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ARTHROPOD MANAGEMENT

Starvation-Induced Morphological Responses of the Boll Weevil, *Anthonomus grandis grandis* Boheman (Coleoptera: Curculionidae)

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ABSTRACT

Status of the boll weevil, *Anthonomus grandis grandis* Boheman, as a pest of cotton (*Gossypium* spp.) in the United States has diminished because of progress by eradication programs. However, this pest remains of critical importance in South America, and intractable populations in extreme South Texas and northern Mexico persistently threaten reinfestation of adjoining eradicated regions. The pheromone trap is an essential tool for detecting boll weevil infestations in eradication and management programs, and captures by the traps are also used to infer aspects of weevil ecology. Information provided by traps might be more interpretable if reliable morphological indicators can be identified from captured weevils, and less ambiguous if appropriate trapping intervals were identified to maximize the usefulness of those characters. Because captured boll weevils are isolated from food, key aspects of internal morphology were observed for informational potential based on their sensitivity to change in response to starvation. Condition of the midgut, presence of well-developed accessory glands in males, and presence of eggs as well as oosorption of vitellogenic oocytes in females, were particularly responsive to starvation. However, utility of these characters in interpretation of trap captures will require assessment of weevils that have been in the traps for no more than 1–2 d. Provided recently captured weevils are assessed, the characters we identify as indicative of a local source of weevils could improve the ecological information inferred from trap captures, and improve the effectiveness and cost-efficiency of remedial actions in eradication programs.

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The boll weevil (*Anthonomus grandis grandis* Boheman) has been eradicated from most of the cotton (*Gossypium* spp.) producing regions of the United States (U.S.), but remains one of the most important pests of cotton in much of South America (Scataglini et al. 2006). Even within the U.S., intractable populations remain in southernmost Texas, and in Mexico (Anonymous 2014). These populations are the putative sources of periodic reinfestations of production regions in the Texas Coastal Bend and Wintergarden areas, and their presence poses a threat to the investments in eradication by the cotton industry.

The boll weevil pheromone trap plays a key role in population monitoring and detection in eradication and management programs. In addition, these traps have been used in a wide range of ecological investigations including the seasonality of diapause (Graham et al. 1979, Keeley et al. 1977, Paula et al. 2013, Sivasupramaniam et al. 1995), utilization of alternative feeding hosts (Benedict et al. 1991, Hardee et al. 1999, Jones et al. 1993, de Ribeiro et al. 2010), emergence from overwintering and colonization of fields (Bariola et al. 1984, Fuchs and Minzenmayer 1990, Leggett et al. 1988, Stone et al. 1990, Westbrook et al. 2003), and dispersal (Guerra 1986, 1988, Pieters and Urban 1977, Rummel et al. 1975). Interpretations of trap captures that rely on characters of the captured weevils are typically subject to the assumption that observations from captured weevils are representative of the larger population. However, a variety of reports provide evidence that this assumption is questionable or unwarranted. For example, Cate and Skinner (1978) found that persistence of pollen in the weevil gut varied with time since feeding and the method used to preserve captured weevils. Additional trap-based studies of alternate feeding hosts have indicated a relative rarity or absence of cotton pollen (Jones and Coppedge 1999, Hardee et al. 1999) or a relative abundance of non-cotton pollen (de Ribeiro et al.

2010), even though weevils were captured during the cotton production season or even from within cotton fields. Westbrook et al. (2003) found the internal morphology of weevils captured in traps during the spring was not representative of the morphology of weevils recently emerged from overwintering. Graham et al. (1979) reported that whether the incidence of diapause observed in trap-captured weevils was consistent with that observed from field-collected weevils depended on time of year. Finally, Keeley et al. (1977) surveyed field- and trap-collected weevils from several geographical regions. Although they collected diapausing weevils from the field, they failed to observe trap captured weevils exhibiting diapause characters.

The relevance of morphological characters of captured weevils may depend on the trapping interval; it would be useful to know whether a given character developed prior to, or subsequent to capture. This is especially true because many of the informative characters that can occur are induced by isolation of weevils from food, as would occur after capture in a trap. If the usefulness of specific morphological characters and appropriate trapping intervals for their detection were known, then characterization of captured weevils would be informative to management and eradication programs. Because certain characters, such as egg production or pheromone release, are typical of weevil association with a reproductive host (generally fruiting cotton plants), this information may even provide insights into whether captured weevils likely originated from a local population, or from a distant source through dispersal or accidental transportation. Our objective was to evaluate starvation-induced changes in boll weevil characters and identify those most likely to provide ecologically-interpretable information, along with the time course over which those characters can be reliably detected.

MATERIALS AND METHODS

Separate experiments were conducted to evaluate starvation-induced responses of male and female weevils. Weevils of known age were obtained by collecting oviposition-punctured squares from cotton plants in the vicinity of College Station, TX. Weevils were reared to pupation within the infested squares in environmental chambers maintained at 29.4±2°C with a 13:11 (L:D) h photoperiod. Squares were inspected periodically until pupae were noted, after which pupae were harvested daily. Groups of 30–50 harvested pupae held in 100 × 15-mm Petri plates containing moistened vermiculite were inspected daily for adult eclosion. Upon eclosion, weevils were sexed using the tergal notch method (Sappington and Spurgeon 2000). The teneral adults were then confined individually within 100 × 15-mm Petri plates with a short cotton wick saturated with deionized water. Each weevil was fed a fresh, intact square daily for five days. This feeding duration was intended to facilitate development of chorionated eggs in females (Spurgeon and Raulston 1998a) and the accessory glands of males (Spurgeon 2003).

Starvation Responses of Males. Each repetition of the experiment started with a cohort of 100 male weevils. At the end of the five-d feeding period a subsample of 10 males was randomly selected for dissection and remaining males were randomly divided between fed or starved groups. Starved weevils received water only, fed weevils continued to receive a fresh square daily. On each subsequent day, ten males were again selected for dissection from each group until the supply of weevils was exhausted. The fed treatment was included in addition to the starved treatment because male accessory gland condition can be highly variable at five days of age (Spurgeon 2003), and inclusion of the fed treatment facilitated direct comparisons between feeding treatments at the same weevil ages. The experiment was conducted three times, each time with a cohort of weevils arising from a different field collection.

Although respective conditions of the accessory glands and midgut were of primary interest, testis size and development, seminal vesicle condition, and fat body condition were also examined. Accessory glands were classed as undeveloped or developed (combined classes one and two, and classes three and four, respectively, of Spurgeon 2003; illustrated in Spurgeon et al. 2003) according to their association with pheromone production. Undeveloped glands were small and transparent. A developed gland was conspicuously larger than an undeveloped gland and contained visible contents in its lumen. Testis size classes were modified from those reported by Spurgeon and Raulston (1998b), and included 'normal' (long-axis length of combined lobes >1/2 the combined length of the meso- and metathorax and abdomen) or 'small' (long-axis length <1/2 the combined length of the meso- and metathorax and abdomen). Conditions of other organs were adapted from ratings used by Spurgeon and Raulston

(1998a). Testis development was rated as either early- or late-reproductive, seminal vesicles were rated as filled with sperm or not filled, and midguts were rated as containing solid contents or not (solid contents, combined classes of full and intermediate; no solid contents, combined classes of trace and empty; described by Spurgeon and Raulston 1998a). Midguts rated as containing solid contents are typical of actively feeding weevils. Fat bodies were rated as lean (fat absent, or present with most organs in the abdomen visible) or intermediate (fat partially obscuring other organs) without regard for the type of fat body (reproductive or dormant).

Starvation Responses of Females. Each repetition of the experiment began with a cohort of 60 females. After the five-d feeding period a subsample of ten females was randomly selected for dissection. Remaining females were henceforth held with water but without food, and a subsample of ten females was dissected on each subsequent day until the supply of weevils was exhausted. Experiments with females omitted the fed treatment used in male experiments because ovary development and condition of the oocytes are less variable than accessory gland condition in the males (Spurgeon et al., 2003). The experiment involving females was conducted three times, and repetitions were concurrent with those involving males.

Fat body and midguts of females were assessed exactly as described for males. Limited availability of food often results in oosorption of vitellogenic oocytes by the female weevil (evidenced by flocculation or condensation of yolk), egg degradation indicated by vacuolation, and collapsed eggs in the copulatory pouch (Spurgeon et al. 2003). Therefore, presence of these conditions was recorded in addition to presence and numbers of previtellogenic and vitellogenic oocytes, and eggs.

Statistical Analyses. All analyses were conducted using SAS software (SAS Institute 2012). The probability of developed accessory glands in males was assessed by a generalized linear mixed model (GLMM) using a binomial distribution and adaptive quadrature estimation. The analysis contained fixed effects of feeding treatment (fed, starved), male age, and their interaction, and the random effect of experimental repetition. To allow direct comparisons of the weevils dissected at the end of the feeding period with starved weevils, the five-d-old weevils dissected before assignment to feeding treatments were coded as being starved zero days. Initial analyses did not yield model convergence because of complete separation (none of the weevils starved for four days exhibited developed accessory glands). The infinite likelihood caused by this separation was avoided by recoding each accessory gland classification from zero to 0.05 for weevils starved for four days. A significant interaction between male age and feeding treatment was explored by examining simple effects. Experiment-wise type I error rate was controlled using the ADJUST=SIMULATE option in pairwise comparisons among weevil ages within classes of feeding treatments.

Analyses of male testis size and midgut condition were not possible using the GLMM approach because of severe problems with complete separation. Instead, temporal patterns of the respective responses were examined separately for fed and starved weevils using Mantel-Haenszel tests of correlation in contingency tables stratified by experimental repetition (Stokes et al. 2012). The five-d-old weevils dissected before assignment to feeding treatments were included in each analysis. Because sample sizes were unequal, Ridit scoring was used. In addition, asymptotic binomial standard errors were calculated for each combination of feeding treatment and weevil age.

Starvation-induced responses indicated by fat bodies, testis ratings, and seminal vesicle ratings were too weak to warrant statistical analysis. Examinations of these responses were limited to calculation of summary proportions and asymptotic standard errors.

Because the design of experiments for females was simpler compared with the experimental design for males, the GLMM approach was applied to more of the analyses of females. Each GLMM used adaptive quadrature estimation and included the fixed effect of female age and the random effect of experimental repetition. The respective probabilities of occurrence of lean fat body condition, presence of solid contents in the midgut, occurrences of vitellogenic oocytes, combined previtellogenic and vitellogenic oocytes, and eggs were analyzed using a binomial distribution. In analyses of midgut condition, observations of weevils that were starved $\geq$ two d were pooled to avoid problems with complete separation. Numbers of previtellogenic oocytes, vitellogenic oocytes, and combined previtellogenic and vitellogenic oocytes were analyzed using the same model as previously described but with a negative binomial distribution. Numbers of eggs were simi-

larly examined but using the Poisson distribution. Choice of negative binomial or Poisson distribution was based on the value of the Pearson chi-square/df (Stroup 2013).

GLMM analyses of the occurrence of previtellogenic oocytes, crushed eggs, degraded eggs, or oosorption did not yield stable variance estimates. Therefore, temporal patterns in the frequencies of these characters were analyzed using the Mantel-Haenszel correlation test with Ridit scoring and asymptotic standard errors as described for similar analyses of male characters.

RESULTS AND DISCUSSION

Starvation Responses of Males. The conditions of the male accessory glands and midgut were highly responsive to starvation. Analyses of accessory gland condition indicated significant effects of both feeding treatment (F = 72.42; df = 1, 260; $P < 0.01$) and duration of the starvation period (F = 8.83; df = 4, 260; $P < 0.01$). However, the interaction between feeding treatment and starvation period (F = 4.31; df = 3, 260; $P < 0.01$; Fig. 1a) indicated the temporal pattern in accessory gland condition depended on feeding treatment. Examinations of simple effects of starvation period within feeding treatment indicated a difference in accessory gland condition among starved weevils of different ages (F = 10.55; df = 4, 260; $P < 0.01$), but not among ages of weevils continuously provided food (F = 0.35; df = 3, 260; P = 0.79). The probability of occurrence of well-developed accessory glands was decreased by more than 35% after a single day of starvation, and ≥85% at starvation durations of two days or longer. Responses of the midgut occurred even more rapidly than those of the accessory glands (Fig. 1b). When males were starved, the occurrence of solid food within the midgut was reduced by ≥85% compared with the fed controls at each interval of starvation (one–four d; Q_{CSMH} = 31.55, df = 1, P = <0.01). In contrast, the proportion of midguts containing solid food was relatively constant over weevil age for males continuously provided food (Q_{CSMH} = 0.05, df = 1, P = 0.83). Although analyses of testis size indicated a significant increase in the incidence of small testes with increased duration of starvation (Q_{CSMH} = 9.50, df = 1, $P < 0.01$), these responses were of much lower magnitude compared with responses of the accessory glands or midgut contents (Fig. 1c). In comparison, we found only weak evidence of age-related changes in testis size for males continuously provided food (Q_{CSMH} = 3.32, df = 1, P = 0.07; Fig. 1c).

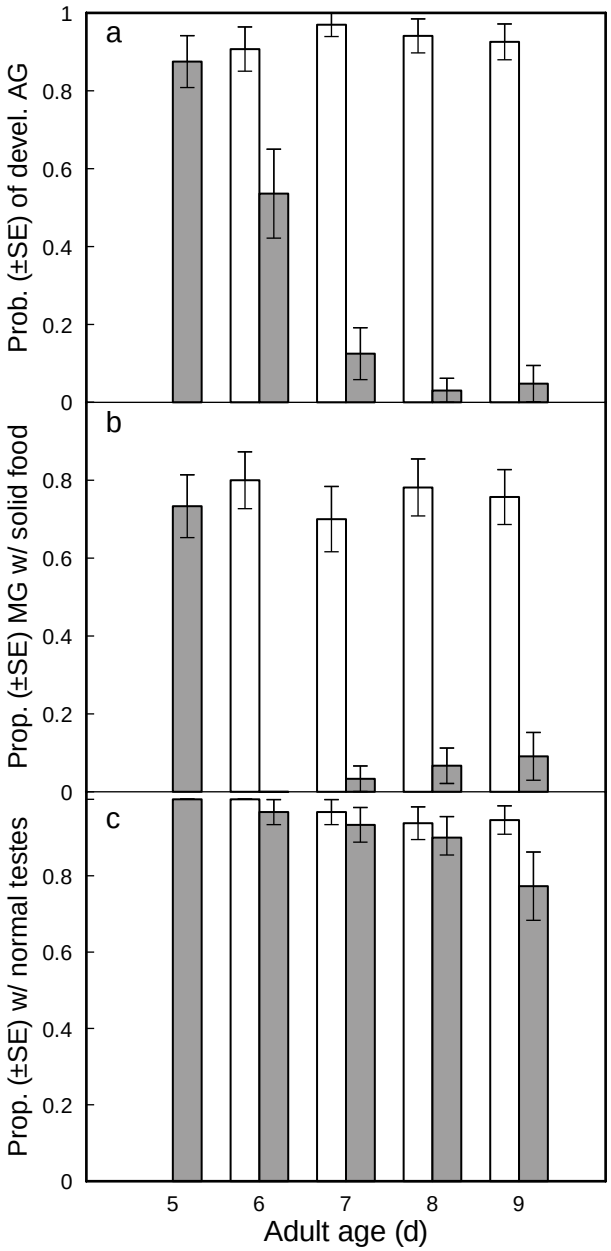

Fig. 1. Mean (±SE) probability of well-developed accessory glands (a) and proportions (±SE) of midguts containing solid food (b) and testes of normal size (c) for male boll weevils starved (shaded bars) or provided food (white bars) for one–four d after feeding for five days after adult eclosion.

The boll weevil typically releases large amounts of pheromone in response to feeding on cotton squares or small or medium sized bolls (Spurgeon 2003; Spurgeon and Suh 2009), and pheromone release is associated with well-developed accessory glands (Spurgeon 2003). Because weevils captured

in pheromone traps do not have access to food, the response of accessory gland condition to starvation suggests the potential to detect recent association with a reproductive host. Also, Suh and Spurgeon (2016) documented the ability of weevils to continue releases of substantial quantities of pheromone for up to two days after they were isolated from food. Therefore, examination of accessory glands of captured weevils may indicate whether they are influencing trap captures by augmenting the pheromone lure. Although condition of the accessory glands changed rapidly in response to starvation, if trap maintenance schedules were appropriately short, the likelihood of detecting recent association with a reproductive host is relatively high. Reproductive development of the boll weevil is highly temperature dependent (Spurgeon and Raulston 1998a). If changes in the condition of the male accessory glands are similarly temperature dependent, trapping environments where temperatures are lower than those used in this study may result in longer durations over which accessory gland condition is informative.

In contrast to results for accessory glands, changes in condition of midguts likely occur too rapidly to routinely provide inferences about the origin of captured weevils, especially considering not all weevils contained midguts with solid food before starvation treatments were imposed. However, probability of detection of solid food in the midgut, or well developed accessory glands, would be increased in situations where, for example, weevils were captured shortly after local volunteer or regrowth cotton plants were destroyed by tillage, herbicide, or intermittent freezing temperatures. In those cases, captured weevils with solid gut contents or well developed accessory glands would almost certainly indicate recent association with a local feeding or reproductive host, respectively.

Although testis size was responsive to starvation duration, temporal changes occurred more slowly compared with accessory glands or midguts. Testis size is also known to respond to type of cotton diet (Spurgeon et al. 2003). These factors make interpretation of testis size difficult even in response to extended periods of starvation. Therefore, utility of testis size in interpretations of trap captures is likely limited.

Conditions of fat bodies or seminal vesicles were unresponsive to starvation durations. The testis ratings of Spurgeon et al. (2003) were similarly unresponsive. The mean proportion (±SE) of male weevils with a lean fat body ranged only from 0.90 ± 0.05 to 0.97 ± 0.03 for starved weevils, and from 0.91 ± 0.05 to 1.00 ± 0.00 for weevils continuously provided food. The lowest proportion of weevils with testes rated as normal, irrespective of feeding treatment or age, was 0.94 ± 0.04. Finally, only a single weevil (starved two days, seven-d-old) was observed with seminal vesicles that were not filled with sperm.

We did not anticipate that fat body, testis, or seminal vesicle condition would be informative because we purposely prepared experimental weevils using a diet that promotes reproductive development (Spurgeon and Raulston 2006). The seminal vesicle ratings are not sensitive to adult diet or physiological status, and we would not anticipate depletion of sperm from these organs in trap captured weevils unless mating occurs within the capture containers. We anticipated that testis and fat body condition would be most useful to indicate the presence of diapausing weevils, as was the case in several trapping studies (Graham et al. 1979, Guerra et al. 1982, Segers et al. 1987, Thompson and Leggett 1978). However, incidence of diapause detected in pheromone traps has not proven a good indicator of incidence of diapause in the field (Graham et al. 1979, Mitchell and Hardee 1974), and some reports indicated few (Sivasupramaniam et al. 1995) or no diapausing weevils were captured in traps (Keely et al. 1977). Regardless, classification of captured weevils as diapausing or not provides few insights into the origin of the captured weevils, especially because of the weevil's dispersal ability. Either classification of diapause or nondiapause could represent a local or distant source.

Starvation Responses of Females. As was observed for the males, the midgut of starved females rapidly emptied of solid food ($F = 23.0$; df = 2, 187; $P < 0.01$; Fig. 2a). The probability of occurrence of solid food decreased from >90% in five-d-old, non-starved females to <15% after a single day of starvation. Comparisons among durations of starvation indicated the probability of solid food in the midgut declined each day from day zero (five-d-old weevils) until day two of starvation (seven-d-old weevils; Fig. 2a; adjusted $P \leq 0.02$). Because of the strong similarity in responses of male and female midguts to starvation, we expect use of midgut condition in interpretation of trap captures of either sex is subject to the same potential and limitations.

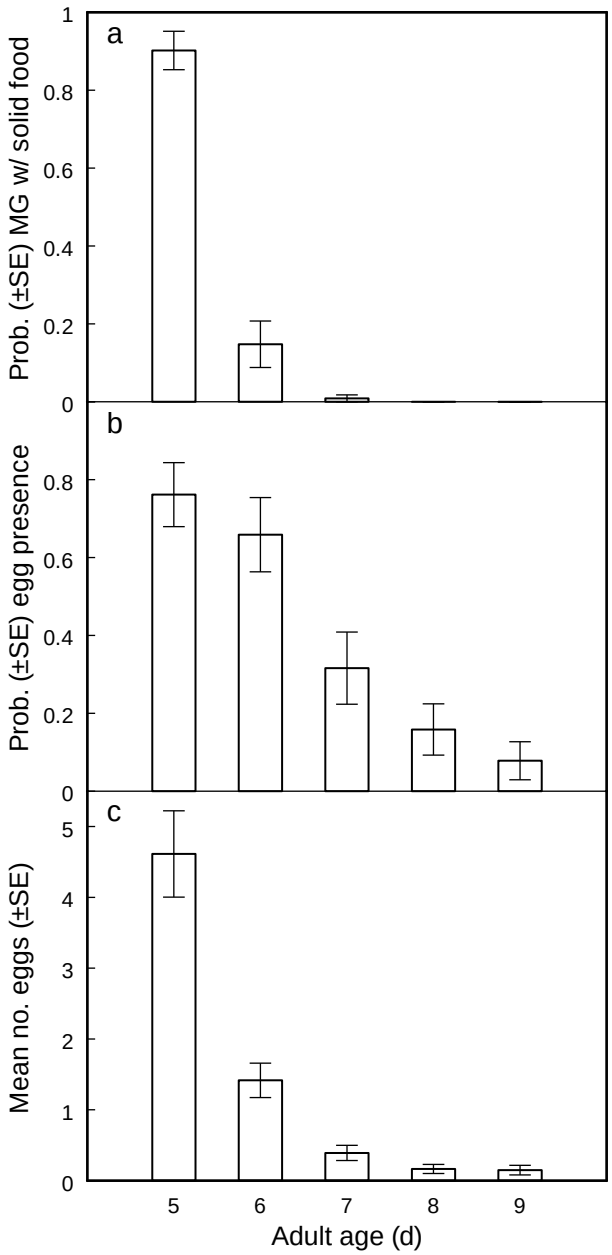

Fig. 2. Mean (±SE) probabilities of midguts with solid contents (a) and presence of eggs (b), and numbers of eggs (c) for female boll weevils starved for one–four d after feeding for five d after adult eclosion.

The occurrence of previtellogenic oocytes (Q_{CSMH} = 0.31, df = 1, P = 0.58), vitellogenic oocytes (F = 0.42; df = 4, 185; P = 0.79), or oocytes of any development (F = 0.33; df = 4, 185; P = 0.86) were not influenced by starvation. The mean proportions (±SE) of females containing previtellogenic oocytes varied from 0.95 (±0.03) to 0.90 (±0.05) among the durations of starvation, whereas the probabilities of vitellogenic oocytes ranged from 0.91 (±0.05) to 0.84 (±0.07). The mean numbers of previtellogenic oocytes (F = 0.97; df = 4, 185; P = 0.42), vitellogenic oocytes (F = 0.81; df = 4, 185; P = 0.52), and combined oocytes per female (F = 0.65; df = 4, 185; P = 0.63) were similarly uninformative. However, both the probability of egg occurrence (F = 10.94; df = 4, 185; P < 0.01) and numbers of eggs were influenced by starvation (Fig. 2b, c, respectively). Pairwise comparisons of the probabilities that a female would contain one or more eggs indicated no response after one day of starvation (t = 1.0, df = 185, adjusted P = 0.85), but the probability was reduced on subsequent days (<0.01 < adjusted P < 0.03; Fig. 2b). The probabilities of egg occurrence at durations of starvation ≥ two days were not distinguishable (0.12 < adjusted P < 0.82). Patterns in the mean numbers of eggs per female were similar to those for egg occurrence (F = 58.80; df = 1, 185; P < 0.01) except the influence of a single day of starvation was distinguishable (five-d-old vs 6-d-old; t = 7.87, df = 185, adjusted P < 0.01). Comparisons among seven to nine-d-old females (starved two to four days) indicated the maximal response observed occurred by two days of starvation (0.28 < adjusted P < 1.0).

The absence of a response to starvation in the presence or numbers of previtellogenic oocytes is not informative, especially considering that diapausing weevils occasionally exhibit previtellogenic oocytes. However, the lack of a response in presence or numbers of vitellogenic oocytes over the duration of starvation is potentially useful. These characters seem relatively persistent indicators of recent association with a reproductive host, which in most cases would be fruiting cotton. In contrast, presence and numbers of eggs declined rapidly in response to starvation. Presence of vitellogenic oocytes or eggs does not preclude a non-local origin of a captured weevil. However, when these characters are combined, a local reproduction-promoting source, such as regrowth or volunteer cotton, is implied particularly if multiple captured weevils exhibit these characters.

As in the case of male weevils, fat body condition in starved females was not informative (F = 0.5; df = 4, 185; P = 0.74). The mean proportions (±SE) of females with lean fat bodies ranged only from 0.91 (±0.05) after two days of starvation, to 0.82 (±0.08) in weevils dissected before the starvation treatments were assigned. The frequency with which crushed eggs occurred in the copulatory pouch was similarly insensitive to starvation (Q_{CSMH} = 0.01, df = 1, P = 0.92), and the proportion of females exhibiting this character ranged from 0.03 (±0.03) in nine-d-old females (starved for four days) to 0.15 (±0.06) in

females starved for two days. However, both the likelihood of females with degraded eggs (Q_{CSMH} = 7.55, df = 1, $P < 0.01$; Fig. 3a) and occurrence of oosorption (Q_{CSMH} = 67.18, df = 1, $P < 0.01$; Fig. 3b) increased with duration of starvation.

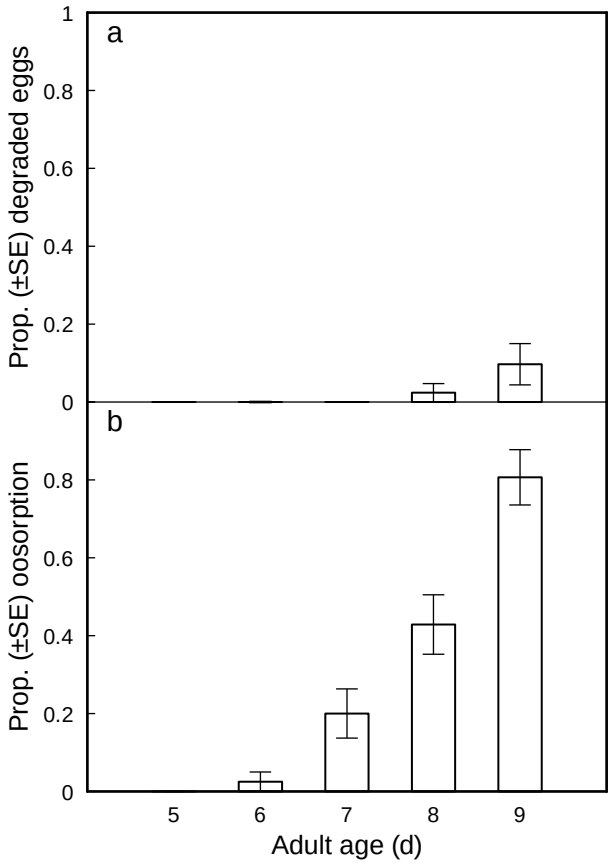

Fig. 3. Proportions (±SE) of ovaries with degraded eggs (a) and exhibiting oosorption (b) for female boll weevils starved for one–four d after feeding for five d after adult eclosion.

As observed for males, the fat body condition of females was not informative because of lack of response to starvation. The frequency of occurrence of crushed eggs in the copulatory pouch was not sensitive to starvation but a response in the frequency of degraded eggs was detected. However, the occurrence of either character was too infrequent to aid in interpreting trap captures. The presence of oosorption within vitellogenic oocytes indicates access to a reproductive host was interrupted for at least a few, and possibly several, days. Therefore, presence of vitellogenic oocytes combined with absence of oosorption would strongly implicate a local reproductive host as the probable source of a captured female weevil. The majority of weevils with vitellogenic oocytes lacked evidence of oosorption even after three days of starvation. Thus, detection of a local reproductive host as the source of trap captured weevils can be indicated with trap intervals as long as two or three days at relatively high temperatures like those in this study. This interval could likely be longer under lower temperatures, but information to describe the temperature-dependence of the onset of oosorption is not available.

Conclusions. Although midgut condition could be informative, response of the midgut to starvation is so rapid that detection of solid contents is unlikely even when captured weevils originate locally. The rapidity of this response suggests that studies to identify potential alternate (non-cotton) feeding hosts should incorporate short trapping intervals to increase relevance of the results. Inappropriately long trapping intervals in previous studies may have been responsible for the absence or rarity of cotton pollen in the guts of weevils captured in the vicinity of fruiting cotton plants. Also, whereas the probability of detecting solid contents in the midgut would be maximized by examining daily trap captures, presence of solid food would not necessarily indicate recent association with fruiting cotton or another reproductive host. This is because the weevil is known to feed on seedling and non-fruiting regrowth cotton plants (Bariola 1984, Esquivel et al. 2004, Rummel and Carroll 1985, Suh and Spurgeon 2006), and there is currently no unambiguous method to distinguish food source based on gut contents. Instead, our results suggest male accessory gland and female ovary conditions offer the most potential for enhancing interpretation of pheromone trap captures and indicating the likely source (local versus distant) of captured weevils. Use of these characters would require short trapping intervals, which should probably not exceed two days. The absence of indicators of association with a recent host (developed accessory glands, lack of oosorption, presence of eggs) in captured weevils does not imply a distant origin because the prior history of captured weevils is generally unknown. This becomes increasingly relevant post-harvest with increased time following thorough elimination of regrowth or volunteer cotton in the local area. However, presence of the criteria provided to detect recent association with a reproductive host almost certainly indicates a local source, which is likely undestroyed cotton. Because the trapping intervals we recommend are shorter than those typically used in field studies or monitoring efforts, utilization of our findings may increase the

quality of ecological information inferred from trap captures. In an eradication context, knowledge provided by this study may improve effectiveness and cost-efficiency of remediation efforts by improving the likelihood of detecting when weevils are arising from a local source.

DISCLAIMER

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