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Acquired preferences for a novel food odor do not become stronger or stable after multiple generations of odor feeding in *Bicyclus anynana* butterfly larvae

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Abstract

Many herbivorous insects have specific host–plant preferences, and it is unclear how these preferences evolved. Previously, we found that *Bicyclus anynana* larvae can learn to prefer novel food odors from eating leaves with those odors and transmit those learned preferences to the next generation. It is uncertain whether such acquired odor preferences can increase across generations of repeated odor feeding and be maintained even in the absence of odor. In this study, we fed larvae with novel banana odor-coated leaves (odor-fed larvae) for five consecutive generations, without selection on behavioral choices, and measured how larval innate preferences changed over time. Then, we removed the odor stimulus from a larval subgroup, while the other group continued to be odor-fed. Our results show that larvae learned to prefer the novel odor within a generation of odor feeding and transmitted the learned preference to the next generation, as previously found. Odor-fed larvae preferred odor significantly more compared to control larvae across five generations of repeated odor or control feeding. However, this led neither to increased odor preference, nor its stabilization. This suggests that when butterfly larvae feed on a new host, a preference for that novel food plant may develop and be transmitted to the next generation, but this preference lasts for a single generation and disappears once the odor stimulus is removed.

KEYWORDS
Bicyclus anynana, epigenetics, Lepidoptera, odor learning, transgenerational inheritance

INTRODUCTION

Both Jean-Baptiste Lamarck and Charles Darwin claimed that any trait or characteristic acquired by an organism during its lifetime could be inherited by its offspring.[1] These initial ideas were later opposed by August Weismann, who proposed the separation of somatic and germ cells and suggested that hereditary information can be carried only by the germline, and that these cells are not influenced by external environments.[2] However, there have been various studies in the past three decades showing that acquired traits can be inherited by subsequent generations.[3–8] In these recent studies, however, and due to the limited number of generations examined, it is still unclear whether such inherited acquired traits can be stabilized and maintained over time to have an impact on trait evolution.

A proposed mechanism for encoding an environmentally induced phenotype into the genome is the mechanism of genetic assimilation. This mechanism was described initially by Waddington in *Drosophila*[9,10] and was replicated later by others in *Drosophila*[11–15] and other systems.[16–20] For instance, environmentally induced phenotypes, such as the production of bithorax flies after exposure of

embryos to ether vapor, could become genetically encoded when flies were subjected to several generations of artificial selection. This means that the phenotype was eventually expressed even in the absence of the causative environmental stimulus. While this type of result is compatible with standard population genetics and evolutionary theory, it is still unclear whether stabilization of an original, environmentally induced phenotype can happen without the action of selection by simply using repeated exposure to the environmental stimulus across several successive generations.

There are several newer studies, however, that support transgenerational inheritance and stabilization of an environmentally acquired trait in the absence of artificial selection. These studies involve the inheritance of a learned response to an odor. For example, mice exposed to the odor acetophenone coupled with a mild electric shock produced a startle behavior upon further exposure to the odor alone. This startle behavior was inherited by F1, as well as F2, offspring, when exposed to the odor alone (i.e., without the shock).[21] When first instar larvae of *Caenorhabditis elegans* were exposed (for 12 h) to dilutions of attractive odors, like benzaldehyde and citronellol, the learned novel odor preference was inherited by their offspring. If those offspring were exposed to the same odor for at least five consecutive generations, the odor preference was inherited for the next successive 40 generations even in the absence of the odor.[22] In a subsequent study, researchers discovered that a longer period of odor imprinting in the first generation of *C. elegans* (from 12 to 60 h) led to a stable inheritance of the odor preference after just one generation.[23] Thus, all these studies support the stabilization of transgenerational inheritance of a learned response independent of selection. Yet, the results appear to depend on the number of generations of exposure to the stimulus, stimulus strength, potentially the stimulus itself (e.g., odor), and the model system used. In Lepidoptera, however, there is barely anything known regarding the possibilities of stabilization and maintenance of learned odor preferences via repeated instances of odor exposure across generations.

In our experiments, we used larvae of the model lepidopteran *Bicyclus anynana* to test whether a stable and fixed preference for a novel odor stimulus can evolve via simple repeated exposure to that odor and without the use of selection. *B. anynana* is an African tropical nymphalid butterfly whose larvae are oligophagous and feed on different grass species.[24–26] Since laboratory domestication in 1988, however, the larvae have been feeding only on corn plants, *Zea mays*.

We chose this species because it is amenable to multiple generations of rearing in the lab and because it has shown to learn preferences for a variety of stimuli after brief exposures to those stimuli, as well as the ability to transmit some of these preferences to the next generation. For example, female *B. anynana* adult butterflies can learn to prefer different wing patterns[27] and manipulated odor signals[28] in mate choice experiments if they are briefly exposed to those signals early in adult life. Female adult butterflies can also learn to prefer novel sex pheromone blends if exposed to these novel blends early in adult life and then pass these learned preferences to their offspring.[29,30] *B. anynana* larvae can also learn to prefer novel odors added to their host plant via the consumption of those odors. In addition, the larvae show an increasing preference for a novel banana odor across their larval development and pass these learned preferences to their offspring, at least across a single generation.[31] In the current series of experiments, we used similar plant odor learning by larvae of *B. anynana* across multiple generations to test whether (1) a preference can evolve for the novel odor in an increasing trend over multiple generations of odor exposure; and (2) whether a stable and fixed preference for a novel larval host plant odor can evolve following the removal of the odor.

We reared groups of *B. anynana* larvae for several generations on two distinct diets that differed by an odorant—a single chemical compound. To test if a preference for this odor can increase with the number of generations of exposure, naive odor preferences were recorded for each generation as soon as the eggs hatched for a total of five generations. Considering the findings from our previous study,[31] we expected the odor-exposed larvae to learn to prefer the new odor after the first generation of exposure. Moreover, we hypothesized that this learned preference increases with additional generations of odor exposure.

To test whether the offspring of five-generation odor-exposed larvae continued to prefer the odor, even in the absence of odor exposure, we replaced the diet of the odor group with ethanol-coated leaves and tested their naive odor preferences across two more generations. This allowed us to investigate if the learned preference had become fixed and long-lasting even after the odor was removed. We hypothesized that five consecutive generations of odor exposure is sufficient to stabilize the learned odor preference, leading larvae to choose the odor for a few additional generations beyond the last exposed generation.

METHODS

Husbandry

B. anynana were reared in a climate-controlled room at 27°C, 60% humidity, and 12:12-h light:dark photoperiod. However, we reared all F0 larvae and the early stages of F1 larvae at 17°C but with the same humidity and photoperiod conditions in the middle of their larval developmental stage. This was due to unavoidable circumstances during the early COVID-19 pandemic period during which we were given restricted access to the campus and needed to slow down larval development. All choice assays, however, were conducted at 27°C. Larvae were fed on organic corn plants (*Z. mays)* that were sourced from Fire Flies and Greenology farms, in Singapore. Butterflies were fed on mashed bananas. Wild-type embryos were collected from corn leaves that were placed inside the adult cages. The eggs were stripped from the leaves and stored in a Petri plate. Upon hatching, the larvae were tested for their innate odor preferences and were randomly separated and assigned to control and odor treatments.

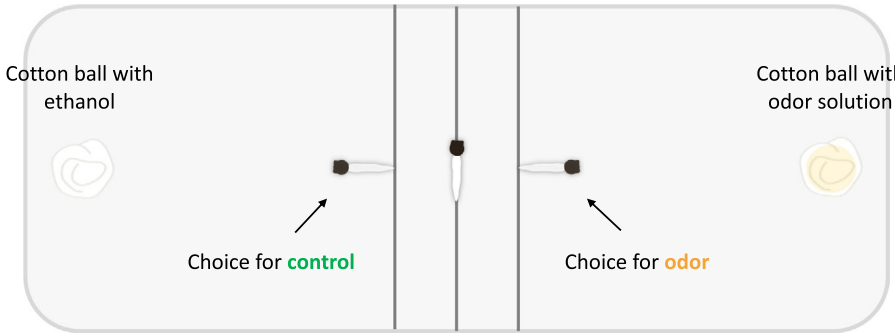

FIGURE 1 Experimental setup for odor choice assay.

Preparation of scented leaves

In previous experiments, we used a "banana odor essence," a food additive of unknown chemical composition as one of our test odors,[31] but in the current experiments, we used a single chemical, isoamyl acetate (IAA), also known as isopentyl acetate, from Sigma-Aldrich (W205532, natural, ≥97%, Food Chemicals Codex, Food Grade) as our experimental odor. IAA is an organic ester compound that is one of the most abundant volatile compounds naturally present in ripened bananas.[32–34] It strongly smells and tastes like banana, and is used in imparting banana flavor in edibles. This common fruit ester is a chemoattractant to the algae *Tetrahymena pyriformis*, *Drosophila melanogaster*, and *C. elegans*[35] and is widely used in odor learning studies.[36–42] IAA is only slightly soluble in water; hence, we used ethanol as the solvent in preparing odor solutions. A 5% IAA odor solution, hereafter called the "odor solution," was prepared by diluting IAA in absolute ethanol (Fisher Chemical, 99.8%, analytical reagent grade). Absolute ethanol was used as the control solution.

We reared two groups of larvae on corn leaves coated with ethanol (control groups feeding on control leaves) and two groups on leaves coated with the IAA solution (odor groups feeding on odor leaves). Corn leaves were coated thoroughly on both sides by rubbing with solution-dipped cotton. Ethanol from the coated leaves was allowed to evaporate completely before feeding those leaves to the larvae. The leaves were replaced with freshly coated leaves every 2 days, or sooner if needed to ensure continuous odor exposure.

Odor choice assay

A preference for either control or banana odor was determined for naive, nonfed larvae within 16 h after hatching using an odor choice assay. All choice assays were performed in the 27°C, 60% humidity, climate-controlled room. The choice assay used a white plastic board as the arena (Figure 1). A line was drawn in the middle of the board. Another line was drawn 3 mm away from the middle line on either side. This length of 3 mm corresponds to the average length of the newly hatched larva. Using a dropper, five drops of control solution were added to a small cotton ball of approximately 1 cm in diameter which was placed 12 cm away from the middle line. Similarly, five drops of odor solution were added to another cotton ball that was placed on the other end. The cotton balls were then left to dry for a few minutes before proceeding with the assay. To determine the odor choice, each larva was placed along the middle line. An almost opaque white box (26.5 cm × 9 cm × 5 cm) was used to cover the behavioral assay setup. This prevented larvae from perceiving any kind of surrounding visual or odor cues. At each inner end of the box, a small piece of green tape was applied to attract the larva to move toward the ends of the box and not toward the sides. Each larva was given 4 min to make a choice. At the end of the 4 min, if the larva had crossed the line nearer to the control side, it was noted as a "choice for control." If it had crossed the line nearer to the odor side, it was noted as a "choice for odor." If it had not made any decision at the end of the 4 min, it was noted as "no choice."

Rearing of larvae on their respective treatments

After the initial choice assay, F0 larvae were randomly separated into two treatments, either control (C) or banana odor (B), with two experimental replicates each (Figure 2). Irrespective of choices made during the choice assay (i.e., in the absence of artificial selection), all the larvae were reared for the next generation. The larvae of each treatment replicate were reared in a cubic net cage (30 cm × 30 cm × 30 cm) that was kept inside a large transparent, air-tight, plastic storage box to prevent any kind of odor interference. Pupae from each cage were transferred to a labeled cylindrical mesh cage for adult emergence. Emerged butterflies from each cage were allowed to mate with other individuals of that same cage. Eggs were collected in labeled Petri plates. After hatching, naive larval odor preferences were determined again and, regardless of the outcome, the larvae continued to be fed the same odor as their parents. Larvae were reared in the respective odor environments for five generations. After five generations, the naive offspring from the odor-fed F5 parents were randomly separated (i.e., regardless of the choice outcome) into a control treatment (banana odor-control group, BC) and a banana odor treatment (B). Larvae of the BC group were fed with control leaves, and B group larvae were continued to be fed on the odor leaf diet. Larvae from these treatments, including those of the control group (C), were reared for two more generations and their naive odor choices were determined at each generation (Figure 2).

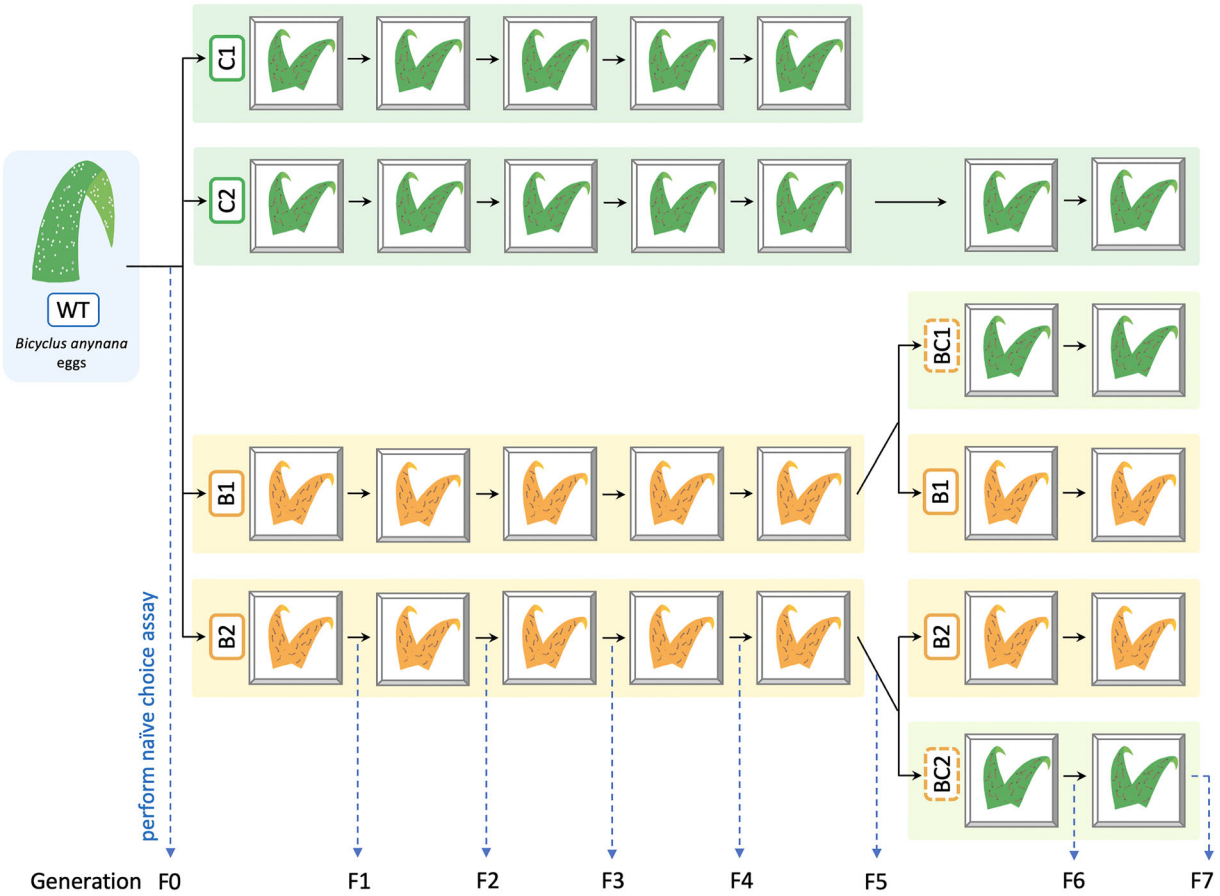

FIGURE 2 Experimental setup for determining how odor learning alters naive odor choice over several generations of odor feeding and whether that learned odor preference is maintained even after the removal of the odor. *B. anynana* eggs were collected and a naive odor choice assay was performed with the newly hatched larvae (F0), where they chose between control and odor. The larvae were then randomly assigned to either control (C) or banana odor treatments (B), with cage replicates C1, C2 and B1, B2, respectively. Note that the leaves are color-coded here in this figure to represent the treatments (control = green) and (odor = orange). Larvae were fed their respective diets throughout development. Adult butterflies from each cage mated among themselves. Eggs were collected on uncoated leaves, the naive odor choice assay was performed on newly hatched larvae, and the generations were continued to be reared in the same way. While C2 was reared till F7, C1 was reared till C5. After F5, B1 and B2 were further split into two treatments (BC1 and BC2, respectively). B1 and B2 continued to be fed on odor leaves, while BC1 and BC2 were fed on control leaves.

Statistical analysis

Pooling replicate cages

To simplify subsequent analyses, we tested whether we could pool data from the two cage replicates performed for each treatment. For this analysis, we initially considered cage as a fixed effect in a binomial generalized linear model (GLM). Upon the initial run of the model, the variable "cage" was found not to have any explanatory power or impact on larval choices of neither C, B, or BC treatments. Thus, in subsequent runs of the model, we used pooled cage replicates data in our main graph (Figure 3). Another graph with unpooled cage replicates can be found in the Supporting Material (Figure S1). The numbers of larvae tested and reared were recorded at each generation (Table 1). For all statistical analyses, we only used data from larvae that made a choice.

Testing for larval odor preferences

Chi-squared test of goodness of fit was used to test if the proportion of larvae belonging to each treatment that choose either control or odor at each generation was significantly different from a 50/50 random choice. If a significant deviation from 50% was found, the choice made was considered a preference. In the following results, initials are used to denote each treatment group at a specific generation. For example, F1B refers to the banana odor treatment larvae at generation F1; F4C refers to the control treatment larvae at generation F4; and F6BC refers to the banana odor-control treatment larvae at generation F6.

Testing for differences in odor choice over generations and between treatments

We tested the effects of larval treatment (C or B), the generations (F1, F2, F3, F4, F5), and their interactions on the larval odor choices using

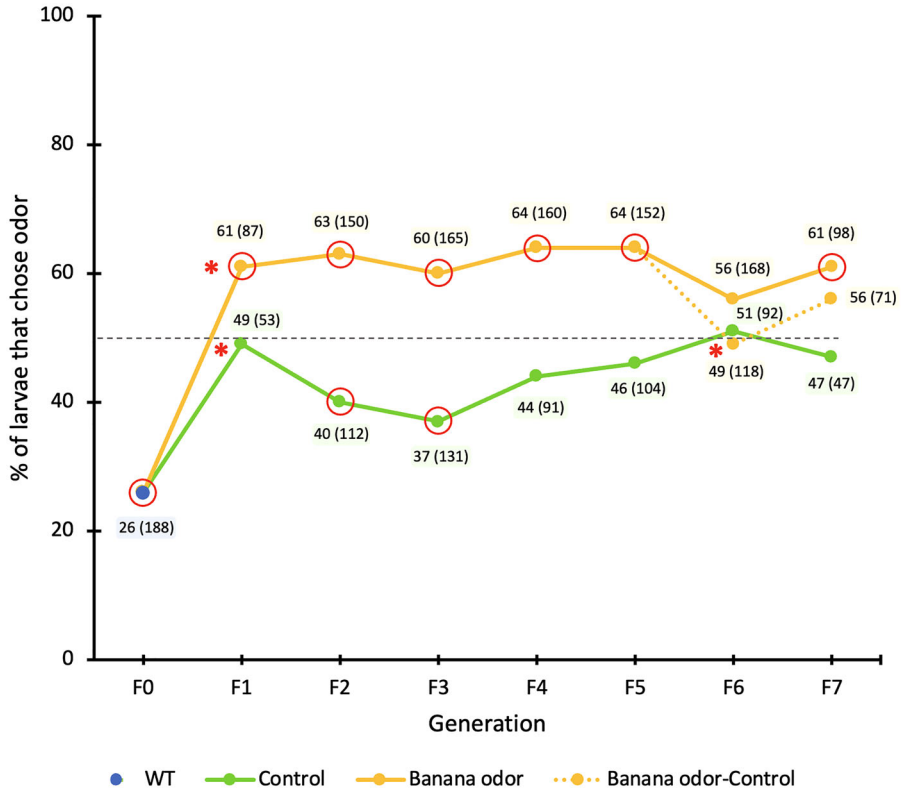

FIGURE 3 Naive preferences of *B. anynana* larvae over the course of several generations of distinct diet treatments. Naive larvae (blue dot) were fed with either control (green line) or odor leaves (orange line). A subpopulation of larvae that were fed on odor leaves were removed from the odor environment after F5 and fed on control leaves (orange dashed line). Percent of larvae that chose odor is denoted near each point and the corresponding total sample size (number of larvae that made a choice) is shown in parentheses. See Table 1 for the numbers of larvae tested and reared each generation. Red circles outlining specific data points represent significant preferences ($p \leq 0.05$; deviations from random choice). Red asterisks near data points denote a significant difference in preference from the previous generation ($p \leq 0.05$).

binomial GLM. We coded choice for control as 0 and choice for odor as 1 in the logit link function. We tested for factors that contributed to explain variation in the dependent variable using likelihood ratio tests (LRT), and subsequently removed the nonsignificant factor and interaction from the final model. The choices made by the naive larvae of both treatments were compared using pairwise post hoc analysis using least square means and logarithms of odds ratio.

Testing for differences in odor choice over generations and among treatments after removal of odor from diet

We tested the effects of larval treatment (C, B, or BC), generation (F6, F7), and their interactions on the larval odor choices using binomial GLM. We coded choice for control as 0 and choice for odor as 1 in the logit link function. We tested for the significance of factors using LRT and subsequently removed all nonsignificant factors and interaction from the final model.

Testing for differences in odor choice between naive F0 and F1, and between F5 and F6 across treatments

We tested for significant differences for either an increase or decrease in the odor choice made by the naive F0 parental generation and the F1 using chi-squared tests. The same analysis was used to compare the odor choices of F5 and F6 larvae across treatments.

All statistical analyses were performed in the R statistical framework (RStudio Team 2022), with the packages lsmeans,[43] lme4,[44] rcompanion,[45] car,[46] multcompView,[47] and Rmisc.[48]

RESULTS

Replicate cages of each treatment exhibited similar odor choice

We first tested if replicate cages of each treatment and at each generation showed different odor choices. Replicate cages of each treatment showed similar odor choices across generations (post hoc comparison, C1–C2 [F1–F5], $p = 0.94$; B1–B2 [F1–F7], $p = 0.73$; BC1–BC2 [F6–F7], $p = 0.74$), and hence were pooled together for the subsequent analyses.

F1 larvae of parents fed on odor showed an innate preference for odor, unlike their parents

We tested whether naive larvae, upon emergence, showed a preference toward either control or odor. Naive F0 larvae that were not exposed to any of the test odors showed a significant preference for

TABLE 1 Dataset showing the numbers of larvae tested and reared in each generation (see also Figure 3 and Figure S1).

Generation	Treatment	Replicate cage	No. of larvae that made a choice	No. of larvae that were used in choice assay	No. of larvae reared[a]
F0	N/A	–	188	400	400
F1	C	C1	51	91	100
		C2	2	4	36
	B	B1	15	33	33
		B2	72	100	100
F2	C	C1	77	100	≥100
		C2	35	47	≥47
	B	B1	81	100	≥100
		B2	69	100	≥100
F3	C	C1	71	100	≥100
		C2	60	100	≥100
	B	B1	82	100	≥100
		B2	83	100	≥100
F4	C	C1	65	88	≥88
		C2	26	30	≥30
	B	B1	84	100	≥100
		B2	76	100	≥100
F5	C	C1	30	37	≥37
		C2	74	100	≥100
	B	B1	85	100	≥100
		B2	67	100	≥100
F6	C	C2	92	100	≥100
	B	B1	74	100	≥100
		B2	94	100	≥100
	BC	BC1	70	100	≥100
		BC2	48	69	≥69
F7	C	C2	47	61	≥61
	B	B1	62	100	≥100
		B2	36	44	≥44
	BC	BC1	69	100	≥100
		BC2	2	3	≥3

[a]The exact number of larvae reared was not recorded from F2 onward, but it was equal to or larger than the number tested that generation.

control (F0: $n = 188$, chi-squared = 45.02, df = 1, $p = 1.95\times10^{-11}$; Figure 3, red circle), supporting previous results.[31]

We next tested if these naive larvae, when fed with odor leaves throughout their larval stages, learned to prefer the odor and transmitted the learned preference to the next generation. The significant preference for control in F0 changed to a significant preference for odor in the F1 generation after larvae were fed on odor leaves for one generation (F1B: $n = 87$, chi-squared = 4.15, df = 1, $p = 0.04$; Figure 3, red circle). In addition, the F1 naive larvae of the odor treatment showed a significant increase in preference for odor when compared to their parental generation F0 (F0: $n_{odor} = 48$, F1B: $n_{odor} = 53$, chi-squared = 30.55, df = 1, $p = 3.26\times10^{-8}$; Figure 3, red asterisk).

We found that the offspring of F0 parents that fed on control leaves (F1C) showed a significant increase in their choice for banana odor relative to their parents (F0: $n_{odor} = 48$, F1C: $n_{odor} = 26$, chi-squared = 9.68, df = 1, $p = 0.002$; Figure 3, red asterisk). However, these F1C larvae did not show a preference for the banana odor, but made a random choice instead (F1C: $n = 26$, chi-squared = 0.02, df = 1, $p = 0.89$; Figure 3).

Learned odor preferences did not increase with multiple generations of odor feeding

To test whether the learned odor preference increased across generations of odor exposure, we examined the choices made by the subsequent generations of odor-fed larvae. Naive larvae of generations F2, F3, F4, F5, and F7 showed a significant preference for odor, as did

TABLE 2 Summary of the binomial generalized linear model likelihood ratio tests.

	df	Deviance	*p*-value
Naive odor choice of F1–F5 larvae			
Full model variables			
Treatment (control, banana odor)	1	48.18	2.33×10^{-12}
Generation (F1, F2, F3, F4, F5)	4	3.24	0.52
Treatment × generation	4	1.44	0.84
Final model variable			
Treatment	1	49.18	2.33×10^{-12}
Naive odor choice of F6–F7 larvae			
Full model variables			
Treatment (control, banana odor, banana odor-control)	2	3.04	0.22
Generation (F6, F7)	1	0.77	0.38
Treatment × generation	2	1.08	0.58
Final model: null model			

Abbreviation: df, degrees of freedom.

F1 larvae (F2B: $n = 150$, chi-squared = 9.63, df = 1, $p = 0.002$; F3B: $n = 165$, chi-squared = 6.6, df = 1, $p = 0.01$; F4B: $n = 160$, chi-squared = 13.23, df = 1, $p = 0.0003$; F5B: $n = 152$, chi-squared = 12.74, df = 1, $p = 0.0004$; F7B: $n = 98$, chi-squared = 4.94, df = 1, $p = 0.03$; Figure 3, red circles). Odor-fed larvae chose the odor significantly more compared to control-fed larvae (post hoc comparison, B–C, $p = <0.0001$; Figure 3 and Table 2). However, a ceiling effect was observed across F1–F5, as the proportions of odor-fed larvae that chose odor was similar across generations.

We also examined whether control-fed larvae developed a preference for control over five generations. Although F1 larvae of the control treatment did not show a preference for control, F2 and F3 control-fed larvae preferred control (F2C: $n = 45$, chi-squared = 4.32, df = 1, $p = 0.04$; F3C: $n = 48$, chi-squared = 9.35, df = 1, $p = 0.002$, Figure 3, red circles). However, subsequent generations of control treatment larvae displayed no preference (F4C: $n = 40$, chi-squared = 1.33, df = 1, $p = 0.25$; F5C: $n = 48$, chi-squared = 0.62, df = 1, $p = 0.43$; F6C: $n = 47$, chi-squared = 0.04, df = 1, $p = 0.83$; F7C: $n = 22$, chi-squared = 0.19, df = 1, $p = 0.66$, Figure 3).

Larvae where odor food was replaced with control food showed a decrease in preference for odor

We then examined whether the larvae that had been fed on odor leaves for five generations maintained the learned preference even in the absence of odor. When the odor-fed larvae were fed with control leaves from F5 onward, they lost their preference for odor at generations F6 and F7 (F6BC: $n = 58$, chi-squared = 0.03, df = 1, $p = 0.85$; F7BC: $n = 40$, chi-squared = 1.14, df = 1, $p = 0.29$; Figure 3). The odor-control larvae chose odor significantly less frequently compared to their parents (F5B: $n_{odor} = 98$, F6BC: $n_{odor} = 58$, chi-squared = 5.78, df = 1, $p = 0.02$). These results suggest that five generations of odor treatment cannot maintain larval preference for odor once the odor stimulus is removed from the diet.

DISCUSSION

Learning novel odor cues of a compatible host plant and passing a preference for those cues to the next generation can be crucial for the survival of both parents and offspring. Here, we confirmed previous results showing that when a novel odor is associated with the typical food plant of a lepidopteran larva, an innate avoidance for that odor can be transformed into a preference in naive larvae of the next generation.[31] However, now we show that five generations of odor exposure/consumption did not lead to an increase in odor preference nor to the stable maintenance of the preference in *B. anynana* larvae. Larvae lost this preference once the odor-laced food of their parents was replaced by control food.

Here, we have used IAA as the novel odor that was added to the typical larval food of corn leaves of *B. anynana*. IAA is abundantly present in ripe bananas. It is important to note that for the last 35 years, standard husbandry practice (including this study) of a banana diet for *B. anynana* adults is common.[49] In spite of this diet, there was no impact on the innate dislike that the larval stages had toward this odor. This indicates that larvae can sense this odor and that this odor was appropriate to use in an experiment examining the development of larval learned preferences. Our results show that most naive larvae were repelled by this odor. But, by adding this chemical compound to the larval diet in the parental generation, the naive offspring developed a preference for this odor. This is similar to what we found in our previous study,[31] where we used a culinary banana essence (with unknown chemical composition) instead of IAA.

It is unclear why F1 control-fed larvae displayed no preference, but we hypothesize that the lower temperatures experienced by F0 and/or

the early F1 larvae might have been responsible for this result. We reared the F0 larvae/adults in a cooler temperature and relocated the F1 offspring to a higher temperature just before performing the choice assays (see Methods). F1 control-fed larvae chose control and odor randomly, and this is different to what we had observed in our previous study where offspring of control-fed larvae still showed a preference for control.[31] Volatility and aerial concentrations of odorants change with temperature,[50] and increased aerial volatility of odors with temperature induces decreased odor sensitivity in *Drosophila*.[51] The peculiar F1 random choices observed in our study might be connected to a similar decrease in odor sensitivity at higher temperatures before a slower physiological adaptation takes place, resulting in larvae choosing the odors randomly. However, odor perception at different temperatures varies among insect species,[52–54] and it is uncertain how *B. anynana* perceived the IAA odor at these different temperatures. We propose, however, that higher temperatures might have ultimately affected our F1 innate odor choice results.

Separately from the temperature effect, the higher proportion of offspring of control-fed larvae that chose odor in the F1–F7 generations relative to their F0 parents may also be due to the brief exposures of their parents to the odor during the choice trial. All these larvae had parents that were subjected (once) to a 4-min exposure to the odor during the choice assay. This single brief exposure to odor, which continued every generation in control animals, might have been enough to alter the baseline preference of these animals relative to the parental generation. This single odor exposure of the parents, however, was never sufficient to create a preference for odor in their offspring. It merely created a lack of preference.

While we demonstrated differences in odor preference across our two treatment groups, the way we conducted the choice assay might have limited the discovery of larger effect sizes for odor preference learning and inheritance. The choice assay arena, when covered with the lid, had a volume of only 1.2 L. This would have resulted in a high aerial concentration of IAA on the odor side that would have declined via diffusion within seconds or minutes. This might have prevented the larvae from having a clear choice of direction to move toward due to the disappearance of the odor gradient. Future choice assay experiments should use the classic Y-maze with continuous air flow in order to maintain a stable difference in odor concentrations between odor and control sides of the maze.

The proportion of odor-fed larvae preferring to move toward the novel odor reached a ceiling after a single generation, even though larvae continued to feed on the odor leaves for four more generations. In our previous experiments that monitored odor preferences of a larval cohort throughout development, larvae increased their preference for the novel odor as they grew. This changed from a 38% preference, in newly hatched first instar larvae, to a 76% preference in fifth instar larvae.[31] Thus, we hypothesized that this increasing preference would also be observed in the newly hatched first instar larvae across generations of continuous exposure of larvae to the new odor. Instead, we found that naive larvae did not show an increase in odor preference across generations. In contrast, the odor preference reached a ceiling of ~61% right after the first generation, with a slight dip and loss of odor preference at F6. An interesting observation to note from Remy's odor imprinting study on *C. elegans* is that both the first and ninth imprinted generations of larvae showed similar migration indices during the chemotaxis assays.[22] This is comparable to what we found in this study, in which both the F1 as well as the F5 odor-exposed larvae showed similar percentages of odor preferences. This indicates that repeated exposures to the odor, across multiple generations, did not increase naive larval preference for that odor, at least in the current setting.

The period of exposure to the novel stimulus and the study system can be important factors in the maintenance and stability of a transgenerationally inherited preference. In our study, and regardless of continuous feeding on the odor for five consecutive generations, naive larvae immediately reverted back to the control preference once the odor stimulus was removed from the feed of their parents. In the odor imprinting study conducted in *C. elegans*, the worms showed a stable inheritance of a novel odor preference after five generations of exposure to the odor.[22] The longer lifespan and a more complex olfactory system of butterflies when compared to *C. elegans* might be responsible for these differences. Alternatively, five generations of odor exposure might not have been enough for this phenomenon to occur in *B. anynana* larvae. Also, it is possible that there are epigenetic factors present in worms that drive this phenomenon but may be absent in butterflies, and this difference might have led to the conflicting findings in odor preference learning and inheritance across these two systems.

A decrease in food quality might have also impacted odor learning observed in the F6 generation, in both control and odor groups. F6 larvae from the odor treatment, in particular, lost their preference for odor, which they regained in the following generation (F7). One possible explanation was the decrease in corn plant quality used during that period. The leaves were smaller and yellower and might have had a different odor. Larval starvation (as many larvae did not feed on these plants as readily) or feeding on this "novel" odor might have confounded the larval choice experiment, making odor-fed F6 larvae lose their preference for odor. Larvae fed on normal quality plants after this generation, and the F7 odor-fed larvae showed a renewed preference for odor. In worms, starvation for 6 days can affect the inheritance of sterility-related acquired traits by reducing the levels of heritable small RNAs.[55] This finding suggests that starvation alone can alter basic physiological functions and may also explain the changes in odor choices observed in both our treatments during times of poor corn plant quality.

Single-generation parental effects, where the environment experienced by the parents alters the phenotype of their offspring,[56,57] likely explain why larval offspring of odor-exposed *B. anynana* parents also preferred odor. Maternal effects impacting offspring's response to odors and fruit volatiles have been observed in many insect species, such as predatory mites,[58] braconid wasps,[59] and apple maggots.[60] Also, the experience of fathers alone can influence the oviposition and odor preferences of the offspring, as found in studies on leaf beetles[61] and honeybees.[62] In our previous study, we also documented a paternal effect. Males that were exposed to the banana odor for a single generation and that mated with naive *B. anynana* females were

able to transmit the learned odor preference to their offspring.[31] In our current experiment, maternal as well as paternal effects might have played a role in transmitting the banana odor preference to their offspring, but we cannot distinguish them as both of the parents were exposed to the odor as larvae.

Mechanistically speaking, epigenetic factors or the odor itself might have been transmitted to the offspring to effect a change in odor preferences. Maternal, paternal, or biparental odor experience might have led to epigenetic modifications, such as DNA methylation, histone modifications, or the transmission of small noncoding RNAs that impacted offspring odor preferences. Any of these factors are capable of regulating gene expression and maintaining heritable variation across several generations.[7,63] Odor exposure and learning can lead to DNA methylation of memory-associated genes in honeybees[64,65] or the inheritance of odor receptor demethylation in mice—potentially changing the sensitivity of offspring to specific odors.[21,66] Noncoding RNAs such as miRNAs and piRNAs that play roles in odor response and pathogen avoidance behavior were found to be inherited in mice[67] and *C. elegans*.[68]

In addition to epigenetic factors, the concept of chemical legacy,[69,70] for instance, can explain a change in larval preference in their host plants. The chemical fingerprint from the host plant can reside in the larval hemolymph, and this "memory" can be passed through the pupal to the adult stage. When adults emerge, they detect minute amounts of the chemical on the surface of the pupae, deposited from the host plant. This influences the oviposition preference of the adult and also induces an olfactory preference for the novel chemical in their larval offspring.[71] In our previous study, however, we found that feeding *B. anynana* larvae on a diet with a novel odor did not affect the adult's oviposition site preference.[31] Yet, it changed the offspring's naive odor preference. The same is observed in our current experiment. It is possible, thus, that IAA molecules passed through the sperm or egg across generations might mediate the observed parental effects of odor learning. Hence, future experiments might explore whether chemical legacy and/or epigenetic factors are involved in the inheritance of learned odor preference in *B. anynana*.

In this experiment, we treated individual larval responses as independent data points, regardless of which replicate cage the individual was reared in. Some researchers, however, may argue that individual larval responses could be a form of pseudo-replication and that the aggregate responses across all individuals in a line should be considered a true replicate. Furthermore, the same researchers might argue that having two replicates that show the same direction of response might simply be due to chance, or to genetic drift, rather than due to the different larval odor treatments. Given that an earlier version of this experiment (run by the same researcher) showed the exact same response (over the course of a single generation of banana odor exposure),[31] this strongly suggests that treatment, rather than genetic drift happening within individual cages, produced the results reported here.

Significance and novelty of this study

We aimed to understand how novel host plant preferences might have evolved in butterflies by investigating how continued exposure to a new odor environment (e.g., a new host plant) alters larval innate preferences for that odor/plant. There are several studies that show that just a few minutes of odor learning can change an insect's innate odor preference in that generation.[72–74] However, we found no study in Lepidoptera that examined whether repeated exposures across generations altered odor preferences in a more extensive way. In this and in our previous experiments, we showed that just one generation of odor exposure was sufficient to alter the offspring's innate odor preference.[31] In the current experiment, however, we showed that this preference does not increase nor stabilize after five generations of odor exposure once the odor is removed. It is possible that learning an odor across a single generation might facilitate host-switching in Lepidoptera, but this needs to be tested in more natural settings. It also remains possible that additional generations of simple odor exposure might lead to an increase in preference or a stable inheritance of the odor preference, as observed in *C. elegans*. This can be tested in the future alongside epigenetic mechanisms of food odor preference learning and transmission across generations.

AUTHOR CONTRIBUTIONS

V.G. and A.M. designed the study. V.G. performed the experiments, collected all the data, made the figures, and analyzed the data. V.G. and A.M. wrote the manuscript and contributed to revisions.

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COMPETING INTERESTS

Both authors declare no competing interests.

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SUPPORTING INFORMATION

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

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