# DIAPAUSE INDUCTION IN SUBTROPICAL BOLL WEEVILS

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## Abstract

Diapause induction in adult boll weevils was revisited using a standard feeding regime developed at this laboratory as a baseline for comparison of the effects of photoperiod, nighttime temperature, and feeding regimes more commonly used in such studies. The baseline feeding regime consisted of a single intact square fed each day to each individual weevil. The more traditional feeding regime consisted of feeding 5 debracted squares each day to a group of 25 mixed sex weevils held in a pint carton. Environmental conditions examined included 11 and 13 h photoperiods, in all combinations with thermoperiods of constant 29°C or 29°C daytime and 10°C nighttime. Assessments of fat body, gonad condition, and diapause were obtained from dissections at 6, 9, and 12 d after adult eclosion. Photoperiod did not influence the incidence of diapause by itself or in combination with any other factor. Cool nighttime temperature slowed the development of diapause characters in males but this effect was not demonstrated in females. Feeding regime was the major factor determining the incidence of diapause characters in both sexes. Results indicated that levels of diapause observed are highly dependent on the criteria used to determine diapause, and that seminal vesicle condition in males, an often used criteria, is of questionable value. Our study indicates the need for researchers to adopt a more standard and consistent experimental approach to examining boll weevil diapause, thereby facilitating comparison of results between laboratories and geographical areas.

## Introduction

Since Brazzel and Newsom (1959) reported the occurrence of a reproductive diapause in the boll weevil, considerable effort has been devoted to understanding the factors involved in diapause induction. Still, the exact nature of the boll weevil diapause and factors controlling it remain uncertain. A survey of the published literature is only marginally informative because of the wide variability in reported results and the inconsistencies in experimental approaches and procedures. Much of the variation in results has been attributed to differences in boll weevil races or related to geographic origin of populations but no study has directly examined differences among populations while using procedures that allow results to be directly compared to those of other studies. This, combined with reports by Guerra *et al*. (1982, 1984) questioning the use of the term diapause to describe boll weevil reproductive dormancy in the tropics and subtropics, prompted us to revisit the diapause phenomenon in subtropical weevils. As a result, recent research at this laboratory has identified a number of potential artifacts common to most diapause induction studies. These artifacts may account for much of the variation reported in previous studies.

Our recent investigations of boll weevil reproductive biology have focused on examining the potential for introduction of experimental artifacts caused by failure to account for differences in physiological ages of weevils at the time of dissection (Spurgeon and Raulston 1997a; Spurgeon and Raulston, 1998), and by use of inadequate feeding regimes (Spurgeon and Raulston 1996; Spurgeon and Raulston 1997b). Although effects of feeding rate and food type on incidence of diapause are well documented, these effects have been poorly quantified. Most studies of diapause induction describe, in at least general terms, the types of food supplied weevils during the feeding or induction period, but few adequately describe the quantities or frequency of feeding. We have found only six reports that describe the numbers of cotton fruit supplied to weevils, which have ranged from 1 square per 2 weevils daily or on alternate days (Earle and Newsom 1964) to 1 square per 10 weevils daily (Lloyd *et al*. 1967). Typical feeding rates for other studies supplying this information have been 1 square per 5 weevils daily or 1 boll per 5 or 10 weevils daily (Harris *et al*. 1969, Jenkins *et al*. 1972, Lloyd *et al*. 1967, Tingle and Lloyd 1969, McCoy *et al*. 1968). Lloyd *et al*. (1967) demonstrated the marked effect of feeding rate on the expression of diapause characters by holding weevils under identical environmental conditions and varying feeding rate. They observed 5% diapause at a feeding rate of 1 square per 5 weevils daily and 75% diapause when feeding rate was reduced to 1 square per 10 weevils daily. In spite of this demonstration, a standardized feeding rate to serve as a baseline "control" in diapause induction studies has not been devised.

Through our previous research regarding feeding regimes we have found a feeding rate of a single intact and undamaged square per weevil daily to serve as an adequate standard for use in reproductive biology studies. While this standard is suboptimal relative to food availability under field conditions, it is a practical feeding rate that results in a very high rate of reproductive commitment (Spurgeon and Raulston 1996, 1997b). Our objective was to use this feeding regime, in comparison to a more typical feeding regime, to reevaluate the effects of photoperiod and temperature conditions on diapause induction of subtropical boll weevils.

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## Materials and Methods

Adult boll weevils were reared from infested squares collected in mid-June and early-July. Only squares which had not yet abscised were collected to minimize the variation in age distribution of weevil immatures and improve the synchrony of adult eclosion. Squares were maintained in 1-ft$^3$ screen cages in an environmental chamber at 29°C and with a 13:11 [L:D] photoperiod. Samples of squares were removed from the cages periodically and dissected to determine the extent of weevil development. When >75% of the larvae had pupated, pupae were harvested.

Harvested pupae were placed in petri dishes each containing a thin layer of moistened vermiculite. Dishes contained 30 to 35 pupae each, and were held in the environmental chamber with the caged squares. Dishes were checked two or three times daily for weevil eclosion, and teneral adults were assigned to experimental treatments. Thus adult weevil age was known and was measured with reference to eclosion rather than from emergence from the square.

Experimental treatments included all combinations of photoperiod (short, 11 h; long, 13 h), temperature regime (warm night, constant 29°C; cool night, 29° for 12 h and 10°C for 12 h), and feeding regime (1 square daily per weevil held individually; 5 squares each day per 25 mixed sex weevils). Thus, eight treatment combinations of photoperiod, temperature regime, and feeding regime were examined.

Only squares that were 6 to 10 mm (mostly 7 to 9 mm) in diameter were fed. Food squares were sorted after collection and any squares that were chlorotic or otherwise discolored, with torn bracts, or infested or damaged by boll weevil or other insects were discarded. Sorted squares were washed with distilled water, drained, and placed in sealable plastic bags for storage in a refrigerator until use. Squares were normally fed within 1-2 days of collection except on weekends, when most squares had been stored for 3 days. Any squares that has become discolored or wilted by the time of feeding were discarded. Our intent was to exercise a high degree of control over the quality and uniformity of food supplied.

Individual weevils were held in petri plates with a short section of water saturated dental wick and food squares were supplied with bracts intact. Weevils held in groups (12 males and 13 females) were confined in pint cardboard cartons with screen lids, supplied water in a 1-oz. plastic cup closed by a paper lid penetrated by a section of dental wick, and fed squares with bracts removed. Removal of bracts reduced the labor associated with changing the food daily. Feeding rates of weevils held in groups were adjusted as required in response to mortality.

Dissections (10 weevils of each sex) were performed at 6, 9, and 12 d after eclosion. The timing of dissections was based on results of Spurgeon and Raulston (1998) and was intended to allow adequate time for disappearance of juvenile fat in both sexes, formation of oocytes containing yolk in most females, and observable sperm accumulation in testes of most males, before dissections began regardless of temperature regime. Fat body condition of both sexes was rated as lean, intermediate, or fat according to the criteria of Brazzel and Newsom (1959). Because weevil size was variable, testis size was assessed in relation to the body size. Testes with a long-axis length >½ the combined length of the meso- and metathorax and the abdomen were classed as normal. Testes <½ as long as this distance were classed as small. Testes <⅓ this length were classed as extra small. Testis condition was based on color and was assigned rating of 'normal reproductive' (center of the testicular lobe opaque white because of sperm) or 'atrophied' (testes opaque because of dense deposits of yellow fat). Translucent testes with opaque centers were assigned a rating normal reproductive even if a tinge of yellow fat was evident. Such testes were previously described as 'light yellow' by Brazzel and Newsom (1959). These classifications were based on observations regarding the chronology of development of small yellow testes reported by Spurgeon and Raulston (1997a). Seminal vesicles were classified as 'full' if they were opaque with sperm. Otherwise they were classed as 'empty', although empty seminal vesicles sometimes were translucent and contained some sperm because they were being refilled following mating. Because ovarioles often became irreversibly elongated during their removal, especially in fat females, female reproductive development was assessed based on the presence or absence of previtellogenic oocytes, oocytes containing yolk, and chorionated eggs.

The experiment was first conducted in early July and repeated in mid- to late-July. Each repetition was used as a replication for analysis of the data. Data for each measured parameter except testis size and condition was analyzed by analysis of variance using the SAS procedure PROC GLM (SAS Institute 1988) with photoperiod, temperature regime, feeding regime, and age at dissection as main effects. The data were analyzed after transformation by $\arcsin\sqrt{p}$, where $p$ is a proportion. The association between testes size and condition was examined using a contingency table, and on the basis of these results, testis rating data were analyzed by considering the proportion of males with extra small yellow testes (subsequently referred to as atrophied testes). In addition, because considerable variation exists among published reports in the criteria used to distinguish diapause, incidence of diapause defined by two separate sets of criteria for each sex was examined by analysis of variance. One set of criteria was relatively lenient (males, fat or intermediate fat body and with atrophied testes; females, fat or intermediate fat body and lacking oocytes containing yolk) while the other set was more stringent (males, fat or intermediate fat body, atrophied testes, and

empty seminal vesicles; females, fat or intermediate fat body and lacking oocytes).

## Results

Overall, male fat body development was not influenced by photoperiod ($F$=0.24; df=1, 24; $p$=0.63) or temperature regime ($F$=0.66; df=1, 24; $p$=0.42), but both feeding regime ($F$=69.24; df=1, 24; $p$<0.01) and age at dissection ($F$=35.02; df=2, 24; $p$<0.01) were influential (Fig. 1). Fat bodies of male weevils fed as a group (5 squares/25 weevils daily) tended to be more developed than those of weevils fed singly (1 square/weevil daily), and fat body development tended to increase with age at dissection. Changes in male fat body condition with increasing age were greater when weevils were fed in a group than when they were fed singly (feeding regime by age interaction, $F$=4.19; df=2, 24; $p$=0.03). Although effects of temperature regime were not significant, the temperature regime by feeding regime ($F$=12.15; df=1, 24; $p$<0.01) and temperature regime by age ($F$=6.16; df=2, 24; $p$<0.01) interactions indicated that cool nighttime temperature slowed male fat body development when weevils were fed as a group.

Inspection of the data for association between testis size and condition indicated that 96.2% of opaque yellow testes were also rated as extra small while 11.5% of normal reproductive testes were assigned to that size class. Thus, an accurate distinction between prereproductive or normal testes and what Brazzel and Newsom (1959) described as atrophied testes could not be made on the basis of testis size alone. These results justified consideration of only extra small yellow testes as being atrophied.

Male testicular rating was not influenced by photoperiod ($F$=0.71; df=1, 24; $p$=0.41), but development of atrophied testes was slowed by cool nighttime temperature ($F$=21.69; df=1, 24; $p$<0.01) and the incidence of atrophied testes increased with age at dissection ($F$=31.03; df=2, 24; $p$<0.01) (Fig. 2). However, the most influential factor was feeding regime ($F$=83.90; df=1, 24; $p$<0.01); atrophied testes were observed less frequently when weevils were fed singly. Effects of nighttime temperature on occurrence of atrophied testes were consistent across other factors, but effects of feeding regime varied with age at dissection ($F$=5.25; df=2, 24; $p$=0.01), probably because of the age dependence of this condition.

The proportion of males with empty seminal vesicles was not influenced by photoperiod whether the entire experiment was considered ($F$=1.62; df=1, 24; $p$=0.22) or data for weevils fed as a group was analyzed separately ($F$=1.20; df=1, 12; $p$=0.29) (Fig. 3). Neither was photoperiod influential in combination with any other factor. Similarly, effects on seminal vesicle condition of temperature regime ($F$=1.05; df=1, 24; $p$=0.31), age at dissection ($F$=0.26; df=2, 24; $p$=0.77), or interactions involving either factor were not demonstrated because dissections were not begun until sufficient time had elapsed to allow filling of the seminal vesicles with sperm. The proportion of males with empty seminal vesicles was influenced by feeding regime ($F$=77.71; df=1, 24; $p$<0.01) because only males fed as a group had access to mates, and this condition was only achieved through transfer of sperm to females during mating.

The occurrence of diapause in males using lenient criteria (fat or intermediate fat body, atrophied testes) was not influenced by photoperiod ($F$=1.07; df=1, 24; $p$=0.31) or any interaction of photoperiod and other factors (Fig. 4). Effects of temperature regime ($F$=20.32; df=1, 24; $p$<0.01), feeding regime ($F$=83.64; df=1, 24; $p$<0.01), age at dissection ($F$=28.88; df=2, 24; $p$=<0.01), and the feeding regime by age interaction ($F$=6.85; df=2, 24; $p$<0.01) on occurrence of diapause were similar to the effects of these factors on occurrence of atrophied testes, indicating that testis condition was more influential than fat body condition in determination of diapause using these criteria.

The occurrence of diapause in males using more stringent criteria (fat or intermediate fat body, atrophied testes, and empty seminal vesicles) differed greatly from that using lenient criteria (Fig. 5). Photoperiod did not influence the occurrence of diapause ($F$=1.15; df=1, 24; $p$=0.30). The incidence of diapause increased with increased nighttime temperature ($F$=19.64; df=1, 24; $p$<0.01) and age at dissection ($F$=9.83; df=2, 24; $p$<0.01), but interaction terms indicated influence of these factors varied between feeding regimes (temperature regime by feeding regime, $F$=19.64; df=1, 24; $p$=0.70; age by feeding regime, $F$=9.83; df=2, 24; $p$<0.01). Effects of feeding regime on occurrence of diapause were dramatic ($F$=94.79; df=1, 24; $p$<0.01) because the classification of diapause required the presence of empty seminal vesicles, which occurred only after mating by weevils fed as a group. Thus, the criterion of empty seminal vesicles used by some authors in studies of diapause appears artifactual and unreasonable.

As in the case of males, female fat body development was not affected by photoperiod ($F$=0.00; df=1, 24; $p$=0.97) or temperature regime ($F$=3.26; df=1, 24; $p$=0.08) but was influenced by feeding regime ($F$=96.80; df=1, 24; $p$<0.01) and age at dissection ($F$=8.29; df=2, 24; $p$<0.01) (Fig. 6). Females tended to be fatter when fed as a group than when fed singly, and female fat body development increased significantly between the ages of 6 and 9 d after eclosion. Also as for males, changes in female fat body with increasing age were more distinct when females were fed as a group than when fed singly (feeding regime by age interaction, $F$=5.04; df=2, 24; $p$=0.01). However, in contrast to males, interactions between temperature regime and feeding regime ($F$=2.91; df=1, 24; $p$=0.10) and temperature regime and age ($F$=3.08; df=2, 24; $p$=0.06) were not significant, although the data indicate trends similar to those observed for males.

Photoperiod also had no effect on reproductive development of female weevils. Photoperiod did not influence the occurrence of previtellogenic oocytes ($F$=0.00; df=1, 24; $p$=0.99; Fig. 7), oocytes with yolk ($F$=0.08; df=1, 24; $p$=0.77; Fig. 8), or chorionated eggs ($F$=0.11; df=1, 24; $p$=0.74; Fig 9). Nor was female reproductive development influenced by the interaction of photoperiod with any other factor. Because adequate time was allowed for considerable development before dissections were initiated, effects of temperature regime (previtellogenic oocytes, $F$=2.22; df=1, 24; $p$=0.15; oocytes with yolk, $F$=1.19; df=1, 24; $p$=0.29; chorionated eggs, $F$=1.87; df=1, 24; $p$=0.18) or age at dissection (previtellogenic oocytes, $F$=0.46; df=2, 24; $p$=0.64; oocytes with yolk, $F$=0.04; df=2, 24; $p$=0.96; chorionated eggs, $F$=0.79; df=2, 24; $p$=0.47) could not be demonstrated. However, feeding regime influenced these parameters dramatically (previtellogenic oocytes, $F$=114.13; df=1, 24; $p$<0.01; oocytes with yolk, $F$=78.86; df=1, 24; $p$<0.01; chorionated eggs, $F$=114.24; df=1, 24; $p$<0.01). In general, results indicated that the degree of reproductive commitment (proportion of females producing eggs) was determined primarily by feeding regime and at an early age, and was severely reduced when females were fed relatively few squares as a group compared to when females were fed individually and at an increased rate.

Female diapause status based on the more lenient criteria (fat or intermediate fat body, absence of oocytes with yolk) would include weevils designated by some authors as "intermediate" diapause, whereas diapause status based on the more stringent criteria (fat or intermediate fat body, absence of oocytes) would include only weevils in "firm" diapause. Based on the more lenient criteria, neither photoperiod ($F$=0.01; df=1, 24; $p$=0.93), temperature regime ($F$=0.26; df=1, 24; $p$=0.62), nor the combination of these factors ($F$=0.13; df=1, 24; $p$=0.72), influenced the frequency of occurrence of diapause characters (Fig. 10). Incidence of diapause tended to increase with age at dissection ($F$=3.84; df=2, 24; $p$=0.04) primarily because of the effects of age on fat body development. Feeding regime was the most influential factor determining diapause ($F$=56.66; df=1, 24; $p$<0.01).

When female diapause was based on the more stringent criteria, effects of photoperiod ($F$=0.02; df=1, 24; $p$=0.90), temperature regime ($F$=0.00; df=1, 24; $p$=0.99), or their interaction ($F$=0.41; df=1, 24; $p$=0.53) again could not be demonstrated. Neither was effect of age at dissection significant ($F$=3.25; df=2, 24; $p$=0.60). Also, the influence of feeding regime was again prominent ($F$=85.77; df=1, 24; $p$<0.01). Although effect of age at dissection was not statistically significant, the feeding regime by age interaction ($F$=3.78; df=2, 24; $p$=0.04) indicated that the proportion of females classed as diapausing increased with age when weevils were fed in groups.

## Discussion

Our results indicate that when applied to adults, neither photoperiod, temperature regime, nor their interaction played significant roles in determining the diapause status of subtropical boll weevils. However, feeding regime was an overwhelming influence on induction of diapause characters. Our observation that low nighttime temperatures slowed the development of diapause characters is in sharp contrast to observations of Earle and Newsom (1964), who reported that high temperatures suppressed diapause. Comparison of our results to those of other investigations are difficult or impossible because of the variability in previous findings. For example, Lloyd *et al.* (1967) reported no effect of photoperiod on the incidence of diapause when weevils were reared on plants at constant temperatures, but that low nighttime temperatures induced diapause. Cobb and Bass (1968) reported no clear cut diapause response to photoperiod, temperature, or food, but observed that certain combinations of these factors resulted in a signficantly greater incidence of diapause. Carter and Phillips (1974) indicated that boll weevil diapause is influenced throughout the season by changes in condition of the host plant while Earle and Newsom (1964) reported that there was no evidence to suggest that diapause induction in the adult stage is influenced by plant maturity. Further, a number of authors have emphasized the influence of photoperiod on diapause induction. Mangum *et al.* (1968) reported that diapause was induced in the adult weevil by exposure to an 11 h photoperiod provided larvae were reared in darkness. Sterling (1972) found that diapause was induced by exposure to photoperiods of 8, 10, 11, 12, 14, and 15 h, and suppressed by photoperiods of 12.5, 13, and 16 h. A more recent work (Wagner and Villavaso 1996) makes the assumption that photoperiod is the primary mechanism for diapause induction across the cotton belt. However, in none of these studies was a feeding regime supplied that was comparable to our standard regime.

Our examinations of the incidence of diapause using different criteria indicate that some differences among the results of previous studies may have been caused by inconsistent application of the various criteria. We also suggest that some of the criteria frequently used may not be appropriate (e.g., empty seminal vesicles). Our data imply that traditionally too much emphasis has been placed on fat body development, and that the classification of 'intermediate diapause' is not sufficiently well understood to be particularly useful.

Our findings indicate that a reexamination of the diapause induction phenomenon in the boll weevil, using a more systematic and controlled approach than previously employed, is warranted. In particular, attention to food sources and potential confounding effects of this factor, as well as better definition of the criteria used to determine diapause status is needed. Design and adoption of a standardized procedure for studying diapause is suggested,

and would facilitate comparisons of results from different geographical regions. In short, much remains to be discovered about the factors controlling boll weevil diapause. Given that the current Boll Weevil Eradication Programs rely heavily on population suppression supplied by diapause treatments, improvements in our understanding of boll weevil diapause could result in improvements in treatment timing and effectiveness, thereby increasing the effectiveness and reducing the costs of suppression programs.

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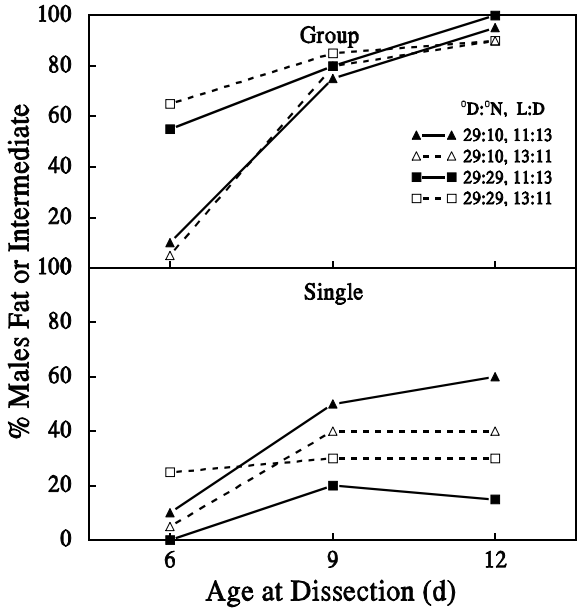


Figure 1. Percentage of male boll weevils with fat bodies rated as fat or intermediate after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

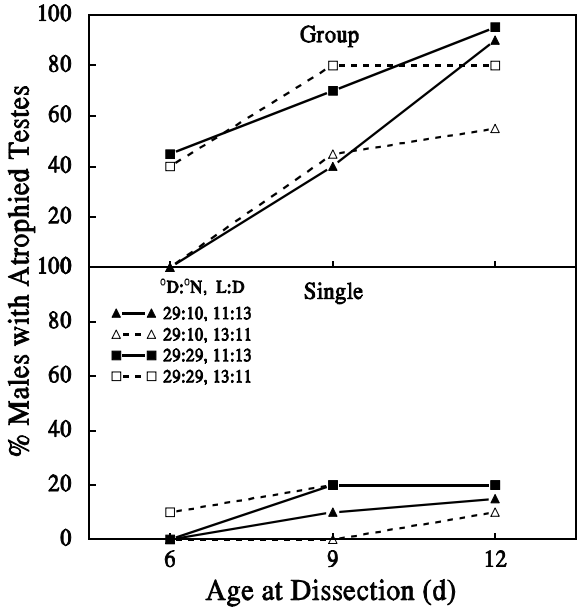


Figure 2. Percentage of male boll weevils with atrophied (extra small, yellow) testes after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

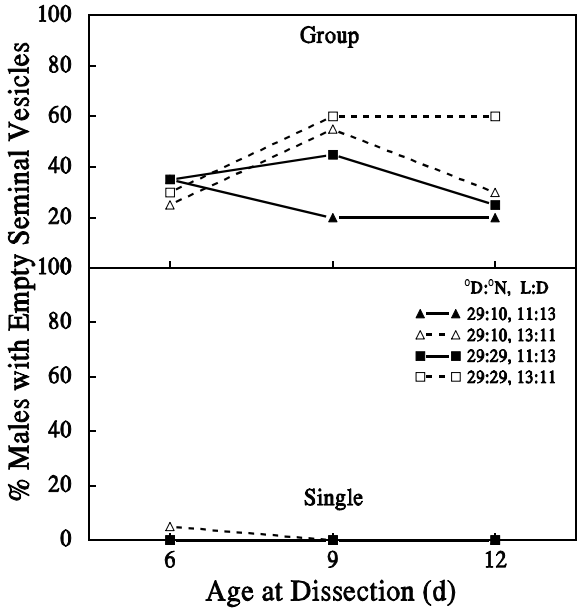


Figure 3. Percentage of male boll weevils with empty seminal vesicles after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

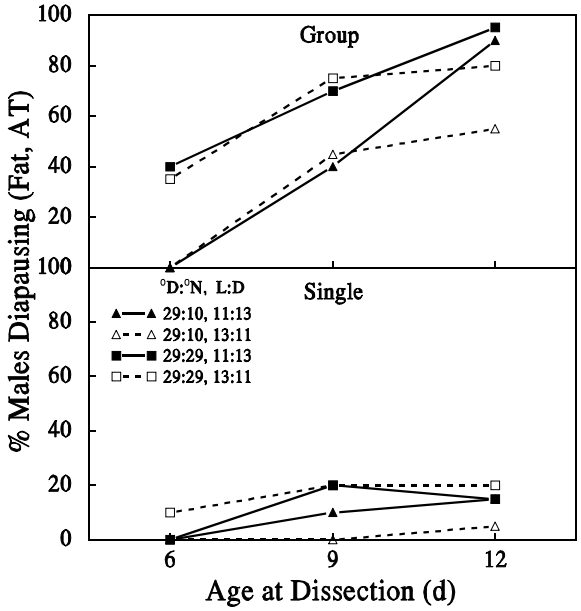


Figure 4. Percentage of male boll weevils in diapause using lenient criteria (fat or intermediate fat body, atrophied testes) after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

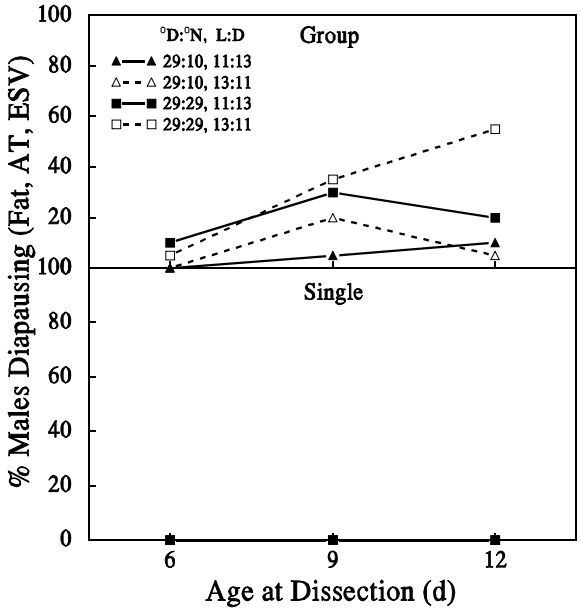


Figure 5. Percentage of male boll weevils in diapause using stringent criteria (fat or intermediate fat body, atrophied testes, empty seminal vesicles) after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

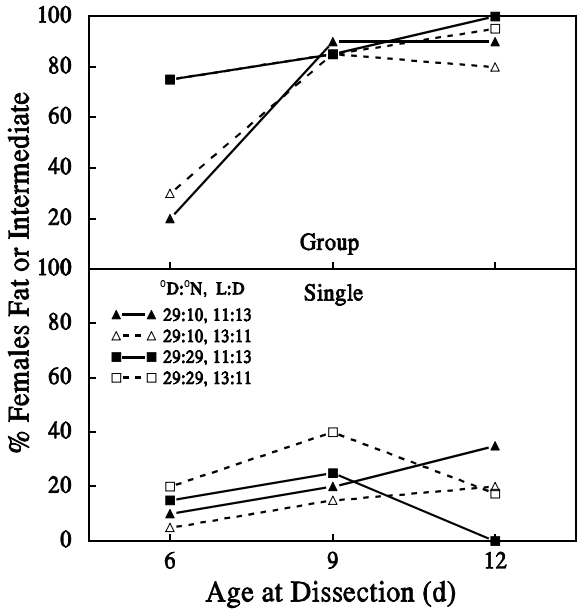


Figure 6. Percentage of female boll weevils with fat bodies rated as fat or intermediate after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

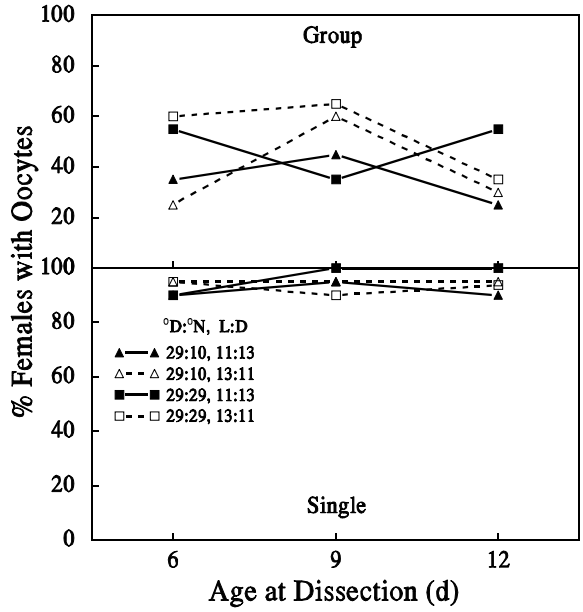


Figure 7. Percentage of female boll weevils containing previtellogenic oocytes after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

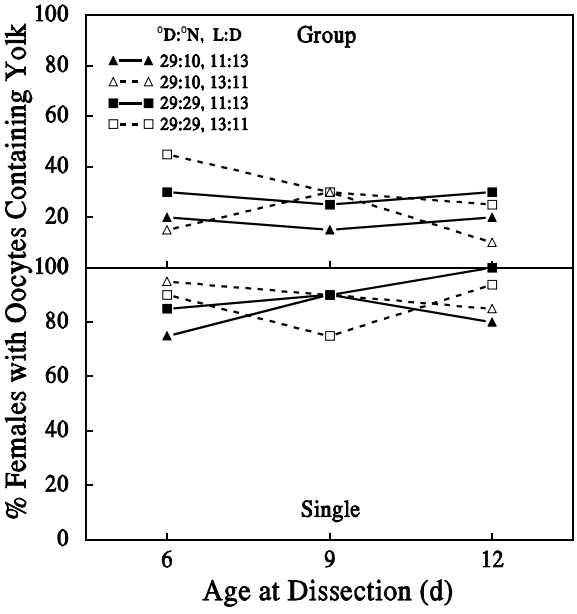


Figure 8. Percentage of female boll weevils containing oocytes with yolk after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

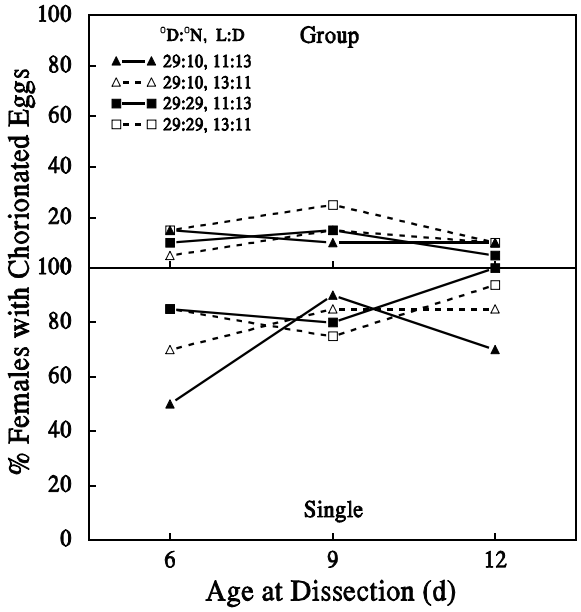


Figure 9. Percentage of female boll weevils containing chorionated eggs after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

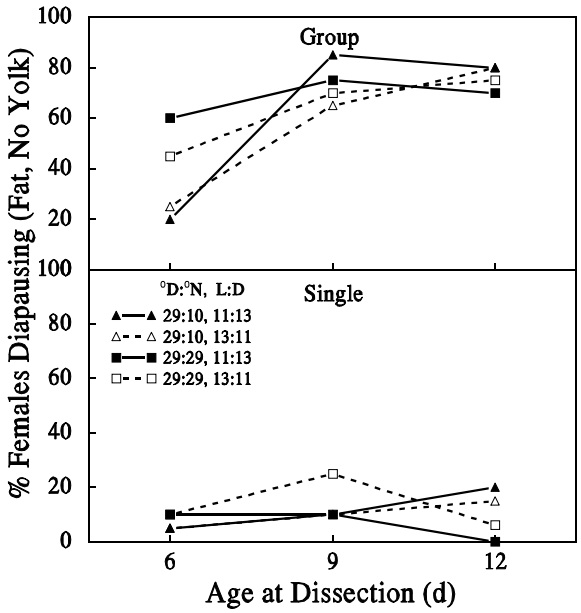


Figure 10. Percentage of female boll weevils in diapause using lenient criteria (fat or intermediate fat bodies, no oocytes with yolk) after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).

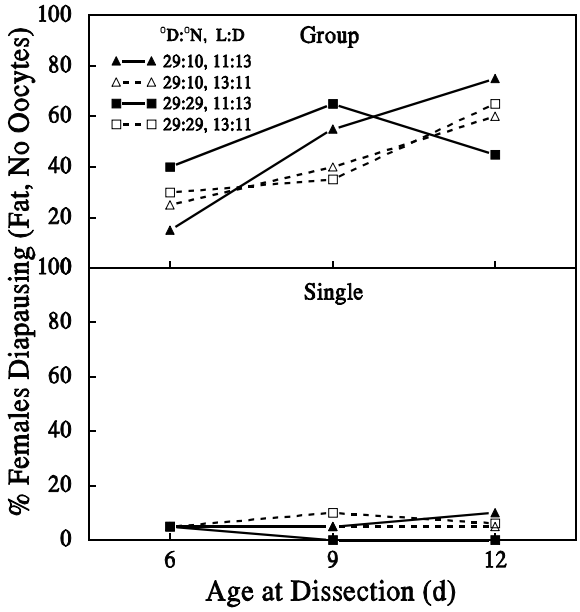


Figure 11. Percentage of female boll weevils in diapause using stringent criteria (fat or intermediate fat bodies, no oocytes) after exposure to combinations of photoperiod, temperature regime, and feeding regime (group, 5 squares/25 mixed sex weevils daily; single, 1 square/weevil daily).