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ASSESSMENT OF FEMALE REPRODUCTIVE STATUS IN *ANASTREPHA SUSPENSA* (DIPTERA: TEPHRITIDAE)

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ABSTRACT

Reliable methods are needed for assessing sexual maturity in field-captured tephritid fruit flies. To provide such a tool for female Caribbean fruit flies, *Anastrepha suspensa* (Loew), this study documented changes in ovarian development over a four-week period following adult eclosion. The ovarian maturation process was classified into six developmental stages. Stages 1-4 described sequential steps in the development of immature ovaries, stage 5 indicated presence of mature oocytes, and stage 6 was the ovipositional phase. For each stage, four morphometric characters were examined-length of ovary, width of ovary, an ovarian index (length of ovary multiplied by width of ovary), and length of terminal follicle. Ovarian characters were compared by stage and correlated with the number of mature oocytes per ovary (egg load). Ovarian index maximized the differences between sexually mature and immature ovaries, and ovary length provided the best separation of immature stages. All four characters were positively correlated with egg load, but ovarian index and ovary width were the two best indicators of mature oocytes. Use of these parameters to assess egg load would eliminate the need to tease apart ovaries and count mature oocytes, thereby providing an efficient method for processing large samples of flies. Classification of female sexual maturity based on an ovary staging system, in conjunction with assessment of egg load in mature stages, would facilitate evaluation of the physiological age structure of a fly population captured in field deployed traps.

Key Words: Caribbean fruit fly, ovary development, sexual maturation, oocyte, egg load

RESUMEN

Se necesitan métodos confiables para apreciar la madurez sexual de las moscas de la familia Tephritidae que han sido capturadas en el campo. Para proveer una medida para las hembras de la mosca de fruta del Caribe, *Anastrepha suspensa* (Loew), éste estudio documenta cambios en el desarrollo del ovario sobre un periódo de cuatro semanas despues de la ecloción del adulto. La madurez del ovario fué clasificada en seis etapas de desarrollo. Etapas 1-4 describieron los pasos en secuencia en el desarrollo del ovario inmaduro, etapa 5 indicó la presencia de oocitos maduros, y la etapa 6 fué la fase oviposicional. Por cada etapa, cuatro carácteres morfométricos fueron examinados—longitud del ovario, anchura del ovario, un índice del ovario (longitud del ovario multiplicado por la anchura del ovario), y longitud del folículo terminal. Los carácteres del ovario fueron comparados por etapa y correlacionados con el número de oocitos maduros por cada ovario (carga de huevos). El índice del ovario aumentó las diferencias entre los ovarios sexualmente maduros e inmaduros, y la longitud del ovario proveyó la mejor separación de los estados inmaduros. Todos los carácteres fueron correlacionados positivamente con la carga de huevos, pero el índice y la anchura del ovario fueron los indicadores mejores de los oocitos maduros. El uso de estos parámetros para apreciar la carga de huevos eliminaría la necesidad de separar los ovarios y contar los oocitos maduros, proveyendo un método eficiente para procesar una muestra grande de moscas. Clasificación de la madurez sexual de las hembras basada en un sistema de etapa de ovario, en conjunción con la apreciación de la carga de huevos en etapa de madurez, facilitaría la evaluación de la estructura de la edad fisiológica de una población de moscas capturada en mosqueros.

Translation provided by the authors.

Tephritid fruit flies in the genus *Anastrepha* are serious economic pests of fruit crops throughout tropical and subtropical regions of the Americas (Aluja 1994). The Caribbean fruit fly, *A. suspensa* (Loew), is a quarantine pest for the citrus industry and a production pest of guava and other fruits in Florida (Greany & Riherd 1993). The Mexican fruit fly, *A. ludens* (Loew) and West Indian fruit fly, *A. obliqua* (Macquart), though not established in Florida, pose additional invasive threats due to proximity of populations in Mexico and the Caribbean (White & Elson-Harris 1992). Traditionally, monitoring programs for tropical fruit flies have relied on McPhail traps containing liquid protein baits, typically hydrolyzed yeast (Steyskal 1977; Heath et al. 1993). Ammonia was

recognized as the primary fruit fly attractant emitted from liquid protein baits (Bateman & Morton 1981), and ammonia-based synthetic lures have been developed for *Anastrepha* spp. including ammonium acetate and putrescine (Heath et al. 1995; Thomas et al. 2001) and ammonium bicarbonate and putrescine (Robacker 1999).

Relative capture of *Anastrepha* fruit flies among traps baited with liquid protein bait formulations and synthetic lures has been highly variable (Epsky et al. 2004). Field trials of *A. suspensa* found that at sites with a high percentage of mated females, flies made choices among the liquid protein bait formulations tested while at sites with lower percentages, flies were less discriminating (Epsky et al. 1993). In laboratory trials, sexually immature females consumed more protein than sexually mature females (Landolt & Davis-Hernandez 1993). Using a combination of electroantennography (EAG) and behavioral bioassays, Kendra et al. (2005a, 2005b) evaluated dose-response of *A. suspensa* to ammonia. EAG recordings from females 1-14-d old showed that antennal response to ammonia was not constant, but varied depending upon the age/sexual maturity of the flies. The antennal response of sexually mature and immature females correlated with differences in behavioral response to ammonia in flight tunnel bioassays (Kendra et al. 2005b). These laboratory results support the hypothesis that the variability seen in field captures may be due, in part, to the physiological age structure of the fly population during the monitoring period.

Female tephritid fruit flies are sexually immature at eclosion (anautogenous) and the ovarian maturation process is dependent upon multiple factors, including temperature, photoperiod, diet (especially protein availability), and chemical cues (Fletcher 1989; Wheeler 1996; Papaj 2000; Aluja et al. 2001). Therefore chronological age is not equivalent to physiological age. Dodson (1982) found that wild *A. suspensa* require at least 14 d to reach sexual maturity, whereas laboratory-reared strains can mature within 7-8 d (Mazomenos et al. 1977; Kendra et al. 2005b). In addition to genetic strain differences, the presence of males has been shown to affect the rate of ovarian development in *A. suspensa* (Pereira et al. 2006). With such variability in maturation rate, reliable methods are needed to ascertain sexual maturity and mating status in female fruit flies, particularly field-collected specimens. The most accurate methods to determine mating status entail examination of the sperm storage organs (spermathecae and ventral receptacle) for presence of spermatozoa (Dodson 1982; Fritz & Turner 2002; Twig & Yuval 2005), and field cage tests found that 100% of sexually mature *A. suspensa* females were inseminated within a 72-h period (Dodson 1982). To differentiate between sexually mature and immature females, studies on *A. suspensa* have used measurements of ovary length (Nation 1972; Dodson 1982), ovary length and width (Dodson 1978) or ovarian index (ovary length multiplied by width, Landolt & Davis-Hernandez 1993). Nation (1972) also confirmed sexual maturity by the presence of mature terminal oocytes that are ~1 mm long and opaque. However, there are decreases in both the percent of sexually mature females with mature oocytes once oviposition starts (Dodson 1982) and in the number of eggs oviposited over the fairly long life span of *A. suspensa* females (Sivinski 1993), making reliance on single factor determinations unreliable for flies trapped in the field. In this report, we critically examine several ovarian morphometric characters, document changes in these characters for 28 d following adult eclosion, and assess how reliably each character serves as an indicator of sexual maturity in *A. suspensa*.

Materials and Methods

Insects

Anastrepha suspensa were obtained from a laboratory colony maintained at the USDA-ARS, Subtropical Horticulture Research Station, Miami, FL. Rearing conditions consisted of a photoperiod of 12:12 (L:D), 70% RH, and ambient room temperature (25 ± 2°C). In preparation for this laboratory study, pupae (12 d old) were removed from the colony, placed on weighing trays, and held in screen cages (30 × 30 × 30 cm). Once adult flies began to emerge, pupal trays were transferred to new cages every 24 h until emergence ceased, typically 4 d. Since females tended to emerge earlier than males, the first cage often contained only females and therefore was discarded. The remaining cages were mixed-sex (~1:1 sex ratio) and contained flies of known age, staged at 1-d intervals. Adult flies were provisioned with water (agar blocks) and food (refined cane sugar and yeast hydrolysate, 4:1 mixture) *ad libitum*. No oviposition medium was provided since the females readily laid eggs on the mesh sleeves of the rearing cages. Known-aged females were collected and stored in 70% ethanol until dissection.

Morphological Studies

Flies were dissected under a stereomicroscope (at 25× magnification), their ovaries were removed, and ovarian development was classified according to the system described for *Bactrocera cacuminata* (Hering) (Raghu et al. 2003). This system identifies six stages in the ovarian maturation process (Fig. 1): previtellogenic phases (stages 1 and 2), vitellogenic phases (stages 3 and 4), appearance of mature oocytes (stage 5), and an ovipositional phase (stage 6). After determining the developmental stage, measurements were

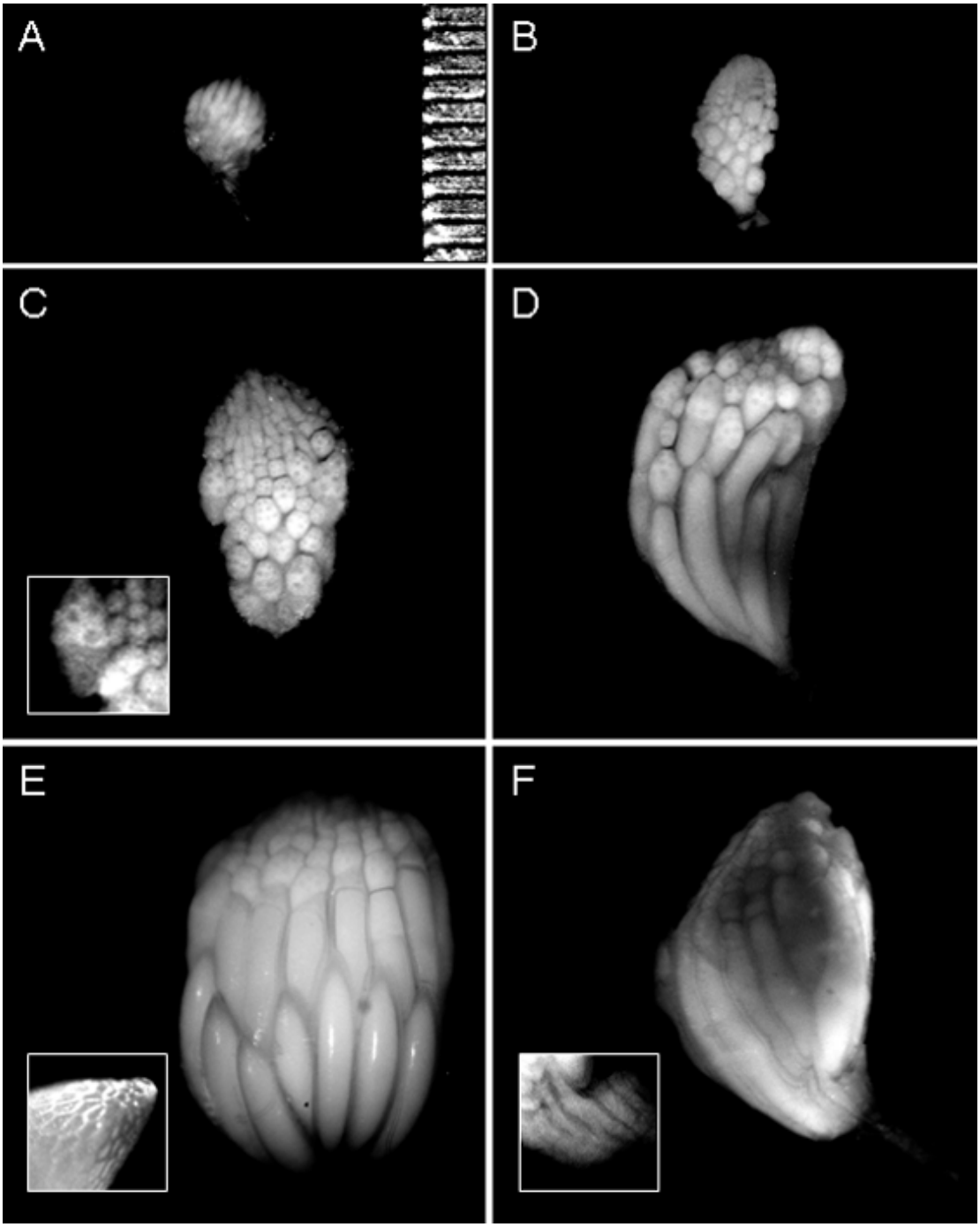

Fig. 1. Stages of ovarian development in adult *Anastrepha suspensa*, adapted from classification system of Raghu et al. (2003). Stages 1 (A) and 2 (B) represent follicles in early and late previtellogenic development, respectively. Stage 3 (C) marks initiation of vitellogenesis, accumulation of yolk in terminal follicles; Inset shows enlarged follicle containing a yolk-filled oocyte (dark lower portion) capped with trophocytes (nurse cells). Stage 4 (D) indicates late vitellogenesis, at which point yolk occupies more than half the follicle. Stage 5 (E) denotes ovaries with mature oocytes, characterized by an intact chorion (eggshell) with a reflective surface and a reticulated pattern (pronounced near the micropyle) visible at high magnification (inset). Stage 6 (F) indicates onset of oviposition, confirmed by presence of residual follicular bodies (corpora lutea) at base of the ovary (enlarged in inset). All ovary images at same magnification, scale unit = 0.1 mm.

taken of the ovary length, ovary width, and length of terminal follicle (i.e., the largest, most advanced follicle). All measurements were made with a hand-held micro-scale (to 0.1 mm; Minitool, Inc., Los Gatos, CA) placed beneath the ovary. Additionally, ovary length was multiplied by ovary width to obtain ovarian index, a standard method for assessing sexual maturation (Landolt & Davis-Hernandez 1993; Kendra et al. 2005b). After the ovaries were measured, they were teased apart carefully with fine insect pins (size 00, Elefant brand, Austria) and the number of mature oocytes (egg load) was counted. To be considered mature, oocytes had to lack accompanying trophocytes (nurse cells, Fig. 1C) and possess a fully developed chorion (eggshell, Fig. 1E), confirmed by the presence of a characteristic reticulated pattern in surface architecture visible at 100X magnification. Finally, the dorsal length of thorax (from anterior edge of mesonotum to posterior end of mesoscutellum) (Sivinski 1993) and length of forewing (from base of costal vein to wing apex where vein R_{4+5} terminates at the margin) were measured as independent indicators of overall female size. Measurements were recorded from females that were 1-28 d post-eclosion, and ten females were dissected for each age class.

Statistical Analysis

Regression analysis was used to describe the relationship between chronological age and ovarian developmental stage using SigmaPlot 8.0 (SPSS Inc., Chicago, IL). Several regression models were tested including polynomial, hyperbolic, logarithmic, and sigmoidal. Differences in response variables (ovarian characters) among the developmental stages were analyzed by one-way analysis of variance (ANOVA) with PROC GLM (SAS Institute 1985) followed by Tukey's test (P = 0.05) for mean separation. The Box-Cox procedure, which is a power transformation that regresses log-transformed standard deviations (y + 1) against log-transformed means (x + 1), was used to determine the type of transformation necessary to stabilize the variance before analysis (Box et al. 1978). Correlations among ovary length, ovary width, ovarian index, follicle length, and number of mature oocytes (egg load) within each developmental stage were determined with two-at-a-time comparisons by PROC CORR. Additional comparisons determined correlation between egg load and the four ovarian characters over the entire 28-d period (all developmental stages combined). Finally, analysis of covariance (ANCOVA) with PROC GLM was used to evaluate effect of differences in size among the sampled females on comparisons among morphometric characters.

Results

Fig. 1 depicts the six stages of ovarian development in adult *A. suspensa*, and comparisons of morphometric characters and egg load among the different stages are given in Table 1. The relationship between ovarian developmental stage and female chronological age was best fit by a sigmoidal model, and this is presented in Fig. 2A.

All ovaries from 1-2 d old adults were classified as stage 1 (Fig. 1A). Stage 1 ovaries were very small and consisted of parallel, previtellogenic ovarioles. Ovary length and width were approximately equal, and these two measurements were positively correlated (r = 0.64238, P = 0.0023). In addition, ovarian index was positively correlated with both ovary length (r = 0.90382, P < 0.0001) and ovary width (r = 0.90204, P < 0.0001) in stage 1 and all subsequent stages; this was not unexpected since ovarian index is a compound character derived from ovary length and width. All 3-d-old and some 4-5-d-old adults had ovaries classified as stage 2 (Fig. 1B). During this stage, separate follicles were first discernible within the ovarioles, but they were still previtellogenic. The ter-

Table 1. Ovarian characters (mean ± sd) at each developmental stage in adult *A. suspensa*.

Stage	*n*	Age (d)	Ovary length (mm)	Ovary width[1] (mm)	Ovarian index[1] (mm^2)	Follicle length (mm)	Egg load[2]
1	20	1-2	0.29 ± 0.06 a	0.27 ± 0.05 a	0.08 ± 0.03 a	0.10 ± 0.00 a	0.0 ± 0.00 a
2	20	3-5	0.58 ± 0.15 b	0.36 ± 0.09 ab	0.21 ± 0.08 ab	0.11 ± 0.03 a	0.0 ± 0.00 a
3	14	4-6	0.90 ± 0.15 c	0.49 ± 0.08 b	0.45 ± 0.12 b	0.26 ± 0.07 b	0.0 ± 0.00 a
4	9	6-7	1.38 ± 0.34 d	0.71 ± 0.12 c	0.97 ± 0.28 c	0.52 ± 0.19 c	0.0 ± 0.00 a
5	21	7-9	1.88 ± 0.22 e	1.29 ± 0.26 e	2.45 ± 0.71 e	1.06 ± 0.12 e	18.2 ± 13.49 c
6	196	9-28	1.56 ± 0.20 d	1.05 ± 0.18 d	1.66 ± 0.43 d	0.96 ± 0.12 d	4.2 ± 3.80 b
	F		279.95	205.08	204.32	479.28	64.01
	df		5, 274	5, 274	5, 274	5, 274	5, 274
	P		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Means within a column followed by the same letter are not significantly different (Tukey's mean separation test [P = 0.05]).
[1]Data were square-root (x + 0.5) transformed prior to analysis; non-transformed means are shown.
[2]Data were log (x + 1) transformed prior to analysis; non-transformed means are shown.

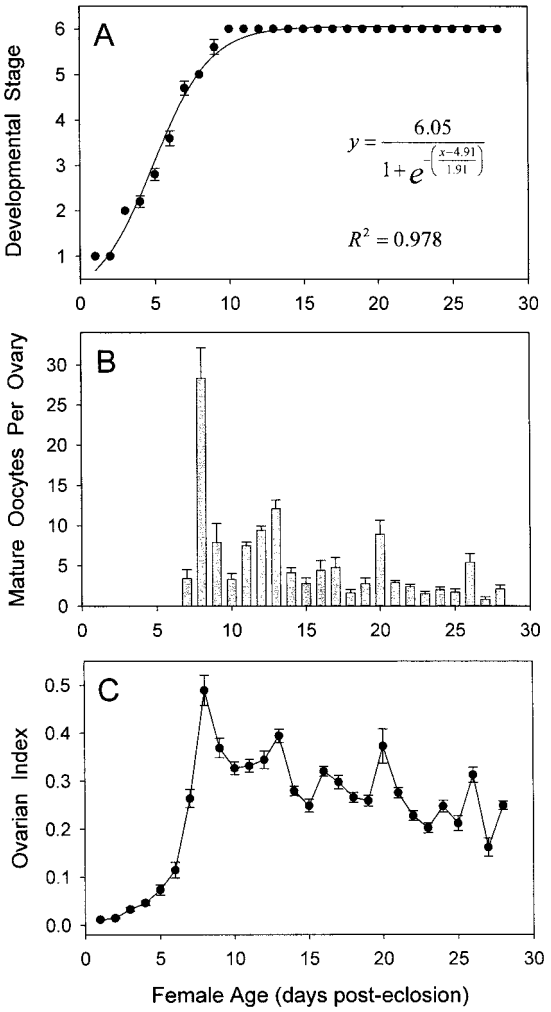

Fig. 2. Three methods for assessing reproductive status in female *Anastrepha suspensa*. (A) Ovarian maturation depicted by developmental stages according to the system of Raghu et al. (2003). (B) Number of mature oocytes per ovary (egg load). (C) Ovarian index (length of ovary multiplied by width of ovary), standardized relative to length of forewing. All three graphs present mean values (±SE) recorded from ovaries dissected 1-28 d after adult emergence, n = 10 females per day.

minal follicles ranged in length from 0.1-0.2 mm. Stage 2 ovaries were longer but not wider than stage 1 ovaries, and there were no correlations among ovary length, width or follicle length during stage 2. Stage 3 (Fig. 1C) was characterized by the onset of vitellogenesis, the accumulation of yolk in the terminal follicles, which occurred in females 4-6 d old. During stage 3 both the ovaries and the terminal follicles were longer than in stage 2, and ovary width and ovarian index were greater than in stage 1. There were positive correlations between ovary length and follicle length (r = 0.54852, P = 0.0422) and between ovarian index and follicle length (r = 0.66225, P = 0.0099), but no correlations between other paired measurements. Observations of asynchronous gonadotrophic cycles were first noted during stage 3. By stage 4 (Fig. 1D), the yolk content exceeded 50% of the terminal follicle, and this stage included adults that were 6-7-d-old. All morphometric characters were greater in stage 4 ovaries than in previous stages, and as was observed for stage 3, there were positive correlations between ovary length and follicle length (r = 0.92467, P = 0.0004) and between ovarian index and follicle length (r = 0.91403, P = 0.0006). Stages 1-4 comprised the classes of sexually immature females, during which egg load remained at zero (Table 1). At the first appearance of mature oocytes, found in flies 7-9-d-old, ovaries were classified as stage 5 (Fig. 1E). The largest egg loads were recorded during stage 5, and accordingly the largest values for all ovary measurements were obtained from females in this stage. There were positive correlations between ovary length and ovary width (r = 0.65385, P = 0.0013), between ovary length and egg load (r = 0.67036, P = 0.0009), between ovary width and egg load (r = 0.91356, P < 0.0001), and between ovarian index and egg load (r = 0.90050, P < 0.0001) during stage 5. Initiation of oviposition marked the transition to stage 6 (Fig. 1F), confirmed by the presence of at least one residual follicular body (corpus luteum) formed after a terminal follicle releases its oocyte. Based on this criterion, ovaries from the majority of sexually mature females (9-28-d-old) were classified as stage 6; however, considerable variation was observed within this age range. Developmental asynchrony increased with age, and was pronounced by late stage 6, giving the ovaries of older females an irregular morphology compared with those of younger mature females. In addition, stage 6 was characterized by an overall decline in egg load (Fig. 2B) and ovary size (Fig. 2C) with increasing age, and mean values of all morphometric characters decreased in stage 6 compared to stage 5 ovaries. Despite this decrease, all measurements except for ovary length were significantly greater in stage 6 than in stage 4. All stage 6 characters were highly and positively correlated when paired with the other characters measured (P < 0.0001).

Mature oocytes were first detected in females 7 d old, and were present in some females sampled each day thereafter up to day 28 (Fig 2B). Mean egg load fluctuated over this period, with maximum number of mature eggs on day 8, and secondary peaks on days 13, 20, and 26. All four morphometric characters were positively correlated with egg load, with the highest correlations obtained with ovarian index (r = 0.78319, P < 0.0001) (Fig. 2C) and ovary width (r = 0.74641, P < 0.0001) from females 1-14-d-old. Correlations decreased with increasing female age throughout weeks 3 and 4.

Forewing length and thorax length were evaluated as characters indicative of overall insect size. As expected, there was no relationship between female age and either measurement, nor were there differences in either measurement among females from the different developmental stages. Wing length (mean ± standard deviation) was 5.92 ± 0.196 mm and measurements ranged from 5.0 - 6.4 mm. Thorax length was 2.44 ± 0.105 mm and ranged from 2.0 - 2.7 mm. The two measurements were positively correlated (r = 0.50896, $P < 0.0001$). However, since the wing is a longer structure, a greater range of length differences could be measured, giving better resolution to size differences among female flies. Therefore, wing length was used to adjust for female size in ANCOVA. The adjustment was not significant for any of the morphometric characters; therefore, a measurement of overall female size did not improve the classification of the females among the stages. The greatest effect was observed in the analysis of ovary length ($F = 2.06$; $df = 5, 268$; $P = 0.0714$), indicating that in tests of flies that are more variable in size, accounting for individual size may improve use of ovary length measurements as an indicator of sexual maturity.

Discussion

The objective of this study was to identify a reliable method by which sexual maturity of female Caribbean fruit flies can be assessed based on morphological evidence. The photographic documentation and morphometric analysis presented in this report indicate that this can be accomplished by classifying ovarian development into six distinct stages, adapting the system proposed by Raghu et al. (2003). As has been reported in *B. cacuminata* (Raghu et al. 2003) and other tephritid species (Fletcher et al. 1978), the gonadotrophic cycles in *A. suspensa* ovarioles were not synchronous. Throughout the early stages of oogenesis, most terminal follicles were observed to be developing in phase; but during the later stages, some follicles were noticeably delayed. Due to this asynchrony, consistent assignment of ovaries to a particular developmental stage was achieved by evaluating the state of the most advanced ovarioles.

The ovaries of *A. suspensa* initially increased in length and then in width during a maturation phase which spanned the first 8-d post-eclosion in our laboratory population. Of the four characters examined, ovary length provided the best separation of immature stages during this maturation phase, but ovary length alone did not discriminate between stage 4 (immature) and stage 6 (mature) ovaries. Distinguishing between these two stages required inspection for residual follicular bodies and assessment of gross ovary morphology. Ovarian index, which combined the contributions of length and width, effectively maximized the differences between immature and mature ovaries. Ovarian index has been used previously for assessment of sexual maturity in this same strain of *A. suspensa*, and Kendra et al. (2005b) concluded that peak EAG response to ammonia occurred in immature flies (4-6-d-old) and peak response to carbon dioxide occurred in sexually mature flies (10-12-d-old). Classification by developmental stage now provides further interpretation of those results. Maximal antennal response to ammonia was measured from females with stage 3 ovaries actively undergoing vitellogenesis (deposition of yolk proteins), and this coincides with the age of peak protein consumption reported by Landolt & Davis-Hernandez (1993). Maximal response to carbon dioxide was found in stage 6 females during the ovipositional phase, which is consistent with the theory proposed by Stange (1999) that carbon dioxide serves as a close-range oviposition attractant for tephritid fruit flies.

The presence of mature oocytes in an ovary is regarded as the definitive character for female sexual maturity (Nation 1972; Aluja et al. 2001). Some 7-d-old females had mature oocytes, but by 8 d of age all females had mature oocytes under laboratory conditions. In a previous study with laboratory reared *A. suspensa*, indicator variable analysis also identified day 8 as the breakpoint between sexually immature and mature females (Kendra et al. 2005b). The transition from maturation phase to oviposition phase is marked by substantial changes both physiologically and behaviorally. The 8-d-old females (stage 5) had the maximum average egg load, and this was followed by secondary peaks at 5-7-d intervals. Approximately 10% of the 9-28-d old females (stage 6) had no mature eggs present in the ovaries. This included 30% of the 18-d-old females and 50% of the 28-d-old females. Fluctuations in egg load versus age suggest that eggs are laid in batches initially, when ovarioles are most synchronous. Over time, the cyclic pattern diminished apparently due to increasing asynchrony in oogenesis. Once a female is sexually mature, with fully developed eggs, she may switch from food-seeking behaviors, which allow her to obtain protein for egg development, to oviposition-site seeking behaviors, which enable her to locate suitable host fruit. Predominance of these two activities may alternate throughout stage 6 as females undergo successive cycles of oviposition. Although food-seeking behavior was thought to be primarily an activity of sexually immature females, the cyclic fluctuation of egg load indicates that, despite being sexually mature, a female might not engage in host-seeking/oviposition behaviors until she possessed an appropriate egg load. Thus, determination of egg load of sexually mature females (especially in stage 6) may provide further discrimination among females captured in field trials.

The six-stage system is a useful means of evaluating female sexual maturity, but its accuracy

for stages 4-6 depends upon assessment of mature oocytes within the ovaries. Without inspection for the presence of a chorion, it is possible to misidentify a full-sized terminal follicle as a mature oocyte, as supported by the lack of correlation between follicle length and egg load in stage 5. Also, stage 6 females may have oviposited all mature eggs at time of capture or mature oocytes may be concealed within the ovary (PK, personal observation). Therefore, the most reliable method for determination of egg load consists of ovary removal, careful separation of ovarioles and counting of mature oocytes, which is very time-consuming. The ideal screening method for field-captured flies would consist of a quick dissection followed by one or two simple measurements. Based on comparisons of the morphological characters examined in this study, ovarian index and ovary width are reliable indicators correlated with egg load. Use of these parameters to assess egg load would facilitate efficient processing of large samples of flies.

Classification of female sexual maturity by ovarian developmental stage, in conjunction with assessment of egg load in the mature stages, would facilitate evaluation of the age structure of a fly population responding to specific lures in field trapping studies. Although a laboratory strain of *A. suspensa* was used for this study, the proposed classification system should have broad applications since it is based on several ovarian characters and reflects female physiological age. In addition, standardization for insect size may improve resolution of ovary measurements as parameters for assessing maturity status in more variable field populations. The utility of this method for wild populations of *A. suspensa* and other tephritid species will need to be addressed in complementary studies.

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