and pass this learned preference to their offspring. We tested this hypothesis by examining larval odor preferences before and after feeding them with leaves coated with control and novel odors and by examining odor preferences again in their offspring. Larvae of the parental generation developed a preference for two of these odors over their development. These odor preferences were also transmitted to the next generation. Offspring of butterflies fed on these new odors chose these odors more often than offspring of butterflies fed on control leaves. In addition, offspring of butterflies fed on banana odors had a significant naïve preference for the banana odors in contrast to the naïve preference for control leaves shown by individuals of the parental generation. Thus, butterflies can learn to prefer novel host plant odors via exposure to them during larval development and transmit these learned preferences to their offspring. This ability potentially facilitates shifts in host plant use over the course of insect diversification.

KEY WORDS: *Bicyclus anynana*, odor learning, oviposition choice, intergenerational inheritance.

The inheritance of learned or acquired traits, an idea promoted by Lamarck and then independently by Darwin, suggested that the use and disuse of traits led to the establishment of variations that could be transmitted to the next generation and that over time these variations would give rise to diversity across species (Burkhardt 2013). These early ideas on the provenance of organismal variations were later largely dismissed by August Weismann with the proposed separation of germline and soma, by the mutational theory of Hugo De Vries, and finally put to rest with the adoption of the modern synthesis (Burkhardt 2013).

Numerous studies during the last 20 years, however, have been reviving and lending support to the inheritance of learned and acquired traits and couching these ideas in the modern field of epigenetic inheritance and also in the less well-studied field of genetic assimilation or genetic accommodation (West-Eberhard 2003; Jablonka and Lamb 2005; Pigliucci et al. 2006; Jablonka and Raz 2009; Xia and de Belle 2017). For instance, mice exposed to a neutral fruity odor while receiving a mild electric shock adopt a startle behavior later in life while only experiencing the odor, and pass on that startle behavior to their children and grandchildren upon sensing the odor without the shock (Dias and Ressler 2014). Even more impressive, whole populations of nematode worms that are exposed (imprinted) during a critical larval stage to a new odor in their food environment, over the course of five successive generations, move toward those odors for the next 40 generations, even without further imprinting, that is, the whole population appears to have genetically assimilated a learned behavioral response toward the novel odor (Remy 2010). These experiments, likely involving the fixation of epigenetic variation through single or repeated exposures to the same novel environmental stimulus,

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highlight how an exposure to a novel environment, in this case a novel odor, helps create novel behavioural responses in whole populations, some of them long term, via a more efficient method than the gradual spread and fixation of behavioural variants, initially at low frequency, across a population via natural selection.

The imprinted behavior of *C. elegans*, in particular, can be advantageous from an ecological perspective. The novel odor is associated with food and a positive response to the novel odor trains the young worms to more easily locate additional sources of the same food later in life. More generally, organisms might occasionally find themselves in new environments where they will perish if unable to locate or accept novel food sources. Being able to develop acceptance or even develop a preference to a novel food source is certainly adaptive in the face of starvation. It is, thus, not surprising that many animals seem to have evolved innate mechanisms that translate early experiences with food into later preferences for that same food (Doherty and Cowie 1994; Punzo 2002; Scherer et al. 2003; Punzo 2004; Darmaillacq et al. 2006; Schausberger et al. 2010; Zrelec et al. 2013; Arenas and Farina 2014; McAulay et al. 2015; Crane et al. 2018). Furthermore, transmitting these learned food preferences to the next generation would also be adaptive as the novel food source is likely to continue to be present in the environment of the offspring. These ideas are exciting but their acceptance and incorporation into mainstream evolutionary biology can benefit from additional tests in different systems, as well as via uncovering the responsible mechanisms of heredity.

Here, we test these ideas in insect herbivore larvae. Herbivore insects are often born on host food plants selected by their mothers at the time of oviposition via specific plant volatile blends (Bruce and Pickett 2011). It is possible, however, that if the typical host plant is not encountered by the mother, she will lay eggs in less optimal hosts, perhaps even novel hosts (Zhang et al. 2007; Anderson and Anton 2014). The ability of her larval offspring to learn and accept a novel food might help these larvae survive as well as successfully forage on the novel host. Then, the ability of these larvae to pass on their novel food preferences to their own offspring would also be advantageous, as it might speed up development. Data indicate that certain Lepidopteran larvae, for instance, that are fed on novel foods/host plants can develop an oviposition preference for that food later in life (Moreau et al. 2008; Anderson et al. 2013; Petit et al. 2015). Most interestingly, noctuid larval exposure to novel foods can impact adult oviposition preference immediately (Petit et al. 2015) or after a few generations of larval feeding on the novel diet (Petit et al. 2018). Variation in the number of generations required for larval diet to impact adult oviposition preference in three species of noctuid moth correlated with typical diet breadth for those species, where one to two generations were required for the polyphagous species and five generations were required for the monophagous species

(Petit et al. 2018). Regardless of whether novel larval food preferences lead to changes in oviposition preferences in adults, it is still unknown whether a novel larval food preference can be transmitted to the next generation and exhibited by naïve larvae of herbivore insect species.

Here, we use the model butterfly *Bicyclus anynana* to test whether larvae can learn to prefer novel food plant odors upon feeding on plants that have been coated with those odors.

Experimental data indicate that *B. anynana* has natural food choice preferences but is also flexible regarding these preferences depending on plant availability. *Bicyclus anynana* is an oligophagus species from the African tropics, but since its laboratory domestication that started in 1988, *B. anynana* larvae have mostly been fed on young maize plants, *Zea mays*. However, when *B. anynana* females in the laboratory, after about 20 generations of domestication, were presented with nine different tropical grass species, including species of *Oplismenus, Ganotia, Setaria, Axonopus*, *Zea, Digitaria, Panicum*, and *Carex*, they laid eggs on all (Kooi 1993). Grass species that were preferred by females for oviposition, however, led to higher larval survival, shorter development time, and higher pupal weight (Kooi 1993). These butterflies also laid eggs on nongrass species, *Cyperus* and *Juncus,* when these were offered in no-choice situations, but when grasses were available, few eggs were laid on these plants. Hardly any eggs were laid on the dicot species tested, *Tradescantia*, *Pisum,* or *Vicia*, in either choice or no choice situations with grasses (Kooi 1993). This flexible behavior indicates that *B. anynana* has potential to evolve novel food preferences, perhaps via short- or long-term exposure to novel host plants and perhaps via odor learning.

Bicyclus anynana is also a species where adults have previously been shown to learn a variety of different wing patterns (Westerman et al. 2012, 2014; Westerman and Monteiro 2013) as well as pheromone odors (Dion et al. 2017). In addition, these latter learned pheromone preferences have been transmitted to the next generation to affect naïve pheromone preferences (Dion et al. 2017). Experiments on larval food preference learning and inheritance, however, have never been conducted in this species. Investigating food-learning abilities in *B. anynana* is useful as the mechanisms of learning and inheritance can later be investigated using a suite of functional genetic tools developed for this species (Marcus et al. 2004; Ramos et al. 2006; Banerjee and Monteiro 2018).

In our experiment, we reared five groups of *B. anynana* larvae. The control group was fed on the standard laboratory food of maize leaves (coated with ethanol), whereas the experimental groups were fed on leaves coated with an artificial plant odor of almond, banana, coffee, or mango (diluted in ethanol). We tested whether exposing larvae to novel odors, while being naturally paired with food, led to the development of a preference toward plants coated with that odor. We also tested whether this larval treatment translated into shifts in adult oviposition preferences

and whether any novel preferences were transmitted to the next generation through both parents and/or via the fathers alone (Fig. 1). The use of artificial odors to distinguish among food types allowed us to focus this investigation on whether changes in simple chemical signals in plants, rather than more complex changes or nutritional differences between plants, could play a role in driving larval food preference diversification.

Methods
HUSBANDRY
Bicyclus anynana was reared in climate-controlled rooms at 27°C, 60% humidity, and 12:12-hour light:dark photoperiod. Wild-type embryos were collected by placing maize leaves inside adult population cages for 3–4 hours and then by stripping the embryos from the leaves into a plastic container. Larvae, upon hatching, were placed in groups of 10 in wax-coated paper containers with plastic lids having multiple holes to facilitate ventilation. These larvae were then divided into three experimental groups for each odor tested (see details below; Fig. 1).

PREPARATION OF SCENTED FOOD
We used four different artificial odors made from commercially available food essences prepared by Gim Hin Lee (Pte) Ltd (Singapore). A 2% essence solution was prepared for each odor by diluting the food essence in 100% ethanol. Tender and young maize leaves were coated thoroughly by dipping each entire leaf into the solution. Leaves dipped in 100% ethanol were used as control. The larvae of control groups were fed with control leaves (control) throughout their development and those of the treatment groups were fed with leaves coated with the diluted essences of almond, banana, coffee, and mango, all named odor leaves (odor), throughout their development. Leaves were replaced every alternate day by freshly coated leaves to ensure the continuous presence of odor. Each odor treatment group had its corresponding control treatment group leading to a total of four different odor experiments, each with a control and an odor treatment group.

ODOR CHOICE ASSAY
For each odor experiment, embryos of the parental generation were collected into a plastic container and were split into three groups. One group was subjected to naïve choice assays, and the other two groups were each assigned to a diet treatment—control versus odor (Fig. 1). Naïve choice assays were performed right after first instar larvae hatched from eggs prior to any feeding. These larvae were not reared further. Odor choice assays were performed at different stages of larval development on the two other experimental groups, on the 5th, 10th, 15th, and 20th day after hatching, to determine the food odor preference of larvae. For these latter assays, larvae were starved overnight for around

15 hours prior to performing the assay because we hypothesized that hungry larvae would move more quickly toward food. Food choice assays for the offspring of these larvae were performed on naïve first instar freshly hatched larvae only, prior to any feeding. Each assay was performed using a Petri dish of 8.8 cm diameter on which a central line was drawn (on the backside) using a permanent marker to facilitate proper alignment of the larva during the assay. Two more lines, equally spaced from the central line, were drawn on either side of it. The distance between these two outer lines was the average length of the larval body at the time of the choice assay. One side of the Petri dish had a small piece of a control leaf coated in ethanol (∼5 cm × 1 cm), whereas the other side had a similarly sized leaf coated with one of the essences (Fig. 1). The Petri dish was placed inside a large green bowl to reduce the influence of surrounding visual signals, like light intensity and colors, which might impact leaf choice. The larvae were picked up using a sanitized paint brush or tweezers, depending on their size, and placed along the central line of the Petri dish. Each larva was given a maximum of 2 minutes to make a choice and move toward its preferred leaf. The larvae whose bodies completely crossed the outer line closest to either leaf, within 2 minutes, were said to perform either a "choice for control" or a "choice for the odor." Larvae that did not cross either of the outer lines were said to make "no choice." The Petri dish was wiped with a tissue after every larval choice assay. Larvae were then returned to their respective food treatments and, five days later, were tested again in a Petri dish. We did not keep track of the choices of individual larvae, only of the whole cohort.

ADULT OVIPOSITION CHOICE ASSAY
Oviposition experiments were performed for the adults that emerged from the banana and mango odor experiments only, where larvae showed a change in their preferences over time. The pupae from the banana or mango experiments (both control and odor-fed groups) were transferred to separate emergence cages for each group. After emergence, female butterflies were numbered on their proximal wings using a permanent marker to help monitor their age. The emerged males and females were then transferred to the mating cage of their respective groups. The butterflies were allowed to mate. Additional cages were set up with virgin males of the banana and mango odor-fed groups to mate with wildtype females. After four days, each female butterfly was removed and placed in a separate test cage. The butterflies were given two options with regard to oviposition site: control leaves or odor leaves (coated with banana or mango odors for groups reared on banana or mango odors, respectively). The leaves were of similar size and color and were replaced every day. After oviposition, and within 12 hours, the embryos were collected in separate labeled containers until hatching.
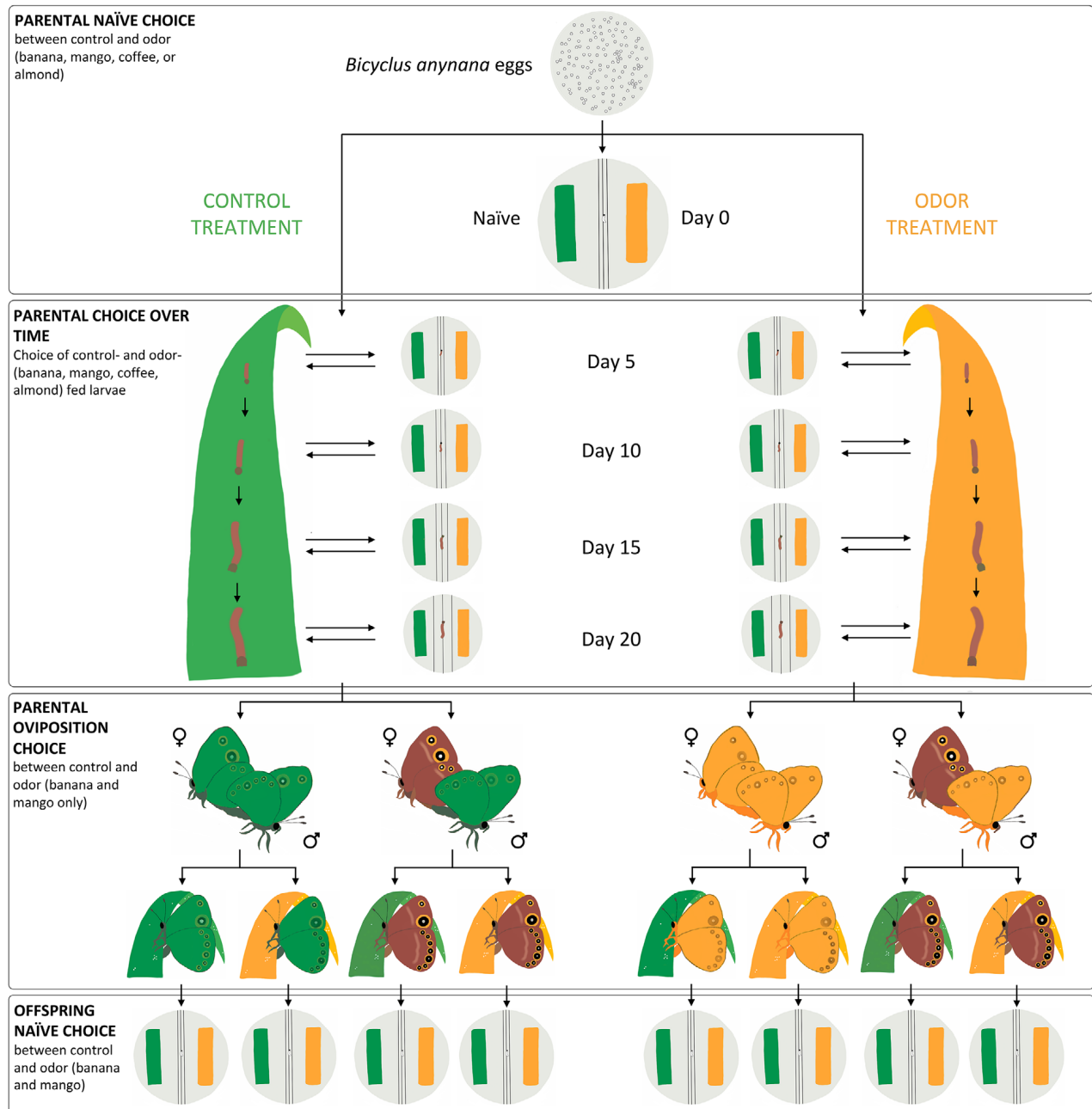
Figure 1. Experimental set-up for examining how odor learning in larvae of *Bicyclus anynana* alters adult oviposition preference and naïve larval odor preference in the next generation. *Bicyclus anynana* eggs were collected and divided into three groups for each odor tested. Larvae from one group, upon hatching and prior to any feeding, were subjected to a choice assay to determine their naïve choice between a control leaf (control—green color) and an odor-coated leaf (odor—orange color). This group of larvae was not used for further experiments. Note that the leaves (and butterflies) are color coded to represent their respective odor treatments but in reality all leaves and butterflies looked wild type. In the control and odor treatment groups, the larvae were fed immediately upon hatching with control and treatment odors, respectively, and throughout their larval stage (parental diet). The larvae of these two groups were tested during choice assays at days 5, 10, 15, and 20, respectively. Leaves were coated with banana, mango, coffee, or almond odors. After day 20, the larvae from the banana and the mango odor experiments only were allowed to develop into adults and used for the subsequent trials. Each treatment was further divided into two groups. In one group, males and females both fed with the control and fruity odors were allowed to mate, whereas in the other, males fed with the fruity odors or control odors were mated with wild-type females. After mating, the females of each group were subjected to a choice assay where they chose between control and odor-coated leaves for oviposition. The eggs were collected and the larvae were tested in a final choice assay immediately upon hatching to examine their naïve odor preferences.

STATISTICAL ANALYSIS

Testing for an appropriate control treatment

Changes in proportions of larvae choosing ethanol-coated leaves over noncoated leaves over larval development (day 5, 10, 15, and 20) were analyzed with a two-tailed Fisher exact test of independence followed by an adjusted pairwise nominal independence post hoc analysis.

Testing for larval odor preferences

We used a chi-squared test of goodness of fit to test if the proportion of larvae choosing the odor leaves over the control leaves was significantly different from random choice (50–50% choice). The test was done separately for each treatment group, generation, and day. A significant deviation from 50% was considered a preference.

Testing for differences in odor choice over time and among diet treatments

We tested the effects of caterpillar age (5, 10, 15, and 20 days after hatching), food treatment (odor or control), and their interaction on larval choice by fitting a binomial generalized linear model (GLM) with the logit link function (choice for the odor was coded 1, whereas the choice for the control was coded 0). We tested the significance of the factors via likelihood ratio tests (LRT), removed nonsignificant interactions and factors from the final model, and performed a post hoc analysis with Tukey adjustment for multiple comparisons. Datasets from each odor experiment (almond, banana, coffee, and mango odors) were analyzed separately. The difference in proportions of caterpillars that chose mango odors and that chose banana odors, on the 20th day after eclosion, was tested with a two-tailed Fisher exact test of independence.

Testing adult oviposition choice

To test for oviposition choices of the odor-fed and control-fed females (for the banana and mango experiments), number of eggs laid by each female on odor-coated leaves were counted and compared with number of eggs laid on control leaves by the same female. These pairs of numbers were compared with a paired *t*-test (if the differences between counts were normally distributed) or with a Wilcoxon signed-rank test (if the differences were not normally distributed). Data normality was tested a priori with Shapiro–Wilk normality tests.

Testing the effect of parental (or paternal) diet and oviposition location on offspring naïve choice

We tested the effect of parental diet (odor or control), number of treated parents (both the mother and father, or the father only), type of plant where eggs were laid (odor or control) (all fixed effects), and all two-way and three-way interactions between these fixed factors, on the naïve food choice of the offspring by fitting a mixed effects logistic regression for binomial data (offspring choice for the odor was coded 1, whereas the choice for control was coded 0). Family identity of the offspring was included as a random factor. A total of 56 and 67 families were counted in the banana and the mango experiments, respectively (Supporting Information File 2). Nonsignificant interaction terms and factors were removed from the final model. The significance of the factors was tested via LRT, and the final model had the lowest Akaike Information criterium (AIC). Differences in the choices made by naïve caterpillars of the parental generation (that are not the parents of the F1 individuals), and by naïve offspring of the next generation were compared using a two-tailed Fisher exact test of independence. A pairwise nominal independence post hoc analysis provided adjusted *P*-values for multiple comparisons. Data from the banana and the mango odor treatments were analyzed separately.

All analyses were performed in the R statistical framework (R Development Core Team 2008; RStudio Team 2016), with the packages Rmisc (Hope 2013), lme4 (Bates et al. 2015), car (Fox and Weisberg 2011), rcompanion (Mangiafico 2018), multcompView (Graves et al. 2015), and lsmeans (Lenth 2016), with the help of McDonald (2014) and Mangiafico (2015, 2016).

Results

We first tested and confirmed that ethanol-coated leaves were an appropriate control treatment to compare with the experimental treatments of odor-coated leaves. Odor-mediated choices, performed at 5, 10, 15, and 20 days after larvae hatched from the eggs, showed that larvae fed on noncoated leaves cannot distinguish an ethanol-coated leaf from a noncoated leaf (Fisher exact test, $n_{\text{day5}} = 181, n_{\text{day10}} = 135, n_{\text{day15}} = 94, n_{\text{day20}} = 64, P\text{-value} = 0.35$) (Fig. 2; Supporting Information. File 1). Ethanol-coated leaves, thereafter named control leaves or just controls, were thus used as the control treatment in all subsequent experiments, whereas leaves coated in novel odors diluted in ethanol, odor leaves, were the experimental treatments.

NAÏVE LARVAE HAD A PREFERENCE FOR CONTROL ODORS ONLY IN THE BANANA CHOICE ASSAY

We next tested whether naïve larvae, upon emergence, showed a preference toward control or odor leaves with four different odors (Fig. 3; Supporting Information File 1). Naïve larvae did not distinguish between control and almond-, coffee-, or mango odors (almond: $n = 100$, chi-squared $= 1.44$, df $= 1$, *P*-value $= 0.23$; coffee: $n = 100$, chi-squared $= 4.00$, df $= 1$, *P*-value $= 0.05$; mango: $n = 267$, chi-squared $= 0.71$, df $= 1, P$-value $= 0.40$) (Fig. 3A, C, and D). However, naïve larvae when given a choice between banana odors and controls significantly preferred controls ($n = 100$, chi-squared $= 5.76$, df $= 1$, *P*-value $= 0.02$) (Fig. 3B).
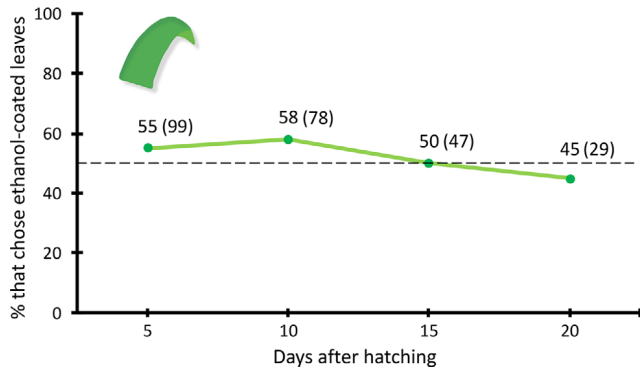
Figure 2. Larvae have no preference for ethanol-coated or non-coated maize leaves and the former were used as control leaves for all remaining experiments. Eggs were collected and the larvae were fed with noncoated leaves. At day 5, 10, 15, and 20, each larva was tested for its choice between uncoated and ethanol-coated leaves using the choice assay. Percentage values are denoted above each point and the corresponding total sample sizes are shown in brackets.

LARVAE FED ON BANANA AND MANGO ODORS ACQUIRED A PREFERENCE FOR THESE ODORS OVER THE COURSE OF DEVELOPMENT

Larval odor preferences, that is, choices that are significantly different from random choices (red circles in Fig. 3), changed over the course of larval development for banana and mango odor-fed larvae (Supporting Information File 1, Fig. 3B, D). In larvae that were fed on banana odor, a significant preference for controls in naïve larvae changed to a significant preference for banana odors on days 5, 15, and 20 (day 5: $n = 123$, chi-squared $= 5.08$, df $= 1$, P-value $= 0.02$; day 10: $n = 83$, chi-squared $= 3.48$, df $= 1$, P-value $= 0.06$; day 15: $n = 69$, chi-squared $= 22.04$, df $= 1$, P-value $= 2.67 \times 10^{-06}$; day 20: $n = 55$, chi-squared $= 8.02$, df $= 1$, P-value $= 0.05 \times 10^{-03}$; Fig. 3B). Naïve larvae did not display a preference for mango odors but at day 10 started showing such a preference after feeding on mango odors (day 5: $n = 110$, chi-squared $= 0.15$, df $= 1$, P-value $= 0.70$; day 10: $n = 118$, chi-squared $= 7.63$, df $= 1$, P-value $= 6.00 \times 10^{-03}$; day 15: $n = 110$, chi-squared $= 8.18$, df $= 1$, P-value $= 4.00 \times$
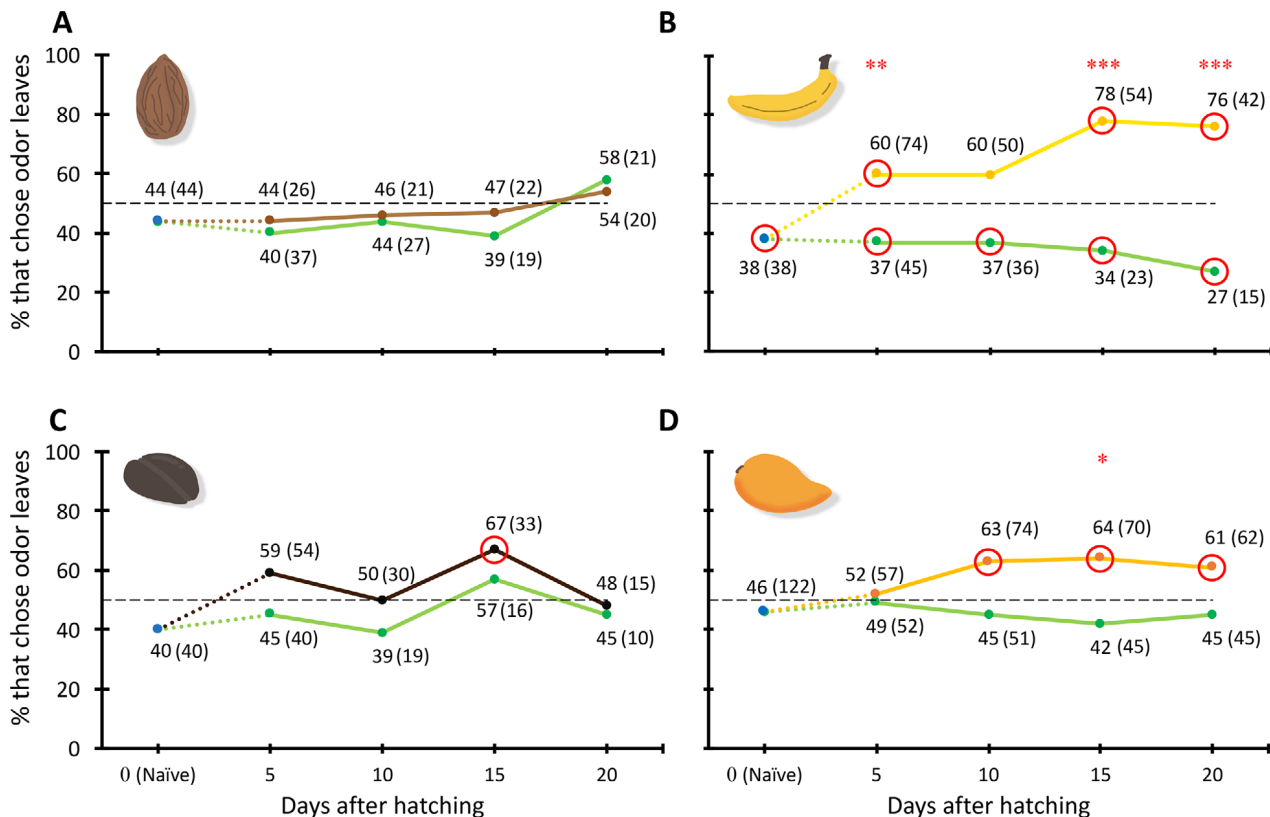

Figure 3. *Bicyclus anynana* larvae learn to prefer banana and mango odor-coated leaves but not almond or coffee odors. In each odor experiment, naïve larvae were either fed control (green lines) or odor leaves (brown, yellow, and orange lines). Each larva was tested for its preference during choice assays for either control leaves or odor leaves at days 5, 10, 15, and 20. A separate group of larvae were tested for their naïve preferences upon hatching, at day 0 (they are connected to the other data points via dashed lines). Percentage values are denoted near each point and the corresponding total sample sizes are shown in brackets. Red circles over plotted points represent significant preferences (deviations from random choice) and red asterisks at the top denote a significant difference between the choices of the odor- and control-fed larvae at a particular day of choice assay.

10^{-03}; day 20: $n = 101$, chi-squared $= 5.24$, df $= 1$, P-value $= 0.02$; Supporting Information File 1, Fig. 3D). Larvae did not display a naïve preference for controls over almond or coffee nor did they develop any preferences over the course of development (with the exception of coffee on day 15) (fed with almond: day 5, $n = 59$, chi-squared $= 0.62$, df $= 1$, P-value $= 0.43$; day 10, $n = 46$, chi-squared $= 0.35$, df $= 1$, P-value $= 0.56$; day 15, $n = 47$, chi-squared $= 0.19$, df $= 1$, P-value $= 0.66$; day 20, $n = 37$, chi-squared $= 0.24$, df $= 1$, P-value $= 0.62$; fed with coffee: day 5, $n = 91$, chi-squared $= 1.33$, df $= 1$, P-value $= 0.25$; day 10, $n = 60$, chi-squared $= 0.00$, df $= 1$, P-value $= 1.00$; day 15, $n = 49$, chi-squared $= 5.90$, df $= 1$, P-value $= 0.02$; day 20, $n = 31$, chi-squared $= 0.03$, df $= 1$, P-value $= 0.86$; Supporting Information File 1, Fig. 3A, C). Larval preference for banana odors at the end of the larval stage, day 20, was not significantly higher than preference for mango odors (Fisher exact test, $n_{banana} = 42$, $n_{mango} = 62$, P-value $= 0.39$).

LARVAE FED ON BANANA AND MANGO ODORS MADE DIFFERENT CHOICES FROM CONTROL FED LARVAE

The different feeding treatments also led to different larval choices over the course of larval development, primarily observed in the banana and mango experiments (Fig. 3). Individuals feeding on either the banana or mango odors chose these odors significantly more frequently than individuals feeding on control leaves, who chose controls more frequently (Table 1) (Fig. 3). Larvae feeding on coffee or almond odors performed similar choices to those feeding on controls (Table 1). The proportion of larvae fed on banana odors that choose the banana odors increased over development time (Table 1), suggesting that the acquisition of banana odor preferences strengthens throughout development. In the mango treatment, however, there were no significant differences between choices performed by larvae on days 5, 10, 15, and 20 (Table 1), suggesting that the gain of mango odor preferences happened, for the most part, early during development and was not reinforced with further exposure to the new odor.

LARVAL DIET DID NOT AFFECT ADULT OVIPOSITION CHOICE

We then examined the effect of larval diet on oviposition choice of adults. For these analyses, we did not include data from wild-type females mated with odor-fed males as these females were not subjected to prior treatments. Females from all treatments laid a roughly similar number of eggs on odor and control leaves, indicating that larval diet did not impact adult oviposition choice, and that this choice was not impacted by the two leaf odors tested, mango and banana (Fig. 4) (paired t-test or Wilcoxon signed-rank tests; banana experiment: control fed: $n = 770$, $t = 0.33$, df $= 9$,
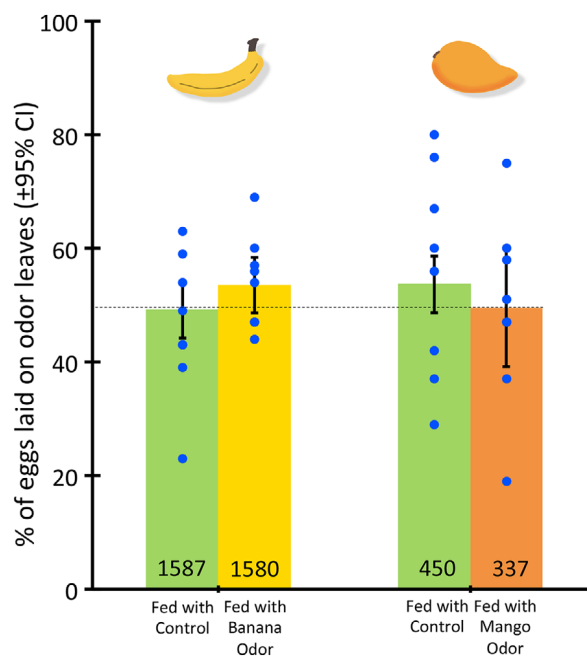

Figure 4. Females that were fed with banana or mango-coated leaves did not lay eggs preferentially on these leaves. Adult oviposition site preference measured via choice assays. Adult butterflies from each odor treatment were allowed to mate among themselves and each female was tested for its preference for either control- or odor-coated leaves for oviposition (only banana and mango odors were tested). The sample size is denoted at the base of each bar in the graph and blue dots are percentage of eggs laid on odor-coated leaves by each female of the treatment.

P-value $= 0.75$; banana odor fed: $n = 819$, $V = 21$, P-value $= 0.56$; mango experiment: control fed: $n = 234$, $t = -0.32$, df $= 8$, P-value $= 0.76$; mango odor fed: $n = 157$, $t = 0.14$, df $= 8$, P-value $= 0.89$).

PARENTAL AND PATERNAL DIET, AND OVIPOSITION LOCATION (FOR BANANA-TREATED PLANTS) AFFECTED THE NAÏVE ODOR CHOICE OF OFFSPRING

We then examined the effect of the larval diet of both the mother and the father ("parental diet"), the effect of larval diet of the father ("paternal diet"), and the effect of oviposition plant on naïve larval food choice of the next generation using the banana and mango odor experiments only. In the banana odor experiment (Fig. 5), both parental diet (banana odor and control leaves) and type of plant where eggs were laid (banana and control leaves) had a significant impact on naive offspring plant choice, with no significant interaction between the two factors (LRT, treatment × oviposition plant, chi-squared $= 0.41$, df $= 1$, P-value $= 0.52$; Table 1, Fig. 5). Further, parental diet and paternal diet alone had similar effects on offspring naive choice (LRT: chi-square $= 0.19$, df $= 1$, P-value $= 0.66$). Offspring of parents fed on banana odors chose banana odors more often than offspring of parents fed on

Table 1. Summary of the GLM likelihood ratio tests output (Figs. 3– 6).

Almond experiment: Food choice of parental larvae fed with almond or control odors

Full model variables	Chi-squared	df	*P*-value
Treatment (fed with almond or control odors)	0.344	1	0.557
Day after hatching	2.595	3	0.107
Treatment × day after hatching	0.071	3	0.791
Final model: null model			

Banana experiment: Food choice of parental larvae fed with banana or control odors

Full model variables			
Treatment (fed with banana or control odors)	67.627	1	$<2.000 \times 10^{-16}$
Day after hatching	2.652	3	0.448
Treatment × day after hatching	10.114	3	0.018
Final model variables			
Treatment	67.810	1	$<2.000 \times 10^{-16}$
Treatment × day after hatching	12.766	6	0.043

Coffee experiment: Food choice of parental larvae fed with coffee or control odors

Full model			
Treatment (fed with coffee or control odors)	4.137	1	0.051
Day after hatching	6.353	3	0.096
Treatment × day after hatching	0.303	3	0.959
Final model: null model			

Mango experiment: Food choice of parental larvae fed with mango or control odors

Full model variables			
Treatment (fed with mango or control odors)	19.164	1	1.199×10^{-5}
Day after hatching	0.703	3	0.872
Treatment × day after hatching	4.304	3	0.231
Final model variables			
Treatment	19.148	1	1.210×10^{-5}

Mango experiment: Choice of offspring of mango odor-fed individuals

Full model			
Treatment (parents fed with mango or control odors)	5.551	1	0.018
Number of parents treated	0.234	1	0.629
Oviposition plant	1.144	1	0.285
Treatment × number of parents treated	0.143	1	0.706
Treatment × oviposition plant	1.005	1	0.316
Number of parents treated × oviposition plant	0.037	1	0.847
Treatment × number of parents treated × oviposition plant	0.085	1	0.770
Final model			
Treatment	5.938	1	0.015

Banana experiment: Choice of offspring of banana odor-fed individuals

| Full model			
Treatment (parents fed with banana or control odors)	44.435	1	2.63×10^{-11}
Number of parents treated	0.192	1	0.662
Oviposition plant	4.342	1	0.037
Treatment × number of parents treated	0.047	1	0.829
Treatment × oviposition plant	0.409	1	0.522
Number of parents treated × oviposition plant	0.013	1	0.909
Treatment × number of parents treated × oviposition plant	0.498	1	0.480
Final model			
Treatment	61.516	1	4.393×10^{-15}
Oviposition plant	4.206	1	0.040
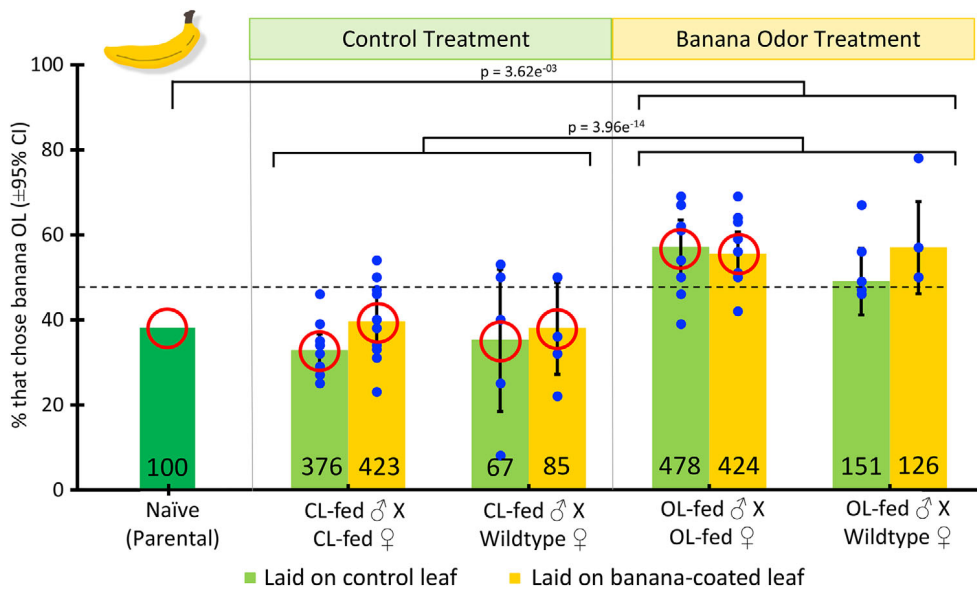
Figure 5. Banana odor preferences of naïve offspring are impacted by parental and paternal diet. Odor choices of naïve larvae of the parental generation (*X*-axis: naïve (parental)) and of naïve larvae of the offspring generation, whose parents underwent different diet treatments. These treatments, represented along the X-axis, include parental diets (control versus banana odor—green and yellow labels on the top of the graph), number of treated parents (adults fed the same diet were mated with each other or males were mated with a wild-type reared female—bottom labels along X-axis), and oviposition site (whether the eggs were laid on odor leaves [yellow bars] or control leaves [green bars]). The sample size is denoted at the base of each bar in the graph. Red circles on plotted bars represent a significant preference (random choice is depicted by the horizontal dashed line). Blue dots represent percentage per family. *P*-values from the Fisher test are displayed.
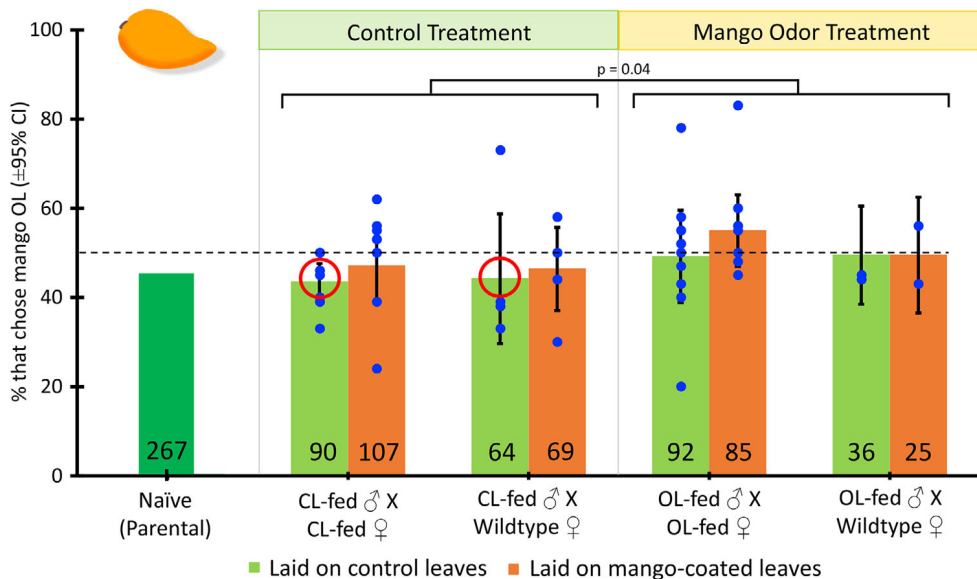

Figure 6. Mango odor preferences of naïve offspring are impacted by parental and paternal diet. Odor choices of naïve larvae of the parental generation (*X*-axis: naïve (parental)) and of naïve larvae of the offspring generation, whose parents underwent different diet treatments. These treatments, represented along the *X*-axis, include parental diets (control vs. mango odor—green and orange labels on the top of the graph), number of treated parents (adults fed the same diet were mated with each other or males were mated with a wild-type reared female—bottom labels along *X*-axis), and oviposition site (whether the eggs were laid on odor leaves [orange bars] or control leaves [green bars]). The sample size is denoted at the base of each bar in the graph. Red circles on plotted bars represent a significant preference (random choice is depicted by the horizontal dashed line). Blue dots represent percentage per family. *P*-values from the Fisher test are displayed.

controls who chose controls more often, regardless of the plant they were laid on, or of the number of parents fed on the diet (LRT, treatment alone: chi-squared = 44.45, df = 1, P-value = 4.38 × 10^{-15}; Table 1, Fig. 5). In addition, offspring from eggs laid on the banana odors chose the banana odors significantly more often than offspring from eggs laid on controls, who chose the controls more often, regardless of parental diet or number of parents fed on the diet (LRT, oviposition plant alone: chi-squared = 4.34, df = 1, P-value = 0.04; Table 1). Individuals from all families chose similarly (LRT; family: chi-squared = 1.90 df = 1, P-value = 0.17). In the mango odor experiment (Fig. 6), parental diet alone impacted naïve offspring plant choice. There was no effect of oviposition plant type and no interaction between these factors (Table 1, Fig. 6). Parental or paternal diet alone had similar effects on offspring naïve choice (LRT, parental origin: chi-squared = 0.23, df = 1, P-value = 0.63). Offspring of parents fed on mango odors chose mango odors significantly more often than offspring of parents fed on controls (P = 0.048, shown in Fig. 6), regardless of the leaves the eggs were laid on or the number of parents treated on the particular diet (LRT, treatment alone: chi-squared = 5.55, df = 1, P-value = 0.048; Table 1, Fig. 6). None of the interactions between factors were significant (Table 1). Individuals from all families chose similarly (LRT; family: chi-squared = 0, df = 1, P-value = 1).

NAÏVE OFFSPRING OF BANANA-FED PARENTS CHOSE BANANA ODOR MORE OFTEN THAN NAÏVE INDIVIDUALS OF THE PARENTAL GENERATION

Overall, the offspring of parents fed on banana odors made a different food choice from the parental generation, but that was not the case for the offspring of mango-fed parents. Offspring of parents fed with banana odors chose banana odors significantly more often than naïve individuals from the parental generation (Fig. 5) (Fisher exact test, $n_{\text{naive parents}} = 100$, $n_{\text{offspring of banana fed}}$ = 1179, $n_{\text{offspring of control fed}} = 951$, P-value = 2.78 × 10^{-14}; post hoc analysis, naïve parental larvae vs. banana odor-fed offspring, adjusted P-value = 3.62 × 10^{-03}). These Fisher exact tests also confirmed and extended the GLM results above: offspring of parents fed banana odor as larvae chose the banana odors more often than offspring of parents fed control leaves as larvae (adjusted P-value = 3.96 × 10^{-14}), whereas the offspring of parents fed control leaves as larvae chose similarly to the naïve individuals of the parental generation (adjusted P-value = 0.91). In the mango treatment, however, Fisher tests showed that the offspring of parents fed mango or control odors as larvae chose similarly to the naïve individuals of the parental generation (Fig. 6). These tests also confirmed that offspring of parents fed mango odor as larvae chose the mango odors more often than offspring of parents fed controls (Fisher exact test, $n_{\text{naive parents}}$

= 267, $n_{\text{offspring of mango fed}} = 465$, $n_{\text{offspring of control fed}} = 755$, P-value = 0.04; post hoc analysis, mango odor-fed offspring vs. naïve parental larvae, adjusted P-value = 0.25; control-fed vs. naïve parental larvae, adjusted P-value = 0.62; mango odor-fed larvae vs. control-fed larvae, adjusted P-value = 0.04).

NAÏVE OFFSPRING OF BANANA-FED PARENTS HAD A PREFERENCE FOR BANANA ODORS AS OPPOSED TO NAÏVE INDIVIDUALS OF THE PARENTAL GENERATION

Some naïve odor preferences, that is, choices that were significantly different from random, changed depending on diet and number of parents that were fed a particular diet. In the banana treatment, offspring of parents fed with control leaves preferred the control leaves, whatever the number of fed parents or the oviposition plant type (Fig. 5, Supporting lnformation File 1). Offspring of parents fed with banana odors significantly preferred the banana odors, whereas offspring of fathers fed with banana odors did not show a preference for either odor type (Fig. 5). In the mango treatment, only the offspring of both parents and of fathers fed control leaves, from eggs laid on control leaves, had a preference for the control leaves. The offspring from other treatments chose the mango and control leaves randomly (Fig. 6; Supporting lnformation File 1).

Discussion

We paired novel odors with the food plant that *B. anynana* larvae have been consuming in the lab since the beginning of their domestication in 1988, young maize plants (*Zea mais*), and larvae learned to prefer those odors during the course of their larval development. Larvae learned to associate banana and mango odors to food, but did not learn this association for coffee nor almond odors. Naïve larvae initially discriminated against banana odors, but learned to prefer and move toward these odors readily as they fed on banana odors, whereas larvae being raised on control leaves continued to prefer these odors throughout development. Larvae initially showed no discrimination toward mango odors but learned to prefer these odors as they fed on them by the end of larval development, whereas larvae being raised on control leaves continued to show no preference for these leaves at later stages of development.

The learned preferences for two fruity odors (banana and mango) versus lack of learned preferences for coffee and almond odors could be due to different starting concentrations for these odors or due to differences in innate genetic sensitivity toward these odors. Although we used 2% solutions for all odors, we were unable to obtain information about the exact molecular composition and concentration of the chemicals in these food odors from the company that made them, and it is possible that

the banana and mango solutions had higher concentrations of chemicals than the coffee or almond solutions. In addition, for an odor to be detected by a larva, the adequate chemo receptor genes for the different components of the odor have to be present in the genome, and these genes have to be expressed in sensory cells such as in the olfactory neurons that enervate the sensory sensilla in the larvae antennae or in the mouthparts (Hallem and Carlson 2006; Popescu et al. 2013; Gomez-Diaz et al. 2018). Although *B. anynana* larvae do not feed on fruit, odor receptor genes for fruit odors might be expressed in the appropriate organs before the adult stage of development (Gerber and Stocker 2007), when these receptors are actually useful to detect ripe fruit, which is the adult food of these butterflies. *Bicyclus anynana* adults have been feeding on mashed banana in the laboratory since 1988, and this feeding history might have selected for larvae/adults that are more sensitive to changes in this odor relative to the other odors, toward which adults have had relative little exposure. Mango odor might share some similarities to banana and lead to similar, yet less strong, responses in *B. anynana* larvae.

It is unclear whether *Bicyclus* larvae learned a preference for food laced with novel fruity odors via mechanisms of associative learning or simply odor imprinting. In associative learning, the larvae associates the novel odor with a food reward to later seek out the odor, whereas in odor imprinting, a simple exposure to an odor, that is unrewarded, leads to a later preference toward that odor (Schausberger et al. 2010). In our experiment, the two mechanisms cannot be separated because larvae were fed the food with the novel odor simultaneously. However, in experiments with other arthropods, unrewarded odor exposure was sufficient to illicit a preference for a food paired later with that odor in stingless honeybees (Roselino and Hrncir 2012), bumblebees (Molet et al. 2009), and in predatory mites (Schausberger et al. 2010). In the bumblebee experiment, however, associating the odor with sugar improved odor learning (Molet et al. 2009). This suggests that early exposure to a food odor, in isolation or in combination with associative learning, mediates food odor learning in arthropods.

Larval diet, however, had no impact on where the adult female laid eggs. This contradicts the natal habitat preference induction (NHPI) hypothesis, which proposes that adult oviposition should shift to the same plant consumed by the larvae, especially if the plant was of high quality (Davis and Stamps 2004; Moreau et al. 2008; Lhomme et al. 2018). These negative results, however, support those of similar experiments done in other lepidopterans (Zhang et al. 2007; Janz et al. 2009; Hu et al. 2018). The disconnect between feeding and oviposition behaviors might be due to the encoding of the odor learning experiences by two separate groups of glomeruli in the antennal lobe (Bisch-Knaden et al. 2018), or to different behavioral responses resulting from tissue-specific activation of the same set of odor receptors (Joseph and Heberlein 2012). In addition, larval food odor learning is likely

mediated via chemoreceptors present in neurons inside sensilla on the proboscis, labial palps, and antennae (Gerber and Stocker 2007), whereas oviposition choice is often mediated via chemoreceptors present in neurons inside sensilla on the tarsus tips of the legs (Briscoe et al. 2013; Takai et al. 2018). It is also possible that banana odor receptors might not be expressed in the neurons of the leg sensilla, leading to a disconnect between the larval food learning behavior and adult oviposition behavior. Alternatively, host oviposition preference might be aided by odors also sensed by the antennae but that are processed in a different way to larval odor learning (Bisch-Knaden et al. 2018). If this is the case, it is possible that had we reared the F1 generation of larvae to adulthood, we might have observed that those adults that laid on odor-coated leaves might have produced a new generation of adults with an actual preference for laying on these leaves, and different from the 50:50 choice observed in the F0 generation. This could be tested in the future.

Regardless of where the eggs were laid, the larval food experience of the parents, or merely that of the fathers, was transmitted to the next generation. Offspring of banana and mango odor-fed parents, or of banana odor-fed fathers alone, showed different innate choices toward banana and mango odors relative to offspring of control-fed parents. The offspring of banana odor-fed parents no longer discriminated against these food odors as individuals of the parental generation had done, and in one case (in the banana odor treatments of both parents) actually preferred it. These larvae started their lives equally likely or more likely to move toward a banana odor than toward a control odor. This change in innate behavior could be adaptive as larvae that learned to move toward the same food plant experienced by their parents are less likely to starve or delay their development compared to larvae that continue to express fixed aversive behaviors.

Odor preference learning and transmission of the preference was stronger for banana odors than for mango odors but it is unclear why we observed these results. *Bicyclus anynana* adults have been fed on banana in the laboratory since 1988 and individuals with greater banana odor sensitivity may have been under selection in the lab environment ever since that time, as sensitivity to this odor would enable them to find the banana food inside the cage more readily. A greater sensitivity to the particular fruity odor of banana instead of mango may have aided larvae in learning preferences for banana odors more readily, and also in passing on those preferences to their offspring, but this requires further investigation.

How a learned odor preference is transmitted to the next generation also needs to be examined at the mechanistic level. Data from bees suggest that changes in the expression of olfactory receptors and changes in DNA methylation take place during an odor learning experience (Claudianos et al. 2014; Biergans et al. 2017). Data from male mice trained to avoid a fruity odor

showed that the odor receptor became demethylated in sperm cells and the male's offspring had higher sensitivity to the odor (Dias and Ressler 2014). This type of mechanism has not yet been observed in insects, however, data from flies trained on odors, and who transmitted a biased odor approach response to the F1 and F2 generations, showed disruption of this response when both olfactory-receptor and dorsal-paired-medial neuron input into the mushroom bodies was abolished (Williams 2016). In addition, odor molecules themselves or noncoding RNAs might have been transmitted via the cytoplasm of the egg or sperm, or via the spermatophore to somehow impact the naïve behavior of F1 larvae (Bertin et al. 2010; Bohacek and Mansuy 2015).

Interestingly, in the banana odor treatment alone, oviposition plant also had an influence on offspring naïve preference, that is, larvae that hatched on the banana odors chose the banana odors significantly more frequently than larvae that hatched on control odors, regardless of parental diet treatment or number of treated parents. This might be because the outside shell of the embryos, the chorion, was contaminated with the banana odor molecules present on the surface of the leaves, and these molecules, potentially ingested when larvae ate their chorion upon hatching, contributed to odor learning in these larvae. The eggs were in contact with the leaves for up to 12 hours, before they were stripped from the plants, allowing chorion contamination. Chorion contamination, however, cannot explain the significant innate choice shift toward banana odors for those larvae that hatched on control odors but whose parents were fed on banana odors as larvae. Some other transmission mechanism had to be involved there.

Mechanisms of food odor learning and inheritance need to be investigated in future studies, but perhaps using more realistic plant volatiles as "novel odors" and/or pure chemicals that can be mapped to single odor receptors. Future work should also test whether learned odors continue to be inherited past the first generation, or whether these effects are single generation biparental, maternal, or paternal effects. In addition, it will be interesting to test whether odor learning, as demonstrated here, is a possible adaptive response shaped by natural selection. The current work showed transmission of a learned odor preference in larvae of an herbivore insect across a single generation but more experiments with other species are needed, both generalist and specialist herbivores, to further assess how this type of inheritance might impact herbivore host switching and ecological speciation.

AUTHOR CONTRIBUTIONS
FMP designed and performed a pilot experiment for the study. AM designed the final study. VG and AV collected all the data. VG made the figures. ED analyzed the data. AM wrote the manuscript with input from all the authors. AM and ED contributed to the revisions. VG and ED contributed equally to this work. All authors declare no conflict of interest.

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Associate Editor: E. Tibbetts
Handling Editor: D. Hall

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary file 1. Statistical results of preference tests (from Chi-squared test of goodness of fit – see method section of the main article).
Supplementary file 2. Data set.