Acoustic courtship songs in males of the fruit fly *Anastrepha ludens* (Diptera: Tephritidae) associated with geography, mass rearing and courtship success

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Abstract: The Sterile Insect Technique (SIT) has been used successfully to control or eradicate fruit flies. The commonly observed inferiority of mass-reared males, compared with wild males, when they are paired with wild females, is apparently due to their inadequate courtship. *Anastrepha ludens* males produce two types of wing vibration during courtship and mating, the "calling sound" and the "premating or precopulatory sound". There were clear differences in the calling songs between successful and unsuccessful courtships in sterile (irradiated) and fertile Mexican flies. Among sterile flies, successful males produce longer buzzes, shorter interpulses and a higher power spectrum in the signal. Fertile flies showed the same trend. For mating songs a significant difference occurred in two parameters: power spectrum between sterile and fertile flies with respect to the type of song, and the signal duration and intensity were greater in non-irradiated flies. Calling songs of wild flies compared with laboratory grown flies from Mexico had shorter interpulses, longer pulses, and a greater power spectrum. However, in the case of premating songs, the only difference was in the intensity, which was significantly greater in wild males. An unexpected result was not observing pulses during pheromone deposition in wild males from Costa Rica. Comparing the premating songs of wild flies from Costa Rica and Mexico, no significant differences were observed in the duration, and the intensity of the signal was slightly greater in flies from Mexico. Rev. Biol. Trop. 57 (Suppl. 1): 257-265. Epub 2009 November 30.

Key words: fruit flies, Tephritide, Anastrepha, sound production, courtship, mass rearing, Mexico, Costa Rica.

Fruit flies are of major economic importance in nearly all tropical, subtropical and some temperate countries worldwide. Several species have spread around the world and some have increased significantly in importance. Over the last decades, their biology and management have received national and international attention (Calkins 1984)

The success in controlling pest populations of fruit flies using mass reared sterile males depends on the abilities of these males to successfully induce wild females to copulate with them. Current understanding of why some courtships result in copulation, while the majority do not, is only fragmentary. The commonly observed inferiority of mass-reared males, compared with wild males, when they are paired with wild females (Rossler 1975b, Calkins 1984, Shelly *et al.* 1994, Hendrichs *et al.* 1996, Briceño & Eberhard 1998) is apparently due to their inadequate courtship *per se*, rather than to inferior abilities to find and attend leks; or it may be due to their reduced ability to attract females with their pheromones (Shelly *et al.* 1994, Shelly & Whittier 1996, Hendrichs *et al.* 1996, Liimatainen *et al.* 1997, Lance *et al.* 2000).

Tests and standards are needed to improve the quality of rearing and to minimize production costs (Boller *et al.* 1981). Such tests are generally conceded to give only incomplete assessments (e.g. discussion at First Meeting of Western Hemisphere Fruit Fly Working Group 1992, in San Jose, Costa Rica), and it is probable that fly quality is less than optimum (Hibiano & Iwahashi 1991).

The Mexican fruit fly, *Anastrepha ludens* (Loew) is a polyphagous, frugivorous tephritid ocurring from Southern Texas South to at least Costa Rica (Stone 1942) and is related to several other economically important tephritid pests such as the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Due to its economic importance and the extensive use of sterile male releases for its control in Texas and Mexico, studies of the communicative qualities that make this fruit fly sexually competitive or attractive were undertaken (Burk & Calkins 1983), since little is known about these basic features of its biology (Webb *et al.* 1983). Since several species of tephritid fruit flies have been reported to produce special sounds before couple formation, one method to evaluate the quality of the flies is to look for differences in the song characteristics of mass reared and wild males during courtship.

Anastrepha ludens males produce two types of wing vibration. The male first applies the anal pheromone to the substrate, during this time he alternately produces short bursts of rapid wing vibration, accompanied by a forward-backward movement of the wings, thus producing a sound each time he rapidly deflects his wing anteriorly; this is the “calling sound”. Once he has leapt forward and landed on the female, he turns and aligns himself on her dorsum with his mouthparts extended and starts to produce a relatively constant, sustained sound, the “premating or precopulatory sound”. The purpose of these sounds is unknown, but it has been proposed that the function of the movements is to waft pheromones into the airstream toward an approaching female (e.g. Sivinsky *et al.* 1984) since they are often correlated with pheromone release. It has also been proposed that it may play a role in forming aggregations, in establishing male territories, in attracting females or in providing a species recognition cue. Circumstantial evidence also suggests that a female that is judging male quality may use sound intensity to decide whether mounted males will be allowed to copulate (Burke & Webb 1983, Webb *et al.* 1983). Similar songs produced by African drosophilids, *Zaprionus* spp., are also considered factors in intrasexual selection (Bennet-Clark *et al.* 1980). There are thus as yet no conclusive demonstrations that any sounds are functionally important in courtship.

The present research examines the possibility that mass rearing results in changes in the temporal and spectral characteristics of the calling and precopulatory sounds of successful and unsuccessful males of three strains of flies: wild flies from Costa Rica and Mexico, and laboratory grown flies. Songs of radiated and non-radiated flies are also compared.

MATERIALS AND METHODS

Mass reared flies (fertile and sterile) from Mexico were obtained as pupae from a strain which had been initiated using wild flies and maintained for 13 years in the Moscafruit Plant of Metapa, Domínguez, Chiapas, Mexico. Wild flies from Mexico were raised from larvae that emerged from infested sour orange (*Citrus aurantium*) collected near Soconusco Chiapas, México, during October, November and December 2006. Costa Rican wild flies were collected from infested orange fruits in April-March 2006 at the Estación Experimental Fabio Baudrit near Alajuela. Third instar larvae were placed in vermiculite (Strong-lite®, Products Corp. Seneca lllinois) with humidity of 23±1ºC during 14 days, the required time for pupal maturation. The pupae were later placed in wooden cages boxes (30x30x30cm) covered with mesh until emergence. When sexually mature, males and females were placed in separate cages and maintained in laboratory conditions under a photoperiod of 12L:12D (550 ±50lux), 25±1°C and 65±5% relative humidity.

Adult flies of all strains were separated by sexes when they were less than two days old and fed a mixture of sugar and protein hydrolysate. Male and female pairs of wild flies, whose sexual maturation is more delayed, were placed together only after they were 15 days old. The irradiation was carried out on non wild pupae 48h before emergence (color of imaginal disks: emergence process =5% -2=60%) in an irradiador JS 7400 series IR99 at a dose of 140 Greys. Non irradiated laboratory flies served as controls.

Male-female pairs of mass reared flies (fertile and sterile) were placed together for videotaping when they were 10 to 12 days old; male-female pairs of wild flies were placed together when they were 18 to 20 days old. Pairs of flies in Costa Rica were videotaped in 13.7cm diameter and 1.8cm deep mating chamber (clear Petri dishes) on a glass table using a Sony DCR.TRV80 digital camera equipped with +6 close-up lenses. The camera was below the table, allowing recording from below (most courtship occurred on the ceiling of the mating chamber). A small microphone (Sennheiser System MZK 80ZU) was inserted through a hole inside the chamber and connected to the camera. Pairs in Mexico were videotaped using a Sony DCR-TRV820P digital camera in a clear plastic cylinder 100x15mm. Each afternoon, ten minutes after a male was released in a container, a female was introduced and the recording was carried out for 60 to 120min maximum or until copulation was observed. A total of 30 recordings were made per treatment.

Recordings of sounds were imported from video recordings into a Pentium 4 computer using a SoundMax Digital audio card 5.0 (16 bits). The mean duration of buzzes and the interval between buzzes were measured using the real time display in the Raven® program when the cursor marked the beginning and the end of the envelope curve in the main window of the program. Other characteristics that we measured were songs fundamental frequency (Hz) and the power spectrum (db).

Courtship outcome was classified in three classes: no mounting, when the male ceased courting without attempting to mount the female (failure to mount is often associated with failure of the female to align herself properly with the courting male and to remain still; Briceño *et al.* 1999, Briceño & Eberhard 2002); failed mount, when the male mounted but was dislodged when the female struggled; and successful mount, when the male mounted the female and achieved genitalic contact.

All statistical tests were non-parametric Mann Whitney U Test due to the highly skewed distribution of many variables. Means are presented followed by one standard deviation for illustrative purposes only.

RESULTS

In an attempt to understand the possible selective factors which might influence song characteristics, we compared several aspects of calling and premating songs in courtship that led to copulation as compared with those that ended in female rejection of a mount or a failure to mount in different strains (Tables 1, 2). There were differences in the calling songs between successful and unsuccessful courtships in sterile and fertile Mexican flies. In sterile flies a significant difference occurred in the interpulse interval, pulse duration and maximum power; successful males produced longer buzzes, shorter interpulses and a higher power spectrum in the signal, and fertile flies showed the same results (Table 1). No differences were found in the fundamental frequency of the signal for sterile males but were significantly higher for successful fertile males. No differences were found for the two types of males in the number of pulses per second. Combining successful and unsuccessful courtships of both treatments, differences were observed in the duration of pulses, the intensity of the signal and the fundamental frequency, being greater in both cases for fertile flies.

For mating songs a significant difference occurred in two parameters. Differences in power spectrum between irradiated and non-irradiated flies were observed with respect to

TABLE 1

Calling sound parameters in successful and unsuccessful mounts of sterile and fertile mass-reared males from Mexico

	Laboratory sterile		Laboratory fertile		Totals	
	Unsuccessful	Successful	Unsuccessful	Successful	Lab sterile	Lab fertile
Duration (sec)						
Interpulse	1.57±1.74	0.88±0.85***	2.20±3.10	1.28±1.69***	1.76±2.55	1.49±1.59ns
Pulse	0.15±0.10	0.17±0.11**	0.20±0.11	0.26±0.12***	0.16±0.10	0.21±0.12***
Spectral measurements						
Max. Power (db)	54.5±8.69	59.4±4.04***	60.6±5.5	61.8±4.98***	55.7±8.1	61.2±5.3***
Fundamental Freq. (Hz)	142±29	141±14 ns	149.9±7.4	157.3±9.3**	142±2.9	152.4±3.4***
Freq. (number/s)	0.56±0.38	0.48±0.535 ns	0.2±0.3	0.5±0.3 ns	0.55±0.4	0.4±0.35 ns
n pulses	227	71	262	287	280	549
Pairs	25	10	18	18	35	36

*p<0.05, **p<0.005, ***p<0.001.

TABLE 2

Premating sound parameters in successful and unsuccessful mounts of sterile an fertile mass-reared males from Mexico

	Laboratory sterile		Laboratory fertile		Totals	
	Unsuccessful	Successful	Unsuccessful	Successful	Lab. sterile	Lab. fertile
Duration (sec)	15.01±9.13	48.8±16.6**	28.9±16.7	51.03±13.9**	33.8±11.9	45.5±14.6*
Spectral measurements						
Max. Power (db)	65.6±3.07	72±3.79***	68.4±4.98	71.2±4.4*	68.9±4.7	70.9±4.6*
Fundamental Freq. (Hz)	150±26	150±25 ns	145±35	149±37 ns	144±36	148±41 ns
Freq. (number/s)	0.56±0.38	0.48±0.535 ns	0.2±0.3	0.5±0.3 ns	0.55±0.4	0.4±0.35 ns
Number mounts	20	10	18	12	30	30
Pairs	25	10	18	18	35	36

*p<0.05, **p<0.005, ***p<0.001.

the type of song, and the signal duration and intensity were greater in non-irradiated flies.

Calling songs of wild flies were shorter in duration between pulses (interpulses) and greater in duration during pulses, with a greater power spectrum (Table 3, 4, Fig. 1). However, in the case of premating songs, the only difference was in the intensity, which was significantly greater in wild males (Fig. 2).

An unexpected result was not observing pulses during pheromone deposition in wild males from Costa Rica. It was therefore not possible to compare them with those of Mexican flies.

TABLE 3

Calling sound parameters in fertile laboratory and wild males from Mexico

	Lab. Flies	Wild Flies
Duration (sec)		
Interpulse	1.49±1.59	1.55±2.91**
Pulse	0.21±0.12	0.14±0.05***
Spectral measurements		
Max. Power (db)	50.3±5.2	61.2±7.2**
Fundamental Freq. (Hz)	140±25.9	141±10 ns
Freq. (number/s)	0.4±0.36	0.9±0.68 ns
N	549	942
Pairs	35	18

*p<0.05,**p<0.005,***p<0.001.

TABLE 4

Premating sound parameters in fertile laboratory and wild males from Mexico

	Lab. Flies	Wild Flies
Duration (sec)	45.±48.3	13.5±4.7 ns
Spectral measurements		
Max. Power (db)	67.2±2.2	70.9±1.6 *
Fundamental Freq. (Hz)	146±38	148±36 ns
N mounts	60	24
Pairs	35	18

Finally, upon comparing the premating songs of wild flies from Costa Rica and Mexico (Table 5), no significant differences were observed in the duration, and the intensity of the signal was barely different, being greater in flies from Mexico (67.2 vs. 63.9, $p<0.05$).

DISCUSSION

Experiments on fruit flies have shown that females prefer larger, more loudly calling

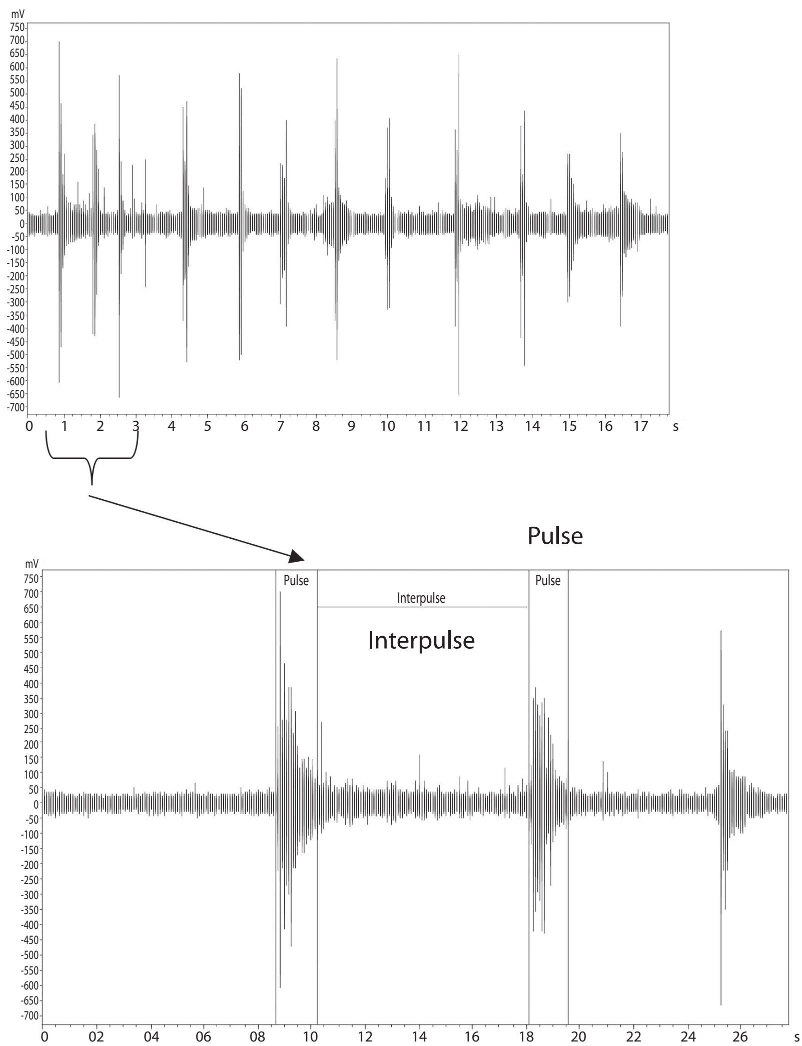

Fig 1. Sounds produced by a male *Anastrepha ludens*. The male applies the anal pheromone to the substrate, alternating with a short burst of rapid wing vibrations, “the calling song”. Talvez la palabra interpulse debe tener tamaño más pequeño de letra para que quepa en el intervalo. Hay posibilidad de ver los números más nítidos?

TABLE 5
Premating sound parameters in fertile wild males from Mexico and Costa Rica

	MEXICO	COSTA RICA
Duration (sec)	13.5±4.7	17.5±2.3ns
Max. Power (db)	67.2±4.6	63.9±3.2*
Fundamental Freq. (Hz)	148±36	146±41 ns
N mounts	24	48
Pairs	18	60

males (Bailey 1991). Sound intensity is an important determinant of female acceptance in the tephritid *Anastrepha suspensa* (Sivinski *et al.* 1984, Webb *et al* 1976), and in phonotatic responses of female mole crickets where males with calling songs 3-5db louder attracted 17 times as many females as males with quieter songs (Forrest 1980). Differences in the power output of precopulatory sound in mounting males of *A. ludens* that were successful vs. unsuccessful in copulating, suggest that power output is another variable that females may use in making mating decisions. Ability to produce loud precopulatory sounds may reflect development of wing musculature, affecting both flight and attracting ability of males. Females that evaluate on the basis of power output might produce fitter offpring. Thus the present data support the idea that song traits like sound intensity can influence courtship success. Furthermore, this study revealed that sterile flies had songs with the shortest pulse, which might indicate a lower fitness of these flies compared with fertile ones. The apparent differences in sound production between successful and unsuccessful males during courtship is only one of many factors that may influence female acceptance in *A. ludens*. Further work will be needed to elucidate female criteria, and how they change under mass rearing conditions.

In addition, mass rearing of sterile males has often been used in attempts to control pest population of medflies, and mass-reared strains have often been conserved for many years. Reproduction in these strains occurs

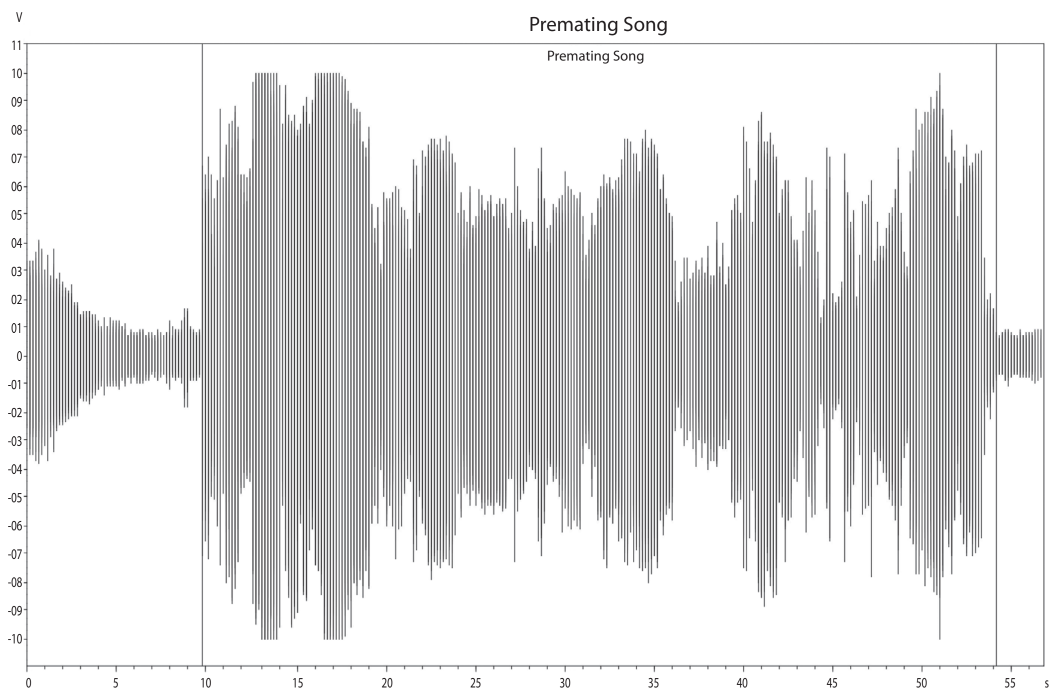

Fig 2. On the female dorsum the male starts to produce a relatively constant, sustained sound, the “premating sound”

under conditions that sharply differ from those in nature in several respects. Old mass-reared strains thus represent the results of inadvertent experiments in which several environmental conditions have been changed. It is not obvious, however, which song traits would be more advantageous under mass rearing conditions (Briceño *et al.* 2002); however, the fact that the duration of the interpulse and pulse, as well as the power spectrum, showed significant differences compared with wild flies, suggests that these variables can also be affected by mass rearing conditions.

The negative effects of irradiation on sexual competitiveness of fruit flies are well documented (Hollbrook & Fujimoto 1970, Hooper 1972, Rossler 1975, Wong *et al.* 1983, Moreno *et al.* 1991). Lux *et al.* (2002) describe in detail these negative effects on courtship. Our results suggest that sterilization could also have an influence on the sound production in *A. ludens*. Significant differences occurred in the fundamental frequency, duration and amplitude of the sounds. Similar effects in sound production in *Glossina pallidipes* have been reported by Bolldorf (2005). As the sound frequency principally depends on the size and weight of the insects (Bennet-Clark 1999, Sueur 2003), the lower frequency in sterilized fruit flies may result from a larger body size or a weaker flight musculature. The fact that the development of the musculature is influenced by irradiation (Langley & Abasa 1970) might also explain the differences in the fundamental frequency of sounds recorded in this study, as sound production is directly correlated with wing beat frequency. The influence of sterilization has been demonstrated in the present work, but the consequences of these differences in sounds on fly behavior have not been shown. Future work needs to focus on determining useful acoustic quality control parameters, which can be applied for fitness testing of flies.

There are several reasons to expect that present-day populations of *A. ludens* may not have uniform courtship songs. The geographic range of the species has increased dramatically. Thus both drift and divergence under sexual selection in geographically isolated populations may have occurred. In *Drosophila mojavenses* Etges *et al.* (2005) found significant geographical variation in mean interpulse interval and mean burst duration, and significant variation in mean burst duration among geographically isolated populations from Baja California and mainland Mexico and Arizona. Thus it is not unusual to find these temporal and spectral differences in isolated populations of *A. ludens*. Moreover, the geographical differences observed in wild flies from Costa Rica (absence of the calling sound) (Briceño, R.D. *et al.* 2002) suggest possible divergence due to founder effects and divergent sexual selection in different populations.

The consistent association between the details of male songs and success argues in favor of a possible role of sexual selection.

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RESUMEN

La técnica estéril del insecto (SIT) se ha utilizado con éxito para controlar o para suprimir las moscas de fruta y su impacto en los cultivos. La inferioridad comúnmente observada de machos criados masivamente, comparada con los machos silvestres, cuando se aparean con las hembras silvestres es al parecer debido a su inadecuado cortejo. Los machos de *Anastrepha ludens* producen dos tipos de vibraciones del ala durante cortejo y el apareamiento, una de “llamada” y “el sonido de pre-apareamiento o precopulatorio”. Se encontraron diferencias claras en las canciones de llamada entre los cortejos exitosos y no exitosos en moscas estériles y fértiles de Mexico. En las moscas estériles, los machos exitosos producen zumbidos más largos, interpulsos más cortos y un espectro de una energía más alta en la señal. Las moscas fértiles mostraron la misma tendencia. Para las canciones precopulatorias hay diferencias significativas en dos parámetros: el espectro de energía entre las moscas irradiadas y no irradiadas también observadas con respecto al tipo de canción, y la duración y la intensidad de la señal fueron mayores en moscas no irradiadas. Las canciones de llamada de las moscas silvestres comparadas con las de laboratorio de México tenían

interpulsos más cortos, pulsos más largos, y un mayor espectro de energía. Sin embargo, en el caso de canciones precopulatorias, la única diferencia estaba en la intensidad, que era perceptiblemente mayor en machos silvestres. Un resultado inesperado fue no observar canciones de llamada durante la deposición de la feromona en machos silvestres de Costa Rica. Al comparar las canciones precopulatorias de moscas silvestres de Costa Rica y de México, no se observó ninguna diferencia significativa en la duración, ni en la intensidad de la señal

Palabras clave: mosca, producción de sonido, cortejo, diferencias geográficas.

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