Gravimetric Method for Determining Stage of an Obligate Internally Feeding Stored-Product Insect Pest, the Cowpea Weevil

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ABSTRACT We conducted two experiments to develop a gravimetric method for determining the stadia of *Callosobruchus maculatus* developing in cowpeas. An important step was to develop regression equations for predicting weight changes in uninfested cowpeas to correct for changes that occur in weight as a result of changing environmental conditions, such as temperature, relative humidity, and barometric pressure. This was vital because we needed to have a way of determining weight changes in infested peas that were caused by the insects developing within them; even the weight of uninfested peas changed over the course of the experiments. The observed weight of each infested pea was corrected based on the proportion weight change in uninfested peas each time weighing was done to get an estimate of the cumulative change in weight caused by the presence of the insect. For each infested pea, cumulative change in weight was plotted against time, and plots were analyzed to determine whether a pattern existed that could be used to consistently distinguish stadia. In the second experiment we used an ultrasonic detector to validate results from the gravimetric method. We found that the gravimetric method provided accurate information on duration of the egg, larval, and pupal stages of *C. maculatus*; however, the gravimetric method could not reliably distinguish the four larval stages of *C. maculatus*. Our paper also discusses practical applications of the gravimetric method for determining the stadia of internally feeding insects in the context of the current and previous research.

KEY WORDS staging insects, *Callosobruchus maculatus*, cowpeas, development stages, stored-product insects

Internally feeding stored-product insects are the most important insect pests of stored grain because they develop and feed inside intact kernels. These pests include the Angoumois grain moth, *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae); the larger grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae); the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae); the granary weevil, *Sitophilus granarius* L. (Coleoptera: Curculionidae); the maize weevil, *Sitophilus zeamais* Motschulsky; and the rice weevil, *Sitophilus oryzae* L. There are also a number of bruchids (Coleoptera: Bruchidae) that are internally feeding pests of legumes; the four most important species (Birch et al. 1985) are the adzuki bean beetle, *Callosobruchus chinensis* L.; the bean seed beetle, *Acanthoscelides obtectus* (Say); the cowpea weevil, *Callosobruchus maculatus* (F.); and the Mexican bean seed beetle, *Zabrotes subfasciatus* (Boheman). Information on stadia of internally feeding stored-product insects is important for the development of simulation models that can be used to optimize control strategies for these pests because development and effects of control strategies differ with stadia. However, there is currently no accurate way to determine the life histories of internally feeding insects that does not involve the use of costly and technologically sophisticated equipment. Ultrasonic detectors that can be used to monitor feeding-generated ultrasonic sounds are an excellent way to study life histories of insects feeding hidden within dry materials (Shade et al. 1990); however, these detectors are not commercially available, thus requiring technical expertise to build and operate. X-rays are very accurate for detecting internally feeding insects (Dobie 1973), but the method is costly and labor intensive. However, destructive dissection is a simple and inexpensive approach to studying the life history of internal feeders, but the shortcomings of this technique are that it is labor intensive and estimates duration of various development stages based on different individuals, which removes individual variability from the data set. Developing an alternative method for staging internally infesting insects is desirable.

A possible alternative is to develop a gravimetric method that can be used to determine the stadia of internally feeding insects by weighing of infested ker-

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nels, as was initially evaluated for *Sitophilus granarius* developing in wheat kernels, as part of a larger study on insect detection methods (Chambers et al. 1984). This study found that weight comparisons between infested and uninfested kernels did not show any perceptible differences until later instars, most obviously characterized by a large weight loss in the last larval instar (Chambers et al. 1984). We wished to further evaluate this concept by using daily weighing without interruption and by using an analytical balance that was 1,000 times more sensitive (0.1 μg compared with 0.1 mg) than that used by Chambers et al. (1984). This balance also has the advantage that weighing is not affected by vibration at the sensitivity required for weighing these internally feeding stored-product pests, so it can be easily used in a laboratory or rearing chamber.

Thus, we hypothesized that the weight of a kernel infested by a single internally feeding insect is going to change in a manner that reveals specific periods of insect activity and inactivity, which presumably would differentiate the different development stages. For example, the weight of an infested kernel during molts would be expected to remain more or less constant but would be expected to drop between molts as the insect feeds on the kernel and uses the nutritional resources acquired for energy production and development. Therefore, kernel consumption by the insect should cause the infested seed to lose weight at a rate that reflects energy production and the inefficiency in converting seed biomass to either frass or insect biomass. Moreover, we expected that insects would lose water to the atmosphere at the beginning of a molt and cease to lose water to the atmosphere after the new cuticular lipids were deposited, with considerable potential for water absorption by the new cuticle, as well. These changes in moisture content were expected to cause a weight loss in kernels when the molt started, followed by an increase in kernel weight later in the molt.

Therefore, we hypothesized that it is possible to study the life history of an internal feeder by monitoring weight changes of singly infested kernels. To test this hypothesis, we determined weight changes in singly infested kernels. To ensure that weight change data were providing correct information on the different developmental stages, in a second study we used an ultrasonic detector to monitor feeding-generated ultrasonic signals from weighed infested kernels to validate the gravimetric method (Shade et al. 1990). Our study describes the detection of weight changes in singly infested cowpeas, *Vigna sinensis* Endl., that are caused by the insects developing within those peas, and we explain how this information can be interpreted to gain understanding of the life cycle of the cowpea weevil and the practical applications of this technique. The cowpea weevil was selected for these studies for two reasons. First, the visible, external eggshell is filled with frass as the neonate bores into the pea, which allows for a visible corroboration of egg hatch. Second, we assumed that the rapid development of this species would make it more suited to the continuous daily weighing that these experiments required.

Materials and Methods

General Procedures. Two experiments were conducted: the first in Savannah, GA, and the second in West Lafayette, IN. In both experiments, peas with a single *C. maculatus* egg were obtained by placing 50 unsexed 0- to 2-d-old *C. maculatus* adults on 50 g of pre-equilibrated (15.4 ± 0.2% moisture content) peas for 24 h in a rearing chamber maintained at 30 ± 0.4°C, 65 ± 3% RH, and 12 L:12 D photoperiod. After the adults were removed, peas with only one egg deposited on the testa were selected and weighed to the nearest 0.1 μg using a Mettler UMT2 balance (Columbus, OH).

We also weighed 100 uninfested peas daily. We used these data to develop a method for correcting weight changes in infested peas for weight changes in uninfested peas that are caused by changing abiotic conditions, such as temperature, relative humidity, and barometric pressure, and to changing biotic conditions, such as seed or microbe respiration. This correction allowed us to determine the amount of actual weight loss caused by the developing insect despite innate fluctuations in weight of the host pea. Cumulative weight change as a result of *C. maculatus* activity within each singly infested pea presumably would follow a pattern in the infested peas that could be used to distinguish the different development stages.

Experiment 1. Twenty infested peas were used for this experiment. Both the infested and uninfested peas were kept in a rearing chamber maintained at 30 ± 0.4°C, 65 ± 3% RH, and 12 L:12 D photoperiod. Infested peas were weighed twice a day at 0800 and 1800 hours, and uninfested peas were weighed once a day at 1200 hours inside the rearing chamber until all adult insects had emerged from the infested peas.

Experiment 2. This experiment was similar to experiment 1 except only 11 infested peas were used, and temperature was 25 ± 0.4°C. In addition, an ultrasonic detector was used to collect *C. maculatus* feeding-generated ultrasonic signals (Shade et al. 1990) from each of the *C. maculatus*–infested peas continuously, except when peas were removed twice daily for weighing. We were limited to 11 peas in this experiment because this is the number of channels available in the ultrasonic detector.

Data Analysis. Regression equations were developed to correct for weight changes in uninfested peas by calculating proportion weight change per day and regressing on the weight of the pea on the previous day. Then, on each day, we calculated the change in uncorrected weight of infested peas, calculated the expected change in weight of infested peas based on the proportion change in weight of uninfested peas (using the regression equations), and subtracted the expected change in weight of uninfested peas from the change in weight of infested peas. The difference is the change in weight of the pea caused by the insect

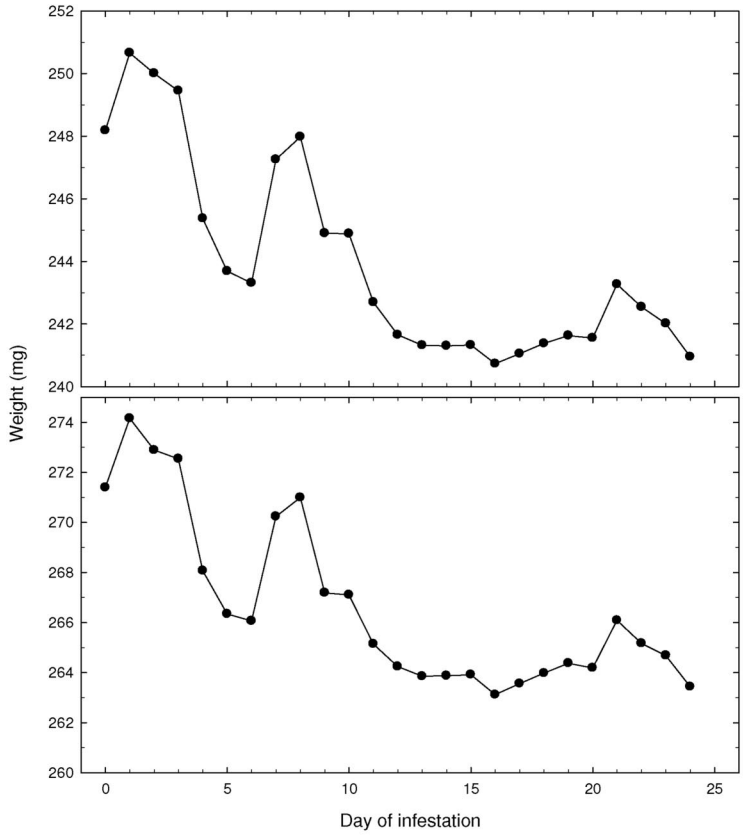

Fig. 1. Fluctuations in uncorrected weights of two uninfested peas in experiment 1 conducted at 30 ± 0.4°C.

and should include any changes in seed or microbe respiration occurring as a result of insect presence.

We also conducted bootstrapping (Blank et al. 2001) using proportion weight change data obtained daily for each of the 100 uninfested peas from experiment 1 to get an idea of the minimum number of uninfested seeds that would need to be weighed each day for the purpose of correcting weight changes in infested peas. We bootstrapped using sample sizes of 10, 25, 50, 75, and 100. In the case of a sample size of 10, for example, we drew a random sample of 10 proportion weight change values from 100 values obtained for a given day; the mean proportion weight change based on these 10 values was calculated. The process of taking 10 values and calculating the mean proportion weight change was repeated 1,000 times. The 1,000 means were arranged in ascending order, in which the 25th and 975th observations represented the 95% CI of the overall mean; the width of this interval was calculated. This process was repeated for sample sizes of 25, 50, 75, and 100 using proportion weight change values that were obtained for each day.

Results

Daily weighing of 100 uninfested cowpeas, in both experiments 1 and 2, indicated that the trends in weight change per day were very consistent across peas of varying initial mass for a given day and were thus predictable (examples of two peas of different initial weights from experiment 1 shown in Fig. 1). However, change in weight of a pea was positively correlated with the weight of the pea [Fig. 2; $r = 0.94$, $P(r > 0) < 0.001$]; thus, we used proportion weight change to correct for change in weight of uninfested peas (Fig. 3). Variances on proportion weight changes were always quite low (Fig. 4). The mean proportion weight change over the course of the experiment was 0.039, the minimum was 0.035, and the maximum was 0.047. The proportion weight change over the course of the experiment showed a weak negative correlation with the maximum weight of each pea during the experiment [Fig. 3; $r = -0.35$, $P(r > 0) < 0.001$].

Weights of uninfested peas over the course of the experiment that were corrected for average weight change in uninfested peas were almost constant over the course of the experiment (Fig. 5). That is, almost all changes observed in weight of uninfested peas could be explained by average changes in weight of uninfested peas that we attributed to abiotic conditions and biotic factors other than insects. Conversely, the corrected weights of infested peas followed a pattern similar to that for uncorrected weights of infested peas (Fig. 5). That is, the greater portion of changes

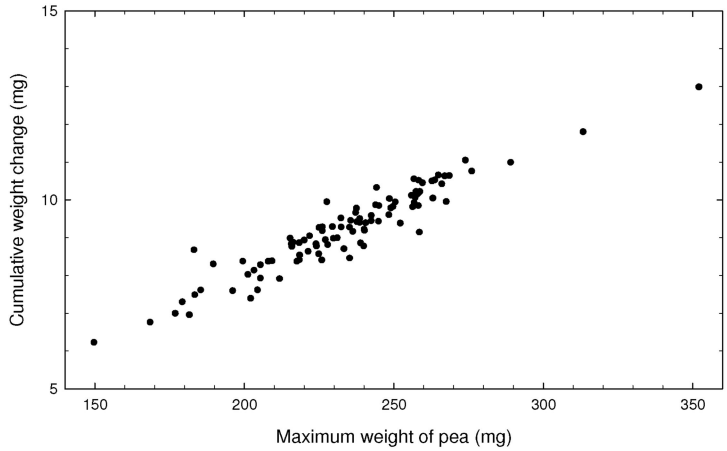

Fig. 2. Correlation between cumulative weight change of each uninfested pea and maximum weight of each uninfested pea during the course of experiment 1 conducted at 30 ± 0.4°C.

observed in weights of infested peas could not be explained by changes in uninfested peas attributed to abiotic conditions. We thus attributed these unexplained changes in weight to the insect feeding inside the pea.

In the first experiment, 17 of 20 insects survived to the adult stage; 5 of 11 survived to the adult stage in the second experiment. Examples of cumulative change in weight caused by *C. maculatus* and cumulative change in weight of an uninfested pea of similar weight, corrected for mean weight change in uninfested peas, are shown in Figs. 6 and 7 for one pea from experiment 1 and the other pea from experiment 2, respectively. Plots of cumulative weight change data obtained using the gravimetric method resulted in a pattern that could clearly distinguish only three potential immature developmental stages: egg, larva, and pupa; the method could not be used to distinguish individual instars. In Figs. 6 and 7, point 1 shows an increase and then a marked decrease in cumulative weight as an egg hatches into a larva, point 2 represents a cessation of rapid decrease in cumulative weight as a larva changes to pupa, and point 3 represents the resumption of rather rapid decrease in cumulative weight when a pupa changes to an adult. The abrupt loss of weight at the end of the experiment is caused by the insect leaving the kernel. Using these criteria, we were able to determine the duration of the egg, larval, and pupal stages of *C. maculatus* used in experiments 1 and 2 (Table 1). The gravimetric method resulted in no clear patterns that could distinguish the four different larval instars of *C. maculatus*, although the ultrasound detection method (Fig. 7) could. Using the ultrasound detection method, we were able to determine the duration of each of the six immature stages of *C. maculatus* (Table 1) and confirm that the weight changes we attributed to the egg, larval, pupal, and adult stages (Fig. 7) were accurate.

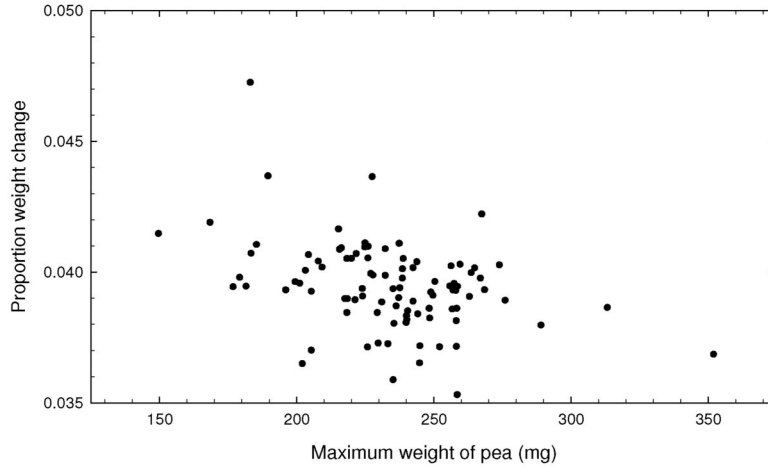

Fig. 3. Correlation between cumulative proportion weight change of each uninfested pea and maximum weight of each uninfested pea during the course of experiment 1 conducted at 30 ± 0.4°C.

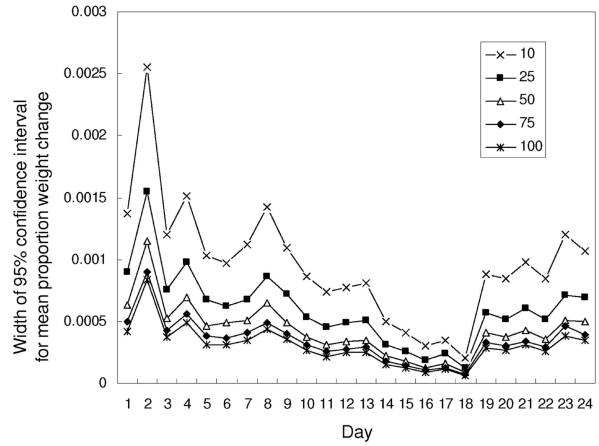

Fig. 4. Widths of 95% CIs for mean proportion weight changes based on bootstrapping using samples of 10, 25, 50, 75, and 100 proportion weight changes taken on each day of experiment 1 conducted at 30 ± 0.4°C.

Discussion

At 25°C and 65% RH (experiment 2), duration of the egg, larval, and pupal stages determined using the gravimetric and ultrasound detection methods were similar (Table 1). Given that the ultrasound detection method is a proven way of staging internally feeding insects (Shade et al. 1990), this indicates that the gravimetric method provides accurate information on the duration of these three immature stages of *C. maculatus.* Similar to the findings with *S. granarius* in wheat (Chambers et al. 1984), we found that the gravimetric method could not reliably distinguish the four larval stages of *C. maculatus.* Our study confirmed that the ultrasound detection method can clearly distinguish the six immature stages of *C. maculatus*, egg, four larval instars, and pupa, corroborating the earlier findings of Shade et al. (1990). El-Sawaf (1956) found that at 25°C and 65% RH, the incubation period and the larval-pupal period of *C. maculatus* were 7.04 and 36.08 d, respectively. His results are similar to our results obtained in experiment 2 at 25°C and 65% RH (Table 1). At 31°C and 65% RH, he found that the incubation period and the larval-pupal period took 3.98 and 20.28 d, respectively. These results also are similar to ours obtained in experiment 1 at 30°C and 65% RH (Table 1). The fact that the incubation period and the larval-pupal period of *C. maculatus* obtained using the gravimetric method, in both experiments 1 and 2, were similar to those obtained by El-Sawaf (1956) further validates the reliability of the gravimetric method for the assessment of the duration of the egg, larval, and pupal stages of internally feeding insects.

Use of the more sensitive ultrabalance in this study resulted in better separation of immature stages than was reported by Chambers et al. (1984). They were able to identify five periods in the life cycle of *Sitophilus granarius* developing in wheat: the egg and first two instars (their time period Q), the third and fourth instars (R), the prepupa and most of the pupal stage (S), the period around the pupal/adult molt (T), and the adult in the kernel (U). We were able to distinguish the egg, larval, and pupal stages and the adult in the kernel.

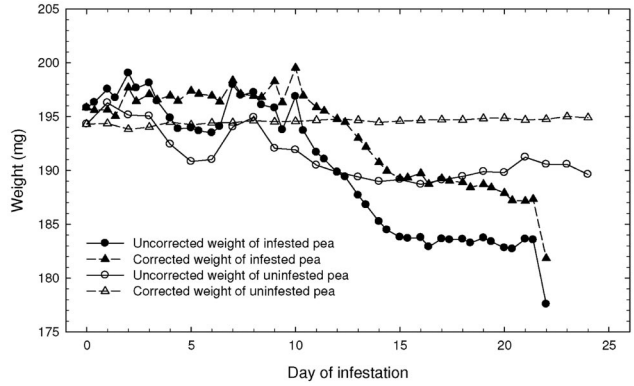

Fig. 5. Uncorrected and corrected (for mean proportion weight changes in uninfested peas) weights of an infested and an uninfested pea in experiment 1 conducted at 30 ± 0.4°C.

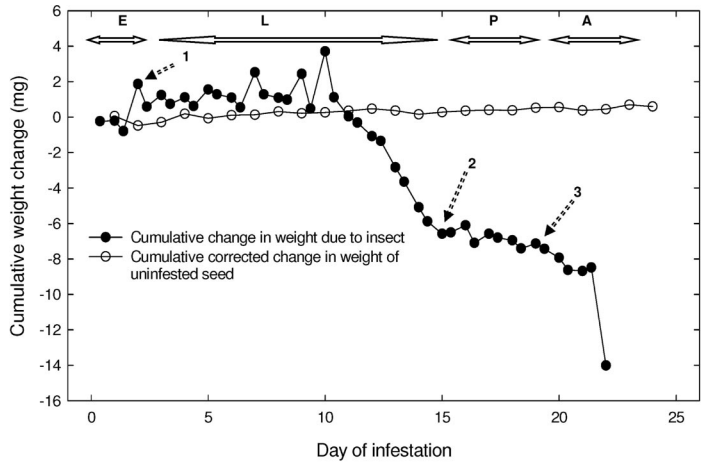

Fig. 6. Cumulative change in weight caused by *C. maculatus* and cumulative change in weight of an uninfested pea, similar in initial weight to the infested pea, corrected for mean weight change in uninfested peas in experiment 1 conducted at 30 ± 0.4°C. These plots based on the gravimetric method can clearly distinguish only four developmental stages shown by the unshaded arrows: egg (E), larva (L), pupa (P), and adult (A). Dotted arrows 1, 2, and 3 represent egg hatch, the end of the larval stage, and the start of the adult stage, respectively.

The increase and sharp decrease in cumulative weight that accompanies the hatching of *C. maculatus* eggs (Figs. 6 and 7, point 1) could be caused by an increase in egg volume from the embryo swallowing air, which allows for diffusion of additional water vapor through the shell (Chapman 1998, Borror et al. 1989). This would result in an increase in the weight of the egg passively, rather than by active absorption of atmospheric water vapor, which has only been reported for a few species (Hadley 1994). As the egg is ruptured, this additional water vapor and other volatile substances could be passively lost. Also, as the hatching process progresses, the active expulsion of air by the neonate would lead to a loss of water vapor and other volatile substances enclosed within the shell. These two processes could contribute to the decrease in cumulative weight observed after hatching.

From Figs. 6 and 7, it can be clearly seen that there is repeated decrease and increase in cumulative weight immediately after hatching until the sustained decrease that leads to point 2 where pupation starts. This pattern may be explained by *C. maculatus* feeding and metabolism, which lead to a decrease in cumulative weight, and by cast skins shed during each larval molt adsorbing moisture and causing an increase in cumulative weight. There may also be hygroscopic

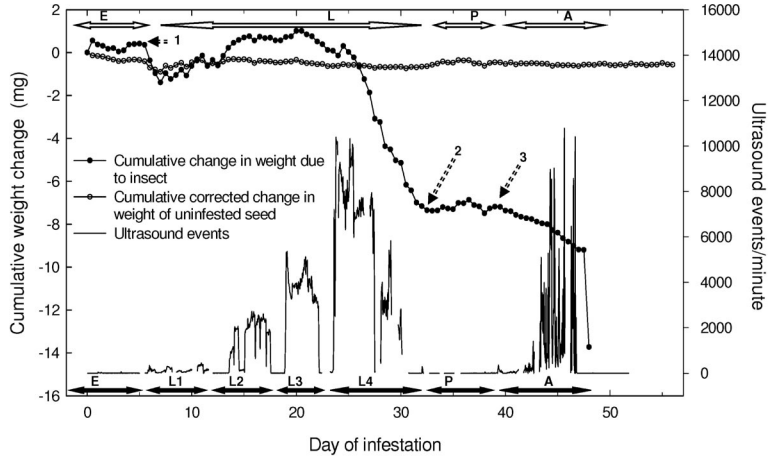

Fig. 7. Ultrasound events, cumulative change in weight caused by *C. maculatus*, and cumulative change in weight of an uninfested pea, similar in initial weight to the infested pea, corrected for mean weight change in uninfested peas in experiment 2 conducted at 25 ± 0.4°C. The ultrasound plot shows that *C. maculatus* goes through seven developmental stages shown by the shaded arrows: egg (E), four larval instars (L1–L4), pupa (P), and adult (A). Only four developmental stages shown by the unshaded arrows (egg, larva, pupa, and adult) can be clearly discerned using the gravimetric method. Dotted arrows 1, 2, and 3 represent egg hatch, the end of the larval stage, and the start of the adult stage, respectively.

Table 1. Duration of egg, four larval instars, pupa, and combined immature stages of *C. maculatus* used in experiments 1 (n = 17) and 2 (n = 5) based on the gravimetric method and the ultrasound detection method

Stage	Duration ± SD (d)		
	Gravimetric method		Ultrasound method (experiment 2)
	Experiment 1	Experiment 2	
Egg	2.06 ± 0.24	6.78 ± 1.29	7.13 ± 1.23
L1	—	—	8.16 ± 2.56
L2	—	—	5.66 ± 0.57
L3	—	—	5.33 ± 0.86
L4	—	—	8.55 ± 0.89
Larva	13.66 ± 1.03	28.82 ± 3.76	27.70 ± 4.55
Pupa	3.95 ± 0.30	7.70 ± 1.86	9.27 ± 0.78
Larva + pupa	17.61 ± 1.10	36.52 ± 4.58	36.97 ± 5.17
Egg to adult emergence from pea	19.67 ± 1.05	43.30 ± 4.54	44.31 ± 4.68

Environmental conditions during experiment 1 were 30 ± 0.4°C, 65 ± 3% RH, and 12 L:12 D photoperiod; in experiment 2, conditions were similar except the temperature was 25 ± 0.4°C.

absorption of moisture by the newly deposited cuticle during each molt (Hadley 1994). These data do not seem to match the discussion of slight, barely perceptible, weight losses in the early development of *S. granarius* feeding in wheat (Chambers et al. 1984). However, close scrutiny of their actual plotted data shows that there are days in which the infested seed weight increased slightly when the larvae were small. Overall trends are difficult to compare because Chambers et al. (1984) did not collect weights continuously (weekends omitted because the intent of their study was not to determine duration of stages), which leaves gaps that show as natural breaks in the plotted data. Moreover, these slight increases also occurred for the uninfested eggs in their study. The size of the plotted symbols in Chambers et al. (1984) is >1 mg, so the subtle increases plotted are ≈0.4 mg/d. This would represent a 0.01 proportional weight gain on a 40-mg wheat kernel [approximate weight estimated from Chambers et al. (1984)], a value that fits in with those we report in Fig. 4. Our actual weight increases are of greater magnitude because the seeds are five- to six-fold heavier, but the trends are the same. Based on ultrasonic data (Fig. 7), the sustained decreases in weight leading to point two that occur during larval development correspond to the later third and the entire fourth larval instar. This is logical because these last larval instars are larger and would be expected to feed voraciously and respire faster as they accumulate resources required for the pupal stage. These late-instar data also closely parallel the trends reported for *S. granarius* (Chambers et al. 1984).

It is important to emphasize that viewing the ultrasonic data allows visualization of the slight increases in weight that occur at the end of each larval molt. Once again, this supports exuvial adsorption or newly deposited cuticular absorption of water by the nonfeeding larvae at the start of each stadium, although these are not discrete for the smaller larvae.

The pupal stage between points 2 and 3 is also characterized by fluctuations in cumulative weight that match those reported for *S. granarius* by Chambers et al. (1984). This should be expected considering that the pupal stage is not a period of inactivity but is dynamic and characterized by processes such as histolysis, histogenesis, and differentiation (Riddiford and Ajami 1973, Wasserthal 1996, Chapman 1998, Dingha et al. 2004). In fact, Bainbridge and Bownes (1981) found that, in *Drosophila melanogaster*, there is a sequence of 45 visible changes that occur during the pupal stage. Some of these changes are most likely responsible for the fluctuations in cumulative weight that are observed during the pupal stage. Dingha et al. (2004) also found that the weight of *Spodoptera exigua* pupae fluctuates over time. The steady decrease in weight after point 3 corresponds to when the adult emerges and begins to respire while still inside the pea, as supported by the ultrasonic data. The sharp drop in weight at the last observation time (Figs. 6 and 7) is a result of the adult leaving the pea.

The gravimetric method we developed can be used to determine the development time for three immature stages of *C. maculatus:* egg, larva, and pupa. It is necessary to emphasize several points in the discussion of the use of the method, in consideration of the earlier published study on *S. granarius* in wheat (Chambers et al. 1984). We hoped that a more sensitive balance would allow us to more readily discern weight changes caused by larval feeding in the early instars. Although we did not achieve that goal, future insight into the practicality of the approach could be gained by using larger species of insects in smaller seeds with a slightly slower development time. This would allow a researcher to offset the limitations identified by this research. The greater weight losses and greater size of *S. granarius* may make weight differences caused by early instars more obvious than for the smaller *C. maculatus*, particularly in a smaller seed. Chambers et al. (1984) reported that initial weights of kernels with their insects averaged 52.3 mg, and average total loss up to the day before emergence was 15.1 mg. In experiment 1 of our study, average initial weight of peas with their insects averaged 234.5 ± 8.6 (SE) mg (n = 17), and weight loss at the last weighing before emergence of the adult from the pea averaged 14.8 ± 0.55 mg (n = 17). Thus, although weight loss was similar in the two studies, percentage weight loss was 29% in the study by Chambers et al. (1984) and 6% in our study. A slower rate of development would provide for more points in each stage than for this study. The rapid progression from egg to adult in *C. maculatus* at 30°C does minimize logistic complications when undertaking this research, but visualization of trends may be more difficult with fewer points in each stage, as observed when comparing the results of experiment 1 at 30°C with those of experiment 2 at 25°C.

In this study, we weighed 100 uninfested peas daily and used these data to develop a method for correcting weight changes in infested peas based on weight changes in uninfested peas. However, we could still

have been able to make the corrections required without seriously compromising accuracy by weighing only 50, or possibly even 25, uninfested seeds (Fig. 4). Therefore, we recommend that, in any future studies that use the gravimetric method to study the development of internally feeding insects on cowpeas, fewer uninfested seeds be used for correcting weight changes in infested peas. This would greatly reduce the amount of work involved.

Overall, this gravimetric method can be used to monitor development in a manner that is less labor intensive and destructive than dissecting seeds, and the gravimetric method has the advantage of maintaining individual variability in the data set. It would also be useful for studying factors that affect development, such as various environmental parameters or insect growth regulators. An additional advantage of using the gravimetric method to stage internally feeding insects is that it uses commercially available equipment and requires no specialized techniques.

Acknowledgments

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