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Moving to Keep Fit: Feeding Behavior and Movement of *Helicoverpa armigera* (Lepidoptera: Noctuidae) on Artificial Diet With Different Protein: Carbohydrate Ratios

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Abstract

Insect herbivores can modify their foraging behavior to obtain a balanced food intake, and they tend to move between food sources with different nutrient values. We investigated this movement in early instar larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) using a putative optimal artificial diet (OP) and high protein (HP) and high carbohydrate (HC) artificial diets based on protein (p) and carbohydrate (c) ratios. Larvae were allowed to choose between the same kind of diet cubes (effectively no-choice), or diet cubes with different p: c ratios. In no-choice tests, we found that first instar larvae remained longest on OP diet and spent the least time on HC diet, while third instar larvae remained longest on HC diet and spent least time on OP diet. First instar larvae moved the most when provided with HC diet, while third instar larvae moved most when provided with OP diet. However, both stages moved the least when allowed to choose between diet cubes with different p: c ratios. The relative growth rate decreased when larvae increased their movement, but this influence was not evident when larvae fed on HC diet. Larvae that fed only on HC diet had the highest relative growth rate, followed by larvae with access to all diets simultaneously, indicating a behavior to mix nutrient intake. We relate these findings to behavior of this major pest species under field conditions.

Key words: larva, nutrient, choice, instar, relative growth rate

Many insect herbivores have the ability to regulate their intake of specific nutrients to meet their physiological demands, thereby enhancing growth and development, as well as fitness and performance (Simpson and Raubenheimer 1999, Lee et al. 2002, Simpson et al. 2004, Deans et al. 2015, Simpson et al. 2015). The movement of insects between food sources to balance intake of different nutrients has been documented in the laboratory (Simpson et al. 2004, Simpson et al. 2015), and changes in these behaviors allow insects to respond to changes in the relative abundance of different food resources (Behmer et al. 2001), as well as changes in their relative proximity (Behmer et al. 2003). This control of food intake suggests regulation of foraging behavior in imbalanced nutritional environments.

Nutritionally imbalanced environments are very common in nature. Host plants offer a highly heterogeneous nutrient landscape at various scales (Woodwell et al. 1975, Eigenbrode and Espelie 1995, Low et al. 2014) even in agricultural monocultures (Deans et al. 2016). The quality of food an insect eats can be different from plant to plant (Sánchez et al. 2004, Tao et al. 2014), tissue to tissue (Deans et al. 2016), and mouthful to mouthful (Shroff et al. 2008), influencing insect movements, development, and survival (Zalucki et al. 2002, Perkins et al. 2013, Zalucki et al. 2017).

In addition to variation in what foods are available, the nutrients needed to complete development in different instars of a given insect herbivore can also be different, and the altered foraging strategies exhibited by some caterpillars as they grow may reflect this changed demand. Cohen et al. (1988) showed that first and second instar *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae tended to feed on silk of maize, but that this tendency was lost early in the third instar when the larvae fed almost exclusively on the kernels. When first instars were offered only silk or only kernels, they molted after approximately 3 d. Subsequently, silk-fed larvae took another 12 d to complete the next three instars while kernel fed larvae took only 5 d. Deans et al. (2018) showed total macronutrient content (soluble protein and digestible carbohydrate content) in corn was always higher in kernels than in silk, which may be the reason why the larvae grow faster when fed on kernels rather than on silk in later instars. Larvae of *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) switch from feeding on leaf sheath to stalks of maize after the third instar (Kantiki and Ampofo 1989). This behavior was ascribed to the high sugar content of the maize stalks as well as the ability of larger larvae to penetrate stalks (Scheltes 1978). Gaston et al. (1991) found that 200 of 1,137 species of British microlepidoptera make a

single marked change in feeding habit as they grow. They suggested a number of reasons that may influence these changes, including food availability, an increase in body size that allows different foods to be exploited, and the risk of being exposed to predators.

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a highly polyphagous insect which can feed and develop on a wide variety of native and cultivated plant species (Zalucki et al. 1986, 1994), this makes it an appropriate model insect to investigate the influence of diet on feeding behavior. Johnson and Zalucki (2007) reported that both first and third instar larvae move extensively on whole *Vigna radiata* ((L.) R. Wilczek) (Fabales: Fabaceae) (mung bean) plants and that they feed at different locations. However, neonates tended to move to, and stay longer on, the terminal leaves and flower buds of a plant where food was soft and moist, while third instars were less likely to stay on the terminal plant parts and were less selective in their choice of food. Third instars fed at fewer sites and in longer bouts than first instars, but spent less time searching and resting (Johnson and Zalucki 2007).

For insect herbivores, protein (p) and carbohydrate (c) are the most intensively studied nutrient groups (Simpson and Raubenheimer 1999, Simpson et al. 2004), but only a few studies have shown that movement of immature Lepidoptera between food resources is related to the nutrient composition of the food available. Cohen et al. (1987) showed final instar larvae of *H. zea* switched between diets more frequently when offered two nutritionally complementary diets in one Petri dish than when only offered nutritionally complete diets. However, the nutritionally incomplete diets used were extreme (p: c ratios were 100: 0 or 0: 100) and unlikely to be found in nature. A less extreme set of diets could lend additional information and insights to the current body of knowledge.

In this study, we examined the influence of defined diets that differ in p: c ratio on the movement of caterpillars in different instars without the confounding influence of other plant characteristics (secondary plant compounds, leaf hairs, waxes etc.). A priori we might expect less movement and more sustained feeding and weight gain on the putative optimized diet and that 'vulnerable' neonates may move more cautiously and feed rapidly for shorter bouts than larger third instars, as described by Johnson and Zalucki (2007). We compared neonates and newly molted third instars on supposed optimized diet and diets defined as sub-optimal based on either their p or c content. Different diets were provided in Petri dishes in either no-choice or choice experiments. The no-choice test investigated how often larvae leave a food resource of a particular nutrient content and the choice test investigated movement and feeding between food resources with different nutrient contents and the consequences of these behaviors, as measured by short-term weight gains, final weight and time to complete development to a given stage.

Materials and Methods

Insects
The *H. armigera* (Mahon et al. 2007) used in the no-choice tests were obtained from CSIRO, Black Mountain Laboratory, ACT, Australia. The same *H. armigera* strain was used in choice tests, but was sourced from the Australian Cotton Research Institute, Narrabri, NSW, Australia. Insects were reared as described by Teakle and Jensen (1985) and later modified in Perkins et al. (2010).

Artificial Diets
For general rearing a standard soyflour-based artificial diet which is described in Perkins et al. (2010) was used. For experiments, the artificial diet used was first developed by Ritter and Nes (1981), and modified by Jing et al. (2013). All diets had a total macronutrient (p+c) content of 42% with different protein to carbohydrate ratios (p: c), while all other nutrients remained the same (Supp Appendix 1 [online only]). Three diets were prepared: the optimal diet (OP), p24: c18 (Tessnow et al. 2018); the high carbohydrate diet (HC), p12: c30; and the high protein diet (HP), p30: c12. The p: c ratios of the HC and HP diets were developed following empirical determination of total macronutrient content (%) and p: c ratios for different cotton tissues grown under both field and greenhouse conditions (Deans et al. 2016).

Feeding and Behavioral Studies in a No-Choice Experiment
Experiments were conducted using neonates and third instar larvae. Neonates were obtained by allowing eggs to hatch in the absence of food and used in experiments within 8 h of hatching. Third instar larvae used in experiments were reared on standard diet until the end of the second instar, and then isolated until they molted. Neonates were weighed (Mettler Toledo Excellence XS Balance) and then directly transferred to diet using a paintbrush. Similarly, third instar larvae were weighed but then cooled on ice before placement on the central cube of treatment diet.

For each developmental stage, five 1 cm³ cubes (1 × 1 × 1 cm) of a given diet were placed on damp filter paper and positioned in the lid of a Petri dish (9 cm diameter); one cube was placed in the center of the lid and four cubes were placed 1 cm away, at the cardinal points. A weighed neonate was then placed on the central cube of diet and the bottom section of the Petri dish then placed over the diet and insects. Forty-five replicates of each diet type (OP, HC, and HP) were prepared and these were divided randomly into three groups of 15 dishes. In the first group, larvae were weighed again 24 h after placement on a diet cube, in the second group larvae were weighed again after 48 h, and the third group larvae were re-weighed as soon as they molted to the next instar. These measurements allowed growth rates (weight gain) to be calculated both within and over the entire instar. All larvae in a group were observed semi-continuously for 6 h (3 h in the morning and 3 h in the afternoon) at approximately 20-min intervals on the day before they were to be re-weighed. The location of each larva (on diet, noting the specific cube; or off the diet) was recorded. A move onto another diet cube or a move off diet cubes was counted as one movement event. After being re-weighed and placed back on the diet, larvae were not subject to further detailed observations.

In total 270 larvae, 135 first instars and 135 third instars, were used. Experiments were conducted under uniform light conditions (L: D [14: 10] h) at 25 ± 2°C. Relative growth rate (RGR) was calculated as the difference in the natural log wet weights between two time periods divided by time elapsed (Kogan and Cope 1974):

$$RGR = (ln(wt_1) - ln(wt_0))/(t_1 - t_0),$$

where wt_1 and wt_0 are the wet weights at times t_1 and t_0.

Feeding and Behavioral Studies in a Choice Experiment
A choice experiment was conducted to investigate how larvae perform in a heterogeneous nutritional environment; three complementary no-choice experiments were run concurrently as controls. To set up each choice arena, three cubes (1 cm³) of each diet (OP, HC, HP) were randomly selected and placed in the upturned lid of a Petri dish (9 cm

diameter) in a 3 × 3 cube 'checkerboard' pattern; within the checkerboard, diet cubes were positioned 1 cm apart and randomized to make sure larvae had equal opportunity to initiate feeding on each diet type. No-choice controls, which were prepared for each diet type, were set up in the same way but contained nine cubes of a single diet type in a Petri dish lid. For the first instars, one larva was placed on each diet cube (nine larvae per dish). There were 10 replicates of mixed diet Petri dishes and a total of nine control dishes (three dishes containing just OP diet, three dishes containing just HC diet, and three dishes containing just HP diet). For the third instars, 30 Petri dishes containing mixed diet were prepared along with 30 dishes containing just OP diet, 30 dishes containing just HC diet and 30 dishes containing just HP diet. A single larva was placed on the central diet cube in a Petri dish. All larvae were observed at 15-min intervals for 6 h a day (3 h in the morning and 3 h in the afternoon) for 2 d. The location of larvae (on diet, noting the diet type and the specific cube, or off the diet) was recorded. All larvae were re-weighed after 2 d to calculate RGR.

Statistical Analyses

Two categories of behavior were evaluated: the presence of larvae on diet and the frequency of larvae transferring between cubes of diet. Only data from the first 2 d were included in the movement analysis to avoid the influence of molting on larval behavior.

All data analysis was conducted in R, version number 3.2.5 (R Core Team 2016). In the no-choice test, a Generalized Linear Model (GLM) based on a binomial response distribution was fitted to the data of the presence larvae on diet, with predictors of diet and instar. A GLM with the same predictors based on a Poisson response distribution was fitted to the data of larval movement between diets. In the developmental test, one-way ANOVA was used to analyze the effect of diet. When appropriate, multiple comparisons were made using Tukey's HSD post-hoc test following ANOVA. Comparisons of regression lines (Fig. 3) for differences were made using ANCOVA, with number of moves as the predictor variable and diet as the co-variate. In choice tests, the proportion of first instar larvae on different diets was analyzed by the Friedman test and then by Wilcoxon test for multiple comparisons. The other movement tests and RGR analysis were the same as in the no-choice tests.

Results

Feeding and Behavioral Studies in a No-Choice Experiment

Movement
We found a significant effect of diet on the presence of first instar larvae on diet. Larvae spent a significantly higher proportion of time on OP diet than on HC diet (glm: $P < 0.01$; Fig. 1A). Diet had a significant effect on the transfer frequency between diet cubes; larvae fed on HC diet alone were more likely to transfer between cubes of diet compared with larvae feeding on OP diet or HP diet alone (glm: OP vs HC, $P = 0.015$; HP vs HC, $P = 0.049$; Fig. 1C).
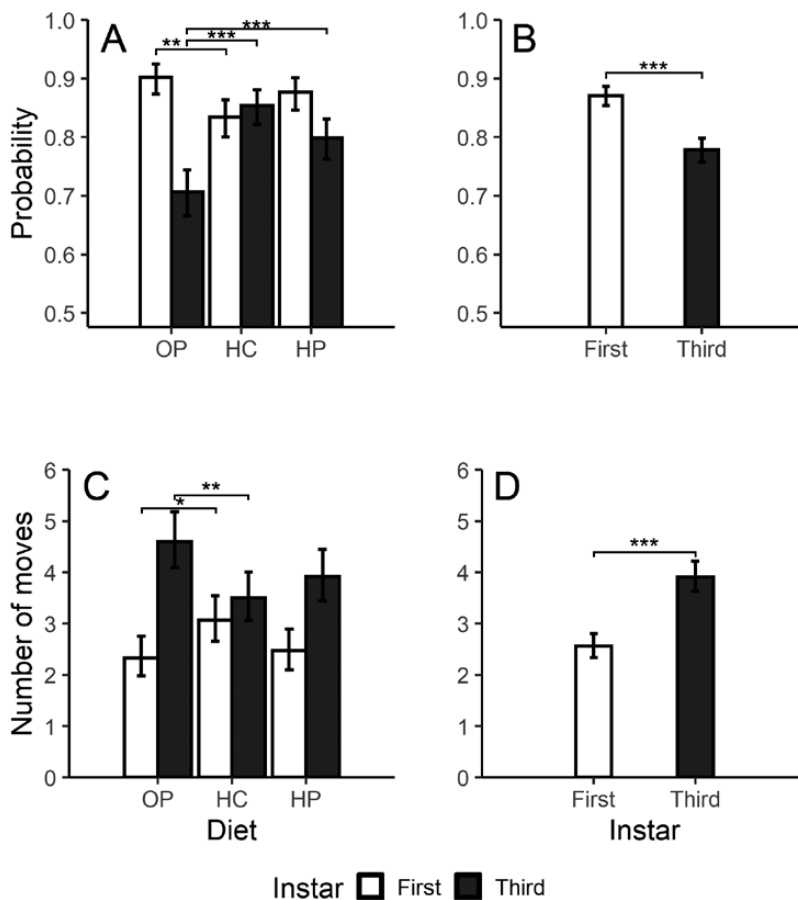

Fig. 1. Probability that first instar and third instar *Helicoverpa armigera* larvae were observed on diet (A, B) and the mean number of moves larvae made between diet cubes (C, D). Asterisks represent significance between groups when tested in GLM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). OP, HC, HP represent optimal, high carbohydrate and high protein diet, respectively; First represents first instar larvae; Third represents third instar larvae.

We found a significant effect of diet on the presence of third instar larvae on diet. Among the three diets, larvae spent the least amount of time on OP diet, and there were significant differences in the time spent on OP diet compared with HC diet and HP diet (glm: OP vs HC, $P < 0.001$; OP vs HP, $P < 0.001$; Fig. 1A). Diet also had a significant effect on the transfer frequency between cubes; larvae on OP diet transferred significantly more frequently than larvae fed on HC diet (glm: OP vs HC, $P = 0.003$; Fig. 1C).

When data of both first instar and third instar were compared, instar significantly affected both the presence of larvae on diet and the transfer frequency between diets; first instar larvae spent more time on diet (glm: first vs third, $P < 0.001$; Fig. 1B) and transferred less between diets (glm: first vs third, $P < 0.001$; Fig. 1D) than third instar larvae.

Development and Growth

Neonates fed on HC diet were significantly heavier at the end of the instar than larvae fed on OP or HP diet (Tukey's HSD test: OP vs HC, $P = 0.045$; HP vs HC, $P = 0.013$; HP vs OP, $P = 0.877$; Fig. 2A), but there was no effect of diet on the weight of larvae completing development through the third instar (ANOVA: $F_{2,38} = 0.754$, $P = 0.477$; Fig. 2B). Larval developmental time in both instars showed no difference between diet treatments (ANOVA: first instar, $F_{2,30} = 2.235$, $P = 0.125$; third instar, $F_{2,38} = 2.059$, $P = 0.142$; Fig. 2A and B).

Diet had a significant impact on RGR of first instar (ANOVA: $F_{2,42} = 4.054$, $P = 0.025$; Fig. 2C) and third instar larvae on the first

day (ANOVA: first day, $F_{2,42} = 4.551$, $P = 0.016$), and on third instar larvae over the first 2 d (ANOVA: $F_{2,42} = 10.94$, $P < 0.001$; Fig. 2D) of the experiment. First instar larvae that fed on OP diet had a significantly higher RGR than larvae fed on HC diet on the first day (Tukey's HSD test: OP vs HC, $P = 0.019$; Fig. 2C). On the first day of the third instar, larvae that fed on HC diet had a significantly higher RGR than larvae that fed on HP diet (Tukey's HSD test: HP vs HC, $P = 0.013$), and over the first 2 d of the third instar, larvae that fed on HC diet had a significantly higher RGR than larvae that fed on OP or HP diet (Tukey's HSD test: OP vs HC, $P = 0.001$; HP vs HC, $P < 0.001$; Fig. 2D).

Developmental stage had a significant impact on RGR of larvae; first instars always had higher RGRs than third instars (ANOVA: over first day, $F_{1,88} = 77.291$, $P < 0.001$; over first 2 d, $F_{1,87} = 74.53$, $P < 0.001$; and over the instar, $F_{1,63} = 96.523$, $P < 0.001$; Fig. 2E).

Number of Moves and RGR

The number of moves made by each individual larva was plotted against its RGR. Larvae in both instars had lower RGRs when they made more moves between cubes of diet (linear regression: first instar, $P < 0.001$; third instar, $P < 0.001$; Fig. 3). When fed on single diets, the regression of number of moves against RGR had the shallowest slope on HC diet for both larval instars, but there was no significant difference when compared with 0 (linear regression: first instar, slope $= -0.022$, SE $= 0.015$, $P = 0.155$; third instar, slope $= -0.012$, SE $= 0.009$, $P = 0.206$). Larvae fed on OP diet
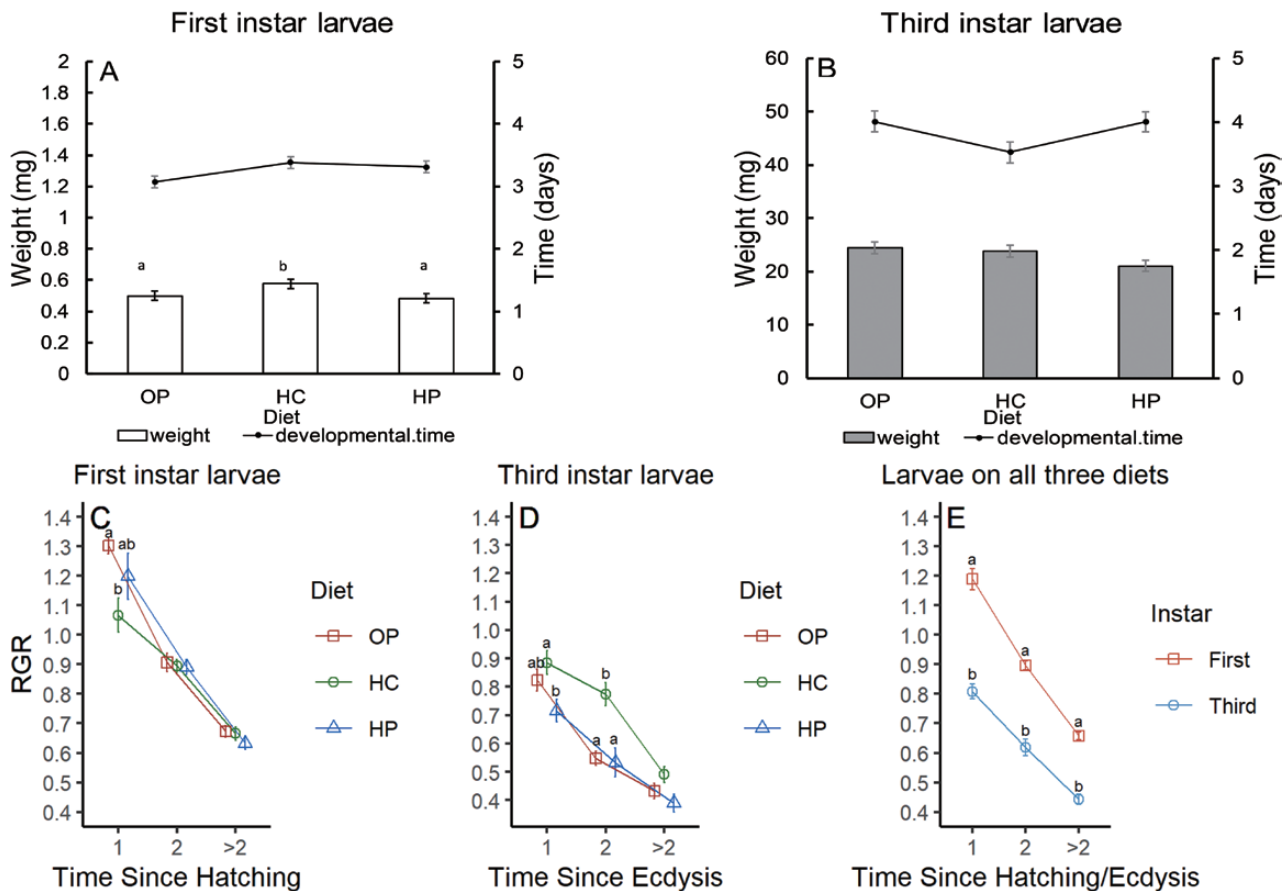

Fig. 2. Larval weight and developmental time when fed on different diets in first (A) and third (B) instars; effect of diet on RGR of (C) the first instar larvae, and (D) the third instar larvae; instar effect on RGR in both first and third instar larvae (E). OP, HC, HP represent optimal, high carbohydrate and high protein diet, respectively. Means with different letters were significantly ($P < 0.05$) different.
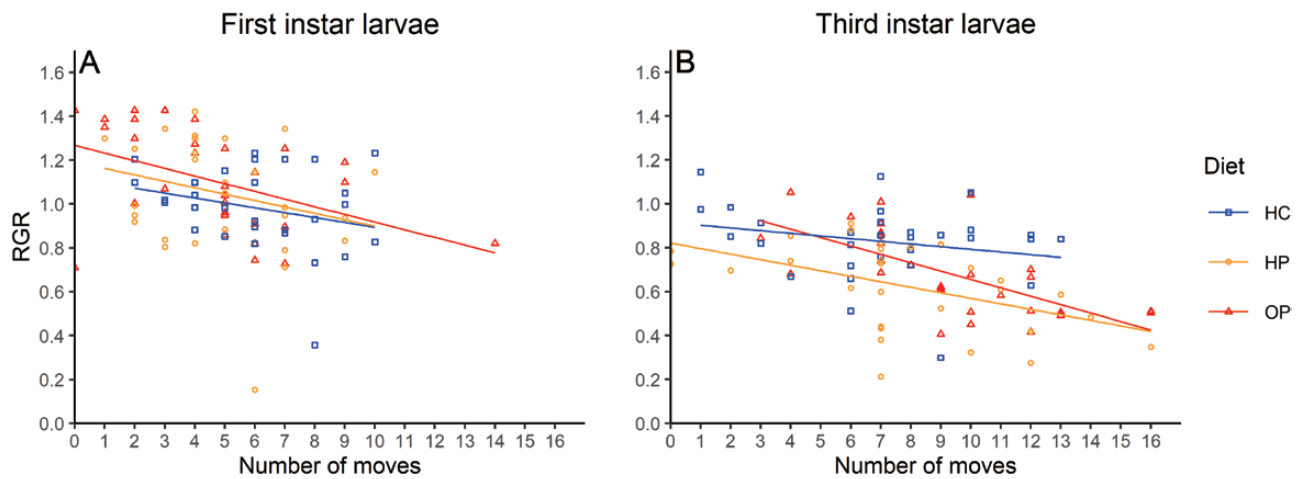
Fig. 3. Scatter plot of individual larval RGR versus the number of moves in the first 2 d. Data from the first instar larvae (A) and the third instar larvae (B) were showed with different shapes and colors to represent different diets. OP, HC, HP represent optimal, high carbohydrate and high protein diet, respectively. Lines indicate regression lines of the same colored data.

had the steepest regression line slope (linear regression: first instar, slope = −0.035, SE = 0.013, P = 0.013; third instar, slope = −0.038, SE = 0.008, P < 0.001). The regression slope for larvae fed on HP diet was intermediate between these extremes (linear regression: first instar, slope = −0.029, SE = 0.022, P = 0.198; third instar, slope = −0.025, SE = 0.009, P = 0.008). The only significant difference between regression lines was for larvae fed on HC diet and larvae fed on OP diet in third instar (ANCOVA: $F_{2,1}$ = 4.356, P = 0.041).

Feeding and Behavioral Studies in a Choice Experiment

Movement

In the first instar, larvae were likely to be on diet most of the time, and in mixed-diet Petri dishes they were more likely be found on OP diet and less likely be found on HC diet (Wilcoxon test: OP vs HC, P = 0.001; OP vs HP, P = 0.038; HP vs HC, P = 0.161; Fig. 4A). When the number of movements across treatments was compared, larvae on HC diet only moved most, and larvae with access to all three diet types (MIX) moved least; this was the only significant difference between treatments (glm: MIX vs HC, P = 0.026; Fig. 4C).

In the third instar (Fig. 4B), larvae were more likely to be found on HC diet than on the other two diets in mixed-diet Petri dishes and OP diet was the least favored amongst the three diets (glm: OP vs HC, P < 0.001; OP vs HP, P < 0.001; HC vs HP, P = 0.007). When the number of movements across treatments was compared, larvae on OP diet only moved most, and larvae on mixed-diet moved least. Significant differences were detected between the OP diet only treatment and the mixed-diet treatment (glm: MIX vs OP, P = 0.001; Fig. 4D).

Relative Growth Rate

When RGRs were compared across different treatments in both instars, larvae presented with mixed-diet had a significantly higher RGR than larvae presented with only HP diet (Tukey's HSD test: first instar, MIX vs HP, P = 0.018; third instar, MIX vs HP, P = 0.012), and larvae presented with only HC diet had significantly higher RGR than larvae presented with only OP diet or only HP diet (Tukey's HSD test: first instar, HC vs HP, P = 0.006; HC vs OP, P = 0.033; third instar, HC vs HP, P < 0.001; HC vs OP, P = 0.001;

Fig. 5A). Overall, there was no difference between the RGR of first and third instar larvae over the first 2 d in the given instar (ANOVA: $F_{1,1}$ = 1.272, P = 0.261; Fig. 5B).

Discussion

Generalist herbivores, such as highly mobile locusts and caterpillars of some species of Lepidoptera, are considered to have many plant food choices in heterogeneous landscapes, and experiments on chemically defined diet have confirmed they can 'maintain' their intake by ingesting complementary nutrients from different food sources (Abisgold et al. 1994, Lee et al. 2002, Simpson et al. 2004, Deans et al. 2015, Simpson et al. 2015). However, why insects make a 'decision' to move between different food sources and how this may change across instars as insects develop, is unclear.

With a simplified single diet environment and a mixed-diet environment, we found a clear correlation between diet and movement in first and third instar *H. armigera* larvae. Whether a larva stayed at a feeding location (cube of diet) and the degree of movement (from one cube to another) were significantly affected by diet treatment (Figs. 1A and C and 4). Normally when larvae stayed longer on a particular type of diet, they were also less likely to change locations. Generally, first instar larvae stayed the longest on OP diet, and the third instar larvae stayed longest on HC diet (Figs. 1A, 4A and B). Accordingly, the frequency with which larvae transferred between diet cubes was the least when they fed on OP diet in the first instar and when they fed on HC diet in the third instar (Figs. 1C, 4 C and D). However, in the choice tests, across different diet treatments, larvae made the least moves in mixed-diet environments in both instars (Fig. 4C and D). This is contrary to the finding of Cohen et al. (1987) who showed that final instar larvae of *H. zea* switched between diets more often when offered nutritionally complimentary food than when offered nutritionally complete food. We assume when offered nutritionally complimentary food, early instar *H. armigera* larvae in our experiment would tend to move less because if the nutrient content is 'complete', there was higher evolutionary risk associated with movement (e.g., predation) than remaining in the same place. However, comparisons with *H. zea* are difficult as the diet, instar, and species, though closely related (Behere et al. 2007), are different.

In the no-choice test, larvae took the least time to complete the first instar when fed on OP diet (Fig. 2A), and the least time to
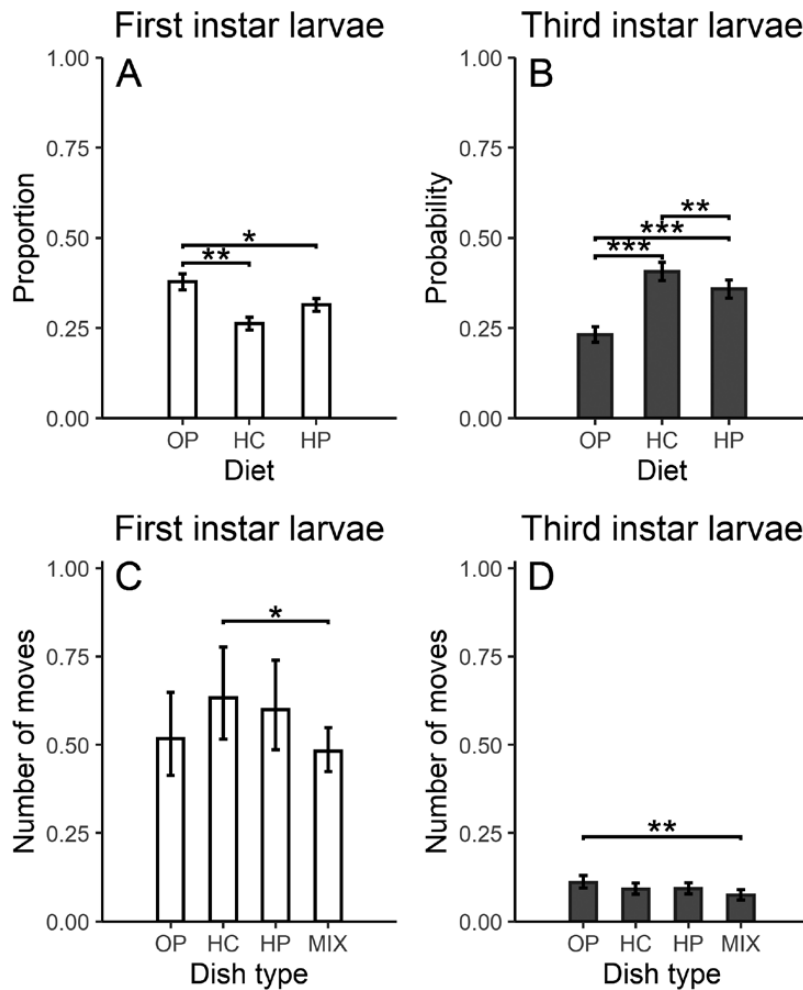
Fig. 4. Proportion of times that first instar (A) and probability of third instar (B) *H. armigera* larvae were observed on diet in choice experiment and the mean number of moves larvae made per dish between diet cubes across different dish types (C, D). OP, HC, HP represent optimal, high carbohydrate, and high protein diet respectively (A, B). OP, HC, HP, MIX represent optimal diet, high carbohydrate diet, high protein diet, and mixed-diet dish types respectively (C, D). Asterisks represent significance between groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

complete the third instar when fed on HC diet (Fig. 2B), which were the diets they were more likely to be found on (Fig. 1). Diet had a significant effect on RGR of first instar and third instar larvae over the first day, and on the RGR of third instar larvae over the first 2 d (Fig. 2A and B). The first instar larvae always had the highest RGR when fed on OP diet, but the differences between diets were less obvious over time (Fig. 2A). However, in the choice test, the first instar larvae had the highest RGR over the first 2 d when fed on HC diet (Fig. 5A), even though they made the highest number of moves (Fig. 4C). This difference may be because of the different cultures kept in the two laboratories that we used in our experiments. The third instar larvae consistently had the highest RGR when fed on HC diet (Figs. 2B and 5A).

Movement and growth rate appear to be related. In general, larvae that move more had lower RGRs (Fig. 3). The effect was least for larvae fed on HC diet; regression of RGR against the number of moves had the lowest slope (Fig. 3); however, the number of moves between cubes of diet were not always the fewest (Fig. 1C). The implication is that larvae that fed on HC diet had more energy to compensate for the respiration costs of high-frequency movement between foods when compared with larvae fed on other diets.

Larvae on all three diets, including the assumed optimal (OP) diet, made movements between diet cubes, indicating other reasons

for this behavior. The supposed OP diet was developed from work on final instar larvae to assess intake target and had not been tested on other instars of *H. armigera* previously. Furthermore, an intake target is not necessarily the same for larvae in all situations, so this 'optimal' diet might not be the preferred choice for all instars. Various studies have demonstrated that larvae in different instars have different demands for nutrients. When larvae of *Lymantria dispar* (Linnaeus) (Lepidoptera: Erebidae) were provided with choices of artificial diets differing in protein and lipid concentrations, their preference shifted away from HP, low lipid cubes toward low protein, high lipid cubes across third instar to final instar (Stockhoff 1993). Cohen et al. (1987) also found that fifth instar *H. zea* increased sucrose intake at the end of the larval period and they suggested that this was due to the metabolic costs of pupation and adult eclosion. The digestive enzymes in the larval mid-gut were shown to be correlated with this behavior (Kotkar et al. 2009, Clissold et al. 2010, Lwalaba et al. 2010, Kotkar et al. 2012, Sarate et al. 2012). First instar larvae of *H. armigera* had much lower amylase levels than third and later instars (Kotkar et al. 2009), which may explain the lack of interest in carbohydrate in first instars. On the other hand, the level of digestive enzymes can be adjusted to meet the nutrient deficiency, by releasing less enzymes for nutrients that are in excess while maintaining or boosting levels
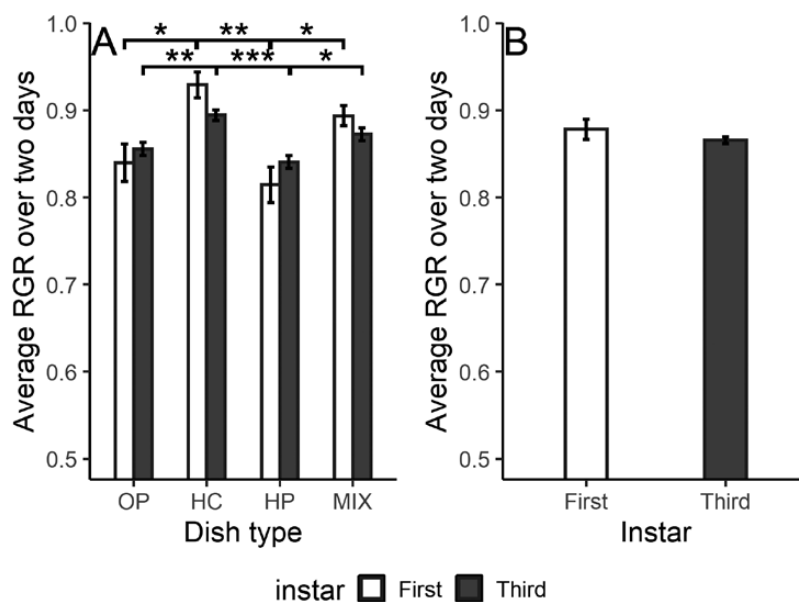
Fig. 5. The influence of dish types (A) and instar (B) on RGR over 2 d. Asterisks represent significance between groups. OP, HC, HP, MIX represent optimal diet, high carbohydrate diet, high protein diet and mixed-diet dish types respectively (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

of enzymes for nutrients that are in deficit (Clissold et al. 2010, Sarate et al. 2012). This may help explain the decreasing difference in RGR between first instar larvae fed on HC and OP diets over time (Fig. 2C).

The macronutrient composition in standard colony diet was similar to that in HP diet (Wang P. unpublished data). Third instar larvae previously raised on standard diet in our experiment had 'experienced' HP diet. However, third instar larvae in choice tests sill preferred HC diet over HP diet (Fig. 4B). In a separate study, when larvae were provided with both diets (p35:c7 and p14:c28) from first to third instar, *H. armigera* larvae changed their preference from HP diet in first instar to high carbohydrate diet in third instar (Katsikis 2019), indicating carbohydrate is a more important nutrient in this stadium.

Sugars are the most important phagostimulants for phytophagous insects, and there is no evidence that herbivores can taste protein directly (Chapman 2003). However, third instars preferred HP diet over OP diet (Figs. 1 A and 4 B), even though OP diet has more sugar than HP diet. Katsikis (2019) and Tessnow et al. (2018) showed a preference of *H. armigera* for p35:c7 over p7:c35 diet in the third and final instars, for p14:c28 over p35:c7 diet in third instar, but no obvious preference when offered p28:c14 and p14:c28 diets in both third and final instars. The choice for diet seems based on a combination of protein and sugar, not sugar alone, but little is known about the physiological basis of how this apparent feedback operates.

The performance of larvae and their behaviors changed when offered diverse diets rather than only one. It has been suggested that feeding on different foods is beneficial for insects because it allows them to obtain target nutrient intake in nutritionally imbalanced environments (Lee et al. 2002, Simpson et al. 2004, Deans et al. 2015, Simpson et al. 2015). Larvae in our experiments had a higher RGR when given access to the three diet types than when they had access to only HP diet or only OP diet, but not when they had access to only HC diet (Fig. 5). HC only diet is normally considered a sub-optimal food because of its low protein content. Roeder and Behmer (2014) also showed high carbohydrate diet slowed down the development of *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) larvae,

and resulted in lower fecundity. By mixing diets in our study, larvae were given the chance to amend a biased nutrient intake to achieve optimized development.

The amount of food that larvae consume varies within each instar. Usually larvae do not feed for a short period after hatching or ecdysis, and this is followed by a period during which food consumption is either constant or, more usually, it increases progressively. The feeding rate will then decline and then cease at some time before ecdysis (Browne 1995). Browne and Raubenheimer (2003) showed increased food ingestion within the fourth instar and fifth instar in *H. armigera*. Johnson and Zalucki (2007) found a decreased RGR with time in the first instar and a relatively stable RGR in the third instar *H. armigera* larvae. Here we found a decreased RGR in larvae exposed to different diet regimes over time within both instars (Fig. 2).

First and third instars showed differences in movement between diets. Contrary to Johnson and Zalucki (2007), the number of times larvae moved between diets changed dramatically from the first instar (5.24 moves/ larva) to the third instar (8.01 moves/ larva) over the first 2 d in the no-choice test. First instar larvae were less likely to move between diets, suggesting a more conservative strategy in food selection compared to the third instar larvae. This may reflect a greater vulnerability in neonates to extreme environments (Terry et al. 1989, Kobori and Amano 2003, Leonard et al. 2016) and predators (Zalucki et al. 2002) than in the third instar larvae. Consistent with other studies (Johnson and Zalucki 2007, Quintero and Bowers 2018), first instar larvae had higher RGR than the larvae in the third instar (Figs. 2C and 5B).

In summary, our study directly associated the movement of caterpillars and the nutrient composition of their diet, in two early instars of *H. armigera* larvae. We present evidence that diet has a clear influence on caterpillar movement, as does instar. However, our study of caterpillar foraging behavior was under relatively simplified laboratory conditions. Singer et al. (2002) suggested that food mixing behavior in nature may frequently involve both nutrient balancing and toxin dilution, and showed that secondary metabolites were more important factors affecting behavior of *Grammia geneura* (Strecker) (Lepidoptera: Arctiidae) larvae than nutrient balancing.

Behmer et al. (2001) showed learning may also influence insect food mixing behavior in fifth instar nymphs of *Locusta migratoria* (Linnaeus) (Orthoptera: Acrididae) (Behmer et al. 2003). Such factors introduce exciting interactions with nutrient which will be explored in future experiments.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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