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Authors: Fritz, Ann H., Dhakal, Preeti, FRITZ, GARY N., and Kirby, Laura A.

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ISOLATING, AMPLIFYING AND QUANTIFYING SPERM DNA IN *ANASTREPHA SUSPENSA* (DIPTERA: TEPHRITIDAE)

ANN H. FRITZ[1], PREETI DHAKAL[2], GARY N. FRITZ[1] AND LAURA A. KIRBY[3]
[1]Department of Biological Sciences, Eastern Illinois University, 600 Lincoln Ave., Charleston, IL 61920

[2]Division of Biology, Kansas State University, Manhattan, KS 66506-4901

[3]V. J. Andrew High School, 9001 W. 171st St., Tinley Park, IL 60487

ABSTRACT

A major obstacle for many studies examining sperm competition and cryptic female choice in insects has been the identification and quantification of sperm stored in the sperm storage organs of females that have mated with two or more males. Historically, sexual selection studies have focused primarily on paternity outcomes for inferring potential underlying mechanisms (e.g., sperm competition and cryptic female choice). We describe a technique for isolating, genotyping and quantifying sperm in *Anastrepha suspensa* Loew, a species that has four sperm storage organs (three spermathecae and a ventral receptacle) that are minute (approximately 80 µm) and exhibit complex interior structures restricting sperm recovery through simple dissection. With our protocol, we were able to isolate and amplify sperm DNA (PCR of microsatellite loci) without contamination from female cells, and quantify sperm contributed to a storage organ by one or more males. Briefly, sperm storage organs are dissected-out of the female abdomen, sonicated to remove female cells, rinsed in saline, crushed between micro-slides (1 × 2 mm), and placed in a microcentrifuge tube for DNA isolation *in situ* using a solution containing 10% chelex, proteinase-K and DTT. After boiling, the DNA is amplified by PCR. With this technique, we have successfully amplified microsatellite loci from as few as 10 ± 3 sperm. Estimates of absolute numbers of sperm stored in sperm storage organs was accomplished by incorporating a reference amplicon standard in each sample during fragment analysis of microsatellite loci. The protocol described in this study enable the localization, identification and quantification of sperm from multiple males stored in female sperm storage organs and, therefore, generates data that can augment interpretations of paternity outcomes.

Key Words: spermathecae, sperm quantification, multiple-mating, sperm storage

RESUMEN

Uno de los mayores obstáculos enfrentando estudios examinando el papel de elección femenina críptica y competencia espermática en insectos ha sido la identificación y cuantificación de esperma en los organos de almacenamiento en hembras copulando con dos o mas machos. Historicamente, estudios sobre la selección sexual han enfocado principalmente en los resultados de paternidad pare inferir la dinámica y el mecanismo de fertilización (por ejemplo, competencia espermática y elección femenina críptica). Describimos una técnica que permite el aislamiento, la determinación del genotipo y cuantificación de esperma en hembras de la mosca *Anastrepha suspensa* Loew, lo cual tiene cuatro órganos de almacenamiento (tres espermatecas y un receptáculo ventral) minusculos (aproximadamente 80 µm) y comportan un interior complejo que restringe el aislamiento de esperma por medio de disección. Con este protocolo, es possible aislar y amplificar el ADN (por medio de PCR de microsatélites) sin contaminación de células femeninas, y cuantificar esperma contribuido a un órgano de almacenamiento por uno o mas machos. En resumen, los órganos de almacenamiento son aislados por disección, las celulas femeninas eliminadas por hondas sónicas, los organos aplastados entre dos portaobjetos minusculos (1 × 2 mm), y luego puesto en un tubo de microcentrífuga para el aislamiento de ADN *in situ* usando una solución conteniendo 10% chelex, proteinasa-K y DTT. Después de hervir, el ADN se amplifica por medio del PCR. Con este protocolo, amplificamos microsatelites de hasta 10 ± 3 esperma. Estimación del número absoluto de esperma en los órganos de almacenamiento fué possible con la incorporación de una referencia interna en el analysis de ADN amplificado y sujeto a un analysis de fragmentos. Las técnicas describidos en este estudio facilitan la localización, identificación y cuantificación de esperma provieniendo de dos o mas machos y almacenado en los various órganos de la hembra, asi generando información que facilita la interpretación de paternidad.

Translation provided by the authors.

Most studies addressing post-copulatory sexual selection have relied on paternity outcomes to infer the relative roles of sperm competition and cryptic female choice and make assumptions regarding the underlying mechanisms of these phenomena (see review by Simmons 2001). What has often been the "black box" for many such studies has been the absence of data on sperm storage patterns in females and how these patterns might affect paternity outcomes (e.g., through female mediation or sperm competition). Since copulation and fertilization are typically temporally removed in insects, the proximate explanation of paternity outcomes may depend on patterns of sperm viability, sperm storage, and sperm use.

Aside from its relevance to sexual selection theory, paternity in insects is particularly important in estimating the feasibility and efficacy of certain biological control systems (e.g., sterile insect technique) and monitoring their control trajectory. An important factor affecting the success of the sterile insect technique, for example, is the competitive fate of ejaculate from laboratory-reared males with that of wild males for paternity outcomes (Hendricks et al. 2002). With species that mate multiply, paternity outcomes may also be affected by the dynamics of sperm storage and use from males of both sources, and the sequence in which they mated.

One of the main reasons for the dearth of data on sperm storage patterns from multiple males has been the difficulty of accurately isolating, identifying, and quantifying sperm from multiple-males inside tiny sperm storage organs of most insects. Also, the difficulty of tagging sperm from different males with visible markers, and without affecting the attributes of the sperm itself, has hampered such studies in the past (Birkhead 2000). These problems are compounded when studying certain insects such as the tephritid fruit fly, *Anastrepha suspensa* (Loew), where the structure, size, and composition of the sperm storage organs make it extremely difficult to isolate and quantify sperm.

Anastrepha suspensa has four sperm storage organs: three spermathecae and a ventral receptacle (Fritz & Turner 2002). Females can mate with one or more males (Sivinski & Heath 1988; A. H. Fritz unpublished data), and sperm storage patterns in singly-mated females have been shown to be highly variable in terms of quantity and location (Fritz 2004); no studies have examined sperm storage patterns in multiply-mated females to date. The spermathecal capsules of *A. suspensa* are relatively hard, sclerotized spheres, and surrounded by layers of maternal cells (Fritz & Turner 2002). Because of their size and shape, it is very difficult to break the spermathecae open and remove sperm (e.g., with micropins). The interiors of the spermathecae are highly sculptured and contain many sclerotized pockets into which sperm are lodged (Fritz & Turner 2002). Consequently, it is not possible to remove a "ball" of sperm from each capsule by simple dissection—many sperm remain lodged and attached to the interior sculpturing of the spermathecal capsules. Any spermatozoa released from the capsule have a propensity to remain in clumps, which often adhere to pieces of the capsule walls. Thus, quantifying sperm within such capsules is extremely difficult, time consuming, and prone to error. Furthermore, the specific identity of the sperm (to one or more males) is indeterminate.

Spermatozoa in the ventral receptacle are equally difficult to isolate and quantify. The ventral receptacle of *A. suspensa* is likely the short-term storage site for sperm (as in *Ceratitis capitata*, Twig & Yuval 2005) and the site of egg fertilization (as in *Batrocera oleae,* Solinas & Nuzzaci 1984). This organ is as small as the spermathecae, is surrounded by a thick layer of osmoregulatory cells and muscle, and is comprised of numerous chitinized and spherical alveoli into which sperm are coiled (Fritz & Turner 2002). Because the sperm are quite intractable in these alveoli, quantification of sperm in this organ is indirect and involves staining the organ with DAPI, and estimating the number of sperm coiled in a subsample of alveoli (Fritz 2004).

The purpose of this study was to develop a methodology that would ameliorate the problems outlined above and allow for future studies focusing on sexual selection and biological control strategies where paternity outcomes are important considerations. The protocols described below also eliminate steps in sperm DNA preparation that would lead to a reduction in DNA copy number, and eliminates any sources of contaminating DNA from the female fly (e.g., during PCR of the sperm genome). Although the methodology described in this study applies specifically to *A. suspensa*, the protocols should be generally applicable to any species (field caught or otherwise) that has similarly small, multiple, sperm storage organs with relatively intractable spermatozoa.

Materials and Methods

The methods described here include 5 major steps: (1) isolation of the sperm storage organs by microdissection, (2) removal of maternal cells from spermathecae (to prevent maternal contamination of DNA during PCR), (3) isolation of sperm DNA from sperm storage organs, (4) PCR amplification of microsatellite loci, and (5) sperm quantification. Flies were reared in a quarantine insectary maintained at 25°C, 55% relative humidity and a 14L:10D photoperiod according to the protocols described by Fritz (2004). All crosses were done with virgin male and female flies that had been separated as teneral flies.

Dissection of Sperm Storage Organs

Except for the ovaries, the reproductive system of female *A. suspensa* is located within the last abdominal segment and the ovipositor sheath. The gross and fine anatomy of the female reproductive system of Caribbean fruit fly have been described by Fritz & Turner (2002). Under a dissecting microscope and using micropins, the reproductive system (including ovaries, oviducts, spermathecae and ventral receptacle) are easily removed under a drop of insect saline (5.5 g NaCl, 0.44 g $CaCl_2$, 0.22 g KCl, 1000 mL H_2O) on a microscope slide. A pair of spermathecae is located to one side of the two lateral oviducts and associated with an accessory gland (Dodson 1978; Fritz & Turner 2002). The third and singular spermatheca is adjacent the opposite lateral oviduct and has a similar association with the remaining accessory gland. The 3 spermathecae, easily identified as round, orange-pigmented structures, are pear shaped and of similar size.

The spermathecae are connected to the bursa by independent ducts, which should be removed at the base of each spermatheca. Although a valve at the base of each spermatheca (Fritz & Turner 2002) helps prevent the loss of sperm during dissection, it is important to dissect flies in insect saline to prevent water from entering the sperm storage organs and expelling any sperm through turgor pressure.

Much of the maternal tissue surrounding each sperm storage organ can be removed with the help of micropins. The ventral receptacle is in the bursa and must be excised by cutting the musculature surrounding it. Although the 3 spermathecae and the ventral receptacle are surrounded by several cell layers of maternal cells, it is not critical that all maternal cells be removed at this juncture, since the next step (below) eliminates any remaining maternal cells from the preparation. A new slide and flamed micropins were used for each dissection in order to prevent contamination from 1 fly to the other.

Removal of Maternal Cells

Microsatellite loci are typically very useful for identifying individuals because of their relatively high levels of polymorphism. When amplifying the sperm from the sperm storage organs of female flies through PCR, it is desirable to remove all traces of the female DNA from the reactants, particularly when the genotypes of the sperm are unknown (as in field-caught female flies). Maternal cell removal from sperm storage organs is particularly important for the following reasons: (1) preventing female DNA from competing with sperm DNA for the production of amplicons (especially when sperm quantities are low), (2) preventing female amplicons from overlapping with sperm amplicons during fragment analysis (particularly important for field-caught samples, where the genotype of the males are unknown), and (3) preventing the generation of amplicons in PCR to that of sperm DNA, thus allowing for estimates of the relative and absolute quantification of spermatozoa through fragment analysis.

The spermathecae are encased in four layers of maternal cells including gland cells surrounding secretory ducts (Fritz & Turner 2002). The ventral receptacle is surrounded by a thick layer of muscle cells overlaying a mitochondria-rich cell layer (Fritz & Turner 2002). In order to remove these sources of maternal cells, each sperm storage organ was transferred (with a fine-tipped micropipette) to a separate 300 µL tube containing 100 µL of 2% Sodium Dodecyl Sulphate (SDS) in insect saline. The SDS helps prevent the sperm storage organ from sticking to the sides of the tube or in a micropipette tip. The lid of each microcentrifuge tube was then held with tweezers and the end of each tube submerged in water held within a micro-cup horn attached to the converter of a sonicator (Fisher Model 60 Sonic Dismembrator). Each tube was sonicated for 10 s at a medium amplitude setting of 10 (of 20 gradations). The micro-cup horn attachment allows sonication *in vitro* without direct exposure to an ultrasonic probe.

The particular sonication variables (time and amplitude setting) used for removing maternal cells described above were established by sonicating sperm storage organs for varying lengths of time and for different amplitude settings. The efficacy of sonication as a means of removing maternal cells (and, thus, removal of maternal sources of DNA) was determined visually by microscopy (at 400× mag.) and by PCR of the sonicated organs for one or more microsatellite loci. The microsatellite primers used to amplify the DNA of *A. suspensa* are listed and described by Fritz & Schable (2004). All 3 sperm storage organs of female flies were subject to PCR along with positive and negative controls (the female fly DNA and DNA from non-sonicated sperm storage organs, versus water, respectively). The absence of visible amplicons from the PCR of the sperm storage organs (with fragment analysis on a Beckman Coulter™ CEQ 8000XL Sequence Analyzer) was evidence that sonication removed all sources of maternal cells successfully.

The effect of sonication on the integrity of spermatozoa contained within the sperm storage organs also was tested. Spermatozoa were dissected out of sperm storage organs (with micropins and by crushing organs under a coverslip) after varying the sonication variables (time and amplitude) and subsequently stained with 100 µM 4'-6-Diamidino-2-phenylindole (DAPI), which forms a fluorescent complex with double stranded DNA). DAPI-stained spermatozoa were then examined

for signs of fragmentation under fluorescent microscopy at 400X magnification. Further tests of sperm fragmentation were performed by simply testing dilutions of spermatozoa in insect saline under different sonication regimes and counting sperm (before and after sonication) with a hemocytometer and DAPI staining.

We tested for the PCR amplification of sperm DNA contained in the sperm storage organs of mated females. These organs were subject to the dissection and sonication protocols described above, and PCR was conducted on the female DNA, the DNA of the male with which she mated, and the DNA of the sperm (contained in her sperm storage organs) for three microsatellite loci (loci 1-3B, 1-5E, 1-1H, Fritz & Schable 2004). The purpose of using 3 microsatellite loci was to maximize the probability of finding mated females that did not share a genotype with either of the males with which they mated. With this *a priori* information, we were able to definitively discern whether or not our protocols eliminated female DNA as a source of error (i.e., whether or not female sources of DNA were contaminating our PCR amplifications of spermatozoa from the female's sperm storage organs). The specifics of the PCR protocol we used to amplify all our samples and subsequent fragment analysis are described below.

Isolation of Spermatozoa from Female Sperm Storage Organs

After sonication, each spermatheca and the ventral receptacle were transferred (by micropipette) to the surface of a glass slide and washed quickly in 3 separate drops of de-ionized water to remove the insect saline, remove female sources of DNA from the cells ruptured by sonication, and remove SDS (which might interfere with PCR). Subsequently, each sperm storage organ was transferred to the surface of a plastic "micro-coverslip" (1 × 2 mm) in 1 µL of sterile, de-ionized water. These "micro-coverslips" were prepared by cutting plastic slide coverslips (from Fisher Scientific) into the appropriate sizes with a razor blade. While viewed under a dissection microscope, a second plastic micro-coverslip of the same size was placed on the surface of the first micro-coverslip, pressed firmly with a probe or tip of tweezers, and simultaneously rotated to completely crush and squeeze-out the contents (sperm) from the storage organ. This "sandwich" of micro-coverslips was then picked-up with fine tweezers and placed into a 300-µL micro-centrifuge tube containing 24 µL of a DNA extraction solution described by Walsh et al. (1991): 5% Chelex (pH = 11) including 1 µL of 10 mg/mL proteinase K and 1 µL of 1M DTT. The tube was then subjected to sonication in a micro-cup horn attached to the converter of a sonicator (as described above) at a setting of 10 for 5 s, centrifuged at 15,000 rpm for 20 s, and incubated at 56°C for 60 min. The tube was subsequently subjected to high speed vortexing for 10 s, centrifuged again at 15,000 rpm for 20 s and placed in boiling water for 8 min. The tube was vortexed once more at high speed for 10 s and centrifuged at 15,000 rpm for 2-3 min.

Amplification and Identification of Sperm DNA

Following the protocol described above, 8 µL of the DNA sample was amplified in a 25 µL PCR reaction for each of 3 polymorphic microsatellite loci mentioned above. In effect, each PCR reaction amplified 1/3 of the sperm contained in a sperm storage organ. One of each pair of primers was fluorescently labeled with D4 WellRED Dye (Sigma-Aldrich®). Fragment analysis of amplicons was by capillary gel electrophoresis on a Beckman Coulter™ CEQ 8000XL Sequence Analyzer with the CEQ DNA Size Standard-400.

The "touchdown" thermocycler program (a program that changes the annealing temperature over various cycles) was as follows: 5 cycles of 96°C for 20 s, the highest annealing temperature for 30 s, and 72°C for 1 min; 21 cycles of 96°C for 30 s, the highest annealing temperature minus 0.5°C per cycle for 30 s, and 72°C for 1 min; and lastly, 10 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 1 min. PCR reactants included: 8 µL of target DNA (from the DNA isolation described above), 0.25 µL of Taq DNA polymerase, 0.5 µL of each primer (each at 20 µm), 12.5 µL of FailSafe™ PCR System buffer B (Epicentre Biotechnologies), and 3.25 µL of water.

Sperm Quantification

Relative Quantification. A capillary DNA sequencer for the fragment analysis of microsatellite loci (Beckman Coulter™ CEQ 8000XL Sequence Analyzer) was used to estimate the relative amounts of sperm contributed by different males to a particular sperm storage organ. The relative contributions of spermatozoa contributed by 2 or more males were estimated by measuring the relative peak heights or peak areas from the output of fragment analysis for a particular locus. Male genotypes for 3 microsatellite loci were determined after males mated with female flies. Sperm storage organs in female flies were subsequently analyzed for the presence, identity, and quantification of spermatozoa; the genotype of each female fly was determined for each of the 3 microsatellite loci (loci 1-3B, 1-5E, 1-1H, Fritz & Schable 2004) used to identify sperm DNA. The rationale for amplifying DNA for three microsatellite loci was to increase the probability of observing female and sperm genotypes that were completely different (making our analysis of contamination, for example, much easier).

Absolute Quantification. The absolute numbers of sperm in sperm storage organs was quantified by comparing sample peak areas produced by fragment analysis against a standard curve generated from peak areas of internal references for known quantities of spermatozoa. Prior to generating a standard curve, standardization of materials and protocols used in quantifying spermatozoa were established considering all the variables that may affect fragment analysis output (e.g., initial quantity and density of sperm in PCR reactions, thermocycler parameters, quality and quantity of PCR reactants, etc.). In addition, the precision of hardware was tested. The precision of our thermocycler (PXE 0.5 Thermal Cycler, Thermo Electron Corporation), for example, was tested by amplifying replicates of the DNA isolated from a particular number of sperm (isolated by our standard protocols outlined above) and by subsequent fragment analysis of mean peak area and variance through fragment analysis on our DNA sequencer. Similarly, we tested the precision of our DNA Sequencer for generating peak area output from each of the 8 capillary gels in a new capillary gel array. For example, amplicons from a single PCR amplification of DNA from a given number of sperm, and for a given microsatellite locus, were analyzed simultaneously by all 8 capillary wells of our DNA Sequencer through fragment analysis (peak height, peak area, and variance).

In order to establish a standard curve of PCR amplicon peaks for different quantities of sperm, it was necessary to obtain different densities of sperm in standardized PCR reactions. Sperm were dissected from the testes of males homozygous for a particular microsatellite locus being considered, pipetted into different volumes of water in a 1.5-µL centrifuge tube, and vortexed to homogenize their distribution. Sperm densities were obtained by examining 6 subsamples of each preparation with DAPI staining and a hemocytometer under fluorescent microscopy. Serial dilutions were then used to establish different sperm densities in equal volumes of liquid.

DNA was extracted from varying densities of sperm (but in equal volumes of liquid) by using the "*in situ*" Chelex based extraction protocol described above (protocols of Walsh et al. 1991) used for extracting DNA from sperm storage organs. DNA was isolated from 10, 20, 50, 150, 250, 350, and 500 spermatozoa in 6 replicates. DNA of each quantity and replicate of sperm was extracted and amplified (PCR) with fluorescently labeled primers for 3 polymorphic microsatellite loci (loci 1-3B, 1-5E, 1-1H, Fritz & Schable 2004) based on protocols as outlined above. PCR amplicons were then analyzed on a Beckman Coulter™ CEQ 2000XL Sequence Analyzer with the Beckman Coulter CEQ DNA Size Standard-400 and the CEQ Fragment Analysis Module Program (Beckman Coulter™, Fullerton, CA) in order to quantify sperm in terms of peak height and peak area.

The internal reference standard for all 3 microsatellite loci used in this study was generated by mass PCR of a fly homozygous for the 4H microsatellite locus (described by Fritz & Schable 2004). An identical quantity (1 µL in our case) of this locus' amplified DNA was added to each sample of amplified sperm DNA (post-PCR) as an internal reference during fragment analysis. To test the precision of this approach, for example, we amplified the DNA of 100 sperm in a single PCR reaction and subjected these amplicons to fragment analysis in all 8 capillary wells simultaneously along with identical quantities of internal reference DNA (1 µl). As mentioned above, estimates of the absolute numbers of sperm in sperm storage organs were generated by comparing sample peak areas produced by fragment analysis of sperm DNA against a standard curve generated from the ratios of peak areas of internal references to peaks produced by given quantities of spermatozoa.

Results

The sonication of sperm storage organs (or of samples of isolated sperm), as described above, was found to be effective in rupturing maternal cells without affecting sperm integrity (Fig. 1) and, therefore, a useful procedure for eliminating DNA from the female. As described above, sperm storage organs are rinsed in insect saline after sonication to remove any traces of female DNA. The subsequent crushing of sperm storage organs between 2 "microslides" to expose spermatozoa, and the isolation of the sperm DNA *in situ* eliminates the problem of sperm DNA copy loss. We obtained measurable amplicon peaks in fragment analysis from as few as 10 ± 3 spermatozoa. Furthermore, amplification of sperm DNA isolated from sonicated storage organs produced no amplicons from the female, thus providing evidence that maternal sources of DNA had, indeed, been excluded from PCR reactions of sperm DNA. In a study with females mated to 2 males (A. Fritz unpublished data), these protocols have successfully identified the sperm from each male in the sperm storage organs of females (and without the confounding amplicon signature of the females) (Fig. 2). Relative contributions of sperm by each male were quantifiable by the proportional heights of amplicons from each male to the total amplicon output (Fig. 2) taking into account any differences in allele amplification efficiency and by genotyping all male and female flies.

Instrument precision is critical for estimates of the absolute quantity of spermatozoa from 1 or more males that have inseminated a particular female fly. We found that the 8 capillary gels of our sequencer produced unsatisfactory variation

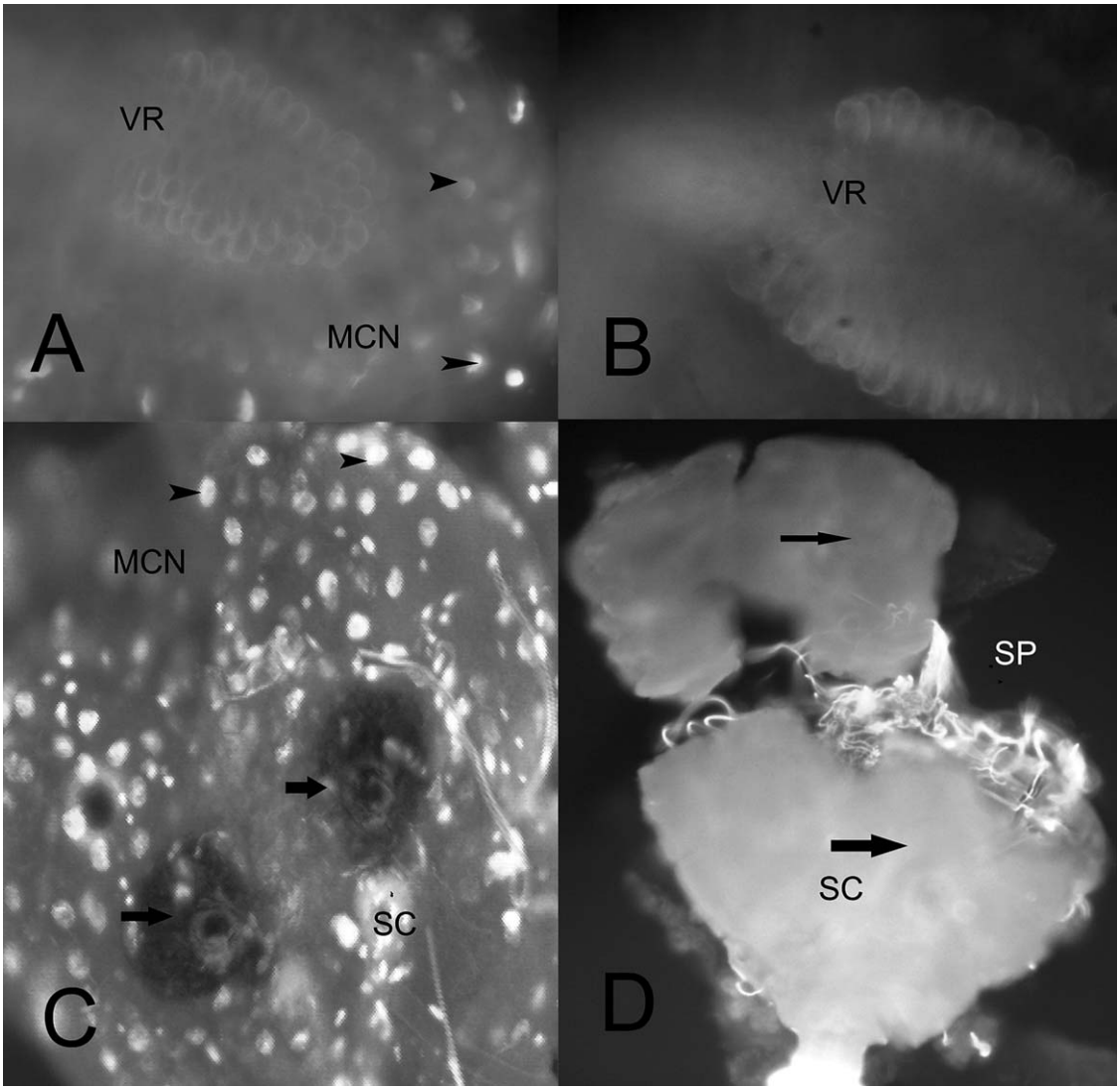

Fig. 1 A: Alveoli of the ventral receptacle (VR) surrounded by maternal cells (arrows point to nuclei) prior to sonication. B: Removal of maternal cells after sonication of the ventral receptacle. C: Spermathecal capsules (SC) surrounded by maternal cells (arrows point to nuclei) prior to sonication. D: Removal of maternal cells after sonication of the spermathecae showing intact spermatozoa emerging from a mechanically crushed capsule.

in output (fragment analysis of peak height and peak area) generated from a single PCR reaction. This variation in output by the CEQ Beckman Sequencer is, apparently, inherent "noise" in the system**.** This source of variation was ameliorated by the development of an internal reference run with each sample of DNA analyzed on the DNA sequencer; with the incorporation of an internal reference, output of fragment analysis becomes the ratio of the peak height (or area) of the sample DNA to the peak height (or area) of the internal reference (Table 1). The mean and standard deviation values indicated, also, that our thermocycler (PXE 0.5 Thermal Cycler, Thermo Electron Corporation) was satisfactorily precise in producing similar densities of amplicons from replicates of the same DNA amplified in random wells of the thermocycler (Table 1).

The relationship between the quantity of DNA isolated from different quantities of sperm versus the ratio of peak area of the internal reference to sample DNA was used to generate a standard curve (Table 2, Fig. 3). Peak area ratios generated for different quantities of sperm DNA showed, as

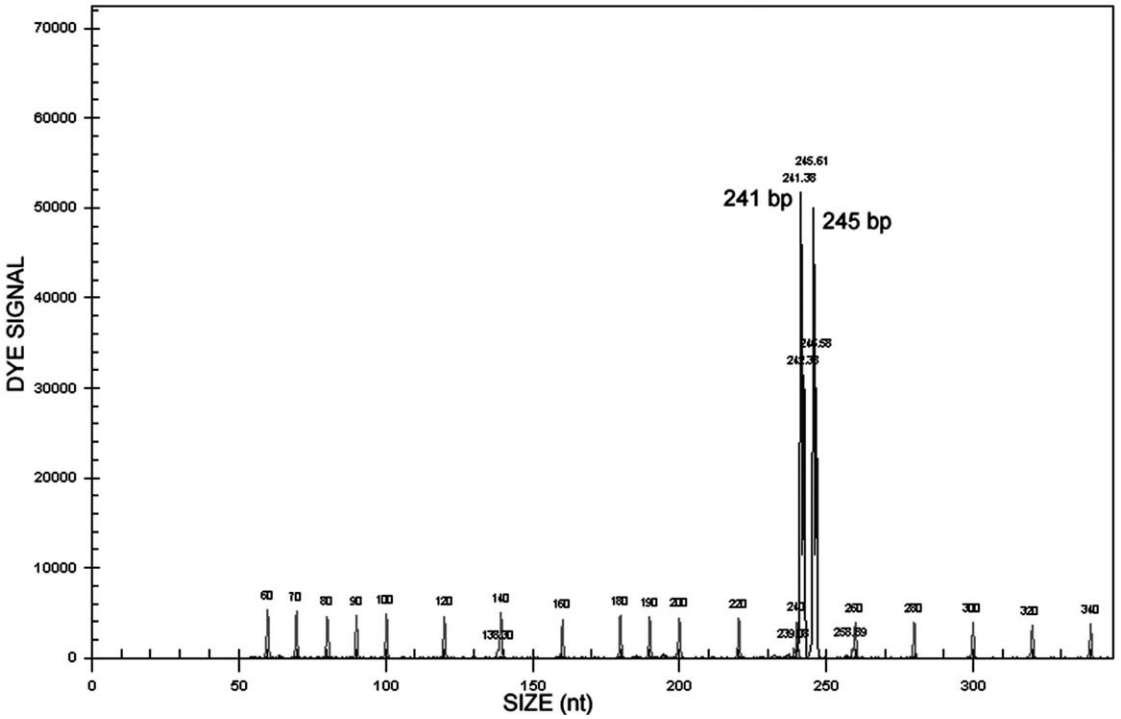

Fig. 2 Fragment analysis showing amplicon peaks (241 and 245 bp) from the sperm of 2 males homozygous for different alleles (241 and 245 bp) and stored in the ventral receptacle of a female fly. The female genotype (which was different from that of the males) is not visibly amplified, demonstrating the absence of contamination of sperm with DNA from female sources.

expected, a proportional decrease in variance with increasingly large samples of sperm DNA (Table 2, Fig. 3). The plot of sperm quantity with peak area ratios produced an approximately linear relationship during the exponential stages of PCR amplification for sperm quantities ranging from approximately 50 to 350 spermatozoa (Fig. 3). For our data and experimental conditions, the best-fit regression line through this range of sperm was $y = 0.024x + 7.69$. In our protocol, each PCR reaction represented 1/3 the sperm contained in a particular sperm storage organ; thus, the total number of sperm estimated in a particular sperm storage organ by the regression line shown above would need to be multiplied by three.

Multiplexing of sperm DNA for the 3 microsatellite loci used in this study was not feasible, since multiple primer pairs were found to compete differentially with reactants (and, therefore, affected differentially the ratio of sample peak area to the internal reference on the capillary sequencer). Thus, all sperm DNA samples were amplified in separate PCR reactions for the 3 microsatellite loci tested in this study.

Discussion

The protocols outlined above allow for the isolation, amplification, and quantification of sperm from minute, sclerotized sperm storage organs containing relatively intractable spermatozoa. The protocols described herein should be applicable to small, sclerotized spermathecae of any arthropod. Our procedure eliminates the task of "chasing-down" spermathecae with micropins to rupture them, eliminates the laborious task of removing relatively intractable sperm from ruptured spermathecae, and eliminates contamination of sperm DNA with DNA from the female genome. No DNA is lost from spermatozoa, since DNA is isolated without incorporating any steps that lead to a loss of copy number (since the release of sperm from their storage organs and the subsequent isolation of DNA occur in a single tube).

By the use of PCR of microsatellite loci, the identity and location of sperm from 2 or more males can be ascertained among the sperm storage organs in female flies. By using microsatellite loci as "non-invasive" markers for each male (as

TABLE 1. REPLICATE RATIOS OF REFERENCE PEAK HEIGHT AND PEAK AREA TO THE PEAK AREA AND HEIGHT OF A GIVEN SAMPLE OF SPERM (E.G., 10) AFTER PCR AND FRAGMENT ANALYSIS ON A DNA SEQUENCER; RFU = RELATIVE FLOURESCENT UNITS.

Ratio of reference standard to sperm sample in terms of peak area (rfu × mm)	Ratio of reference standard to sperm sample in terms of peak height (rfu)
21963/8933 = 2.45	34143/13684 = 2.49
33751/14437 = 2.33	56426/22286 = 2.53
32731/14153 = 2.31	54081/21254 = 2.54
27975/13107 = 2.13	33577/13562 = 2.47
24795/11332 = 2.18	33051/12908 = 2.56
17314/7212 = 2.4	18138/7414 = 2.44
15100/6289 = 2.4	23523/9041 = 2.6
11806/5004 = 2.35	19412/7677 = 2.52
Mean = 2.32; SD = 0.11	Mean = 2.52; SD = 0.05

opposed to labeling sperm from different males), this procedure also allows for the relative quantification and location of spermatozoa from different males in multiple sperm storage organs of female insects. For example, if 2 males homozygous for different alleles mate with the same female and contribute sperm to a particular spermatheca, then the relative peak heights (or areas) provide a good estimate of the relative contributions of each male's sperm. It is convenient if all alleles at a particular locus amplify with approximately equal efficiency, but this relationship can be examined and quantified in order to establish a baseline for interpreting results from actual experiments or field trials.

Estimates of the absolute number of spermatozoa contributed by 1 or more males are also possible, but more problematic. Quantification of sperm by quantifying PCR amplicons (by peak height or area) depends, in part, on the initial quantity and density of target DNA (from sperm) used in a reaction. Other variables include the parameters of the thermocycler and the relative quantities, qualities, and types of reactants of the PCR. Accurate estimates are only possible with the incorporation of an internal reference and the generation of a standard curve. Protocols, reagents, and materials need to be standardized and invariable. For example, we dedicated specific micropipettes to certain steps to prevent aliquot variability; and were careful to use reagents of the same age, batch, and quality (e.g., repeated freezing and thawing of reagents causes degradation). Once protocols are standardized, then a standard curve can be generated of sperm DNA quantity with peak height and peak area.

TABLE 2. THE MEAN ± SD RATIOS FOR EACH OF THE QUANTITIES OF SPERM DNA AMPLICONS TO THE REFERENCE DNA AMPLICONS IN TERMS OF PEAK AREA USED TO MAKE A STANDARD CURVE.

Sperm quantity	Mean ± SD ratios for each quantity of sperm DNA amplicon to the reference standard.
10 sperm	2.69 ± 0.55
20 sperm	6.80 ± 0.74
50 sperm	9.04 ± 0.52
150 sperm	10.84 ± 0.45
250 sperm	14.22 ± 0.59
350 sperm	15.94 ± 0.59
500 sperm	16.13 ± 1.08

Based on the protocol outlined above, the standard curve generated was only approximately linear for quantities of sperm from around 50 to 350 spermatozoa. Most females storing sperm in a sperm storage organ in *A. suspensa* do not exceed 350 sperm (Fritz 2004), which makes this protocol applicable to this species. For other species, however, standard curves would need to be generated using other quantities of spermatozoa and different thermocycler parameters to be useful.

The identification and quantification of sperm stored in females from single or multiple males has long been a hurdle for studies examining sperm storage dynamics and paternity. Methods that have been developed in the past have relied, in one manner or another, on the ability to tag sperm from particular males (e.g., dyes or radioisotopes), and more recent protocols incorporate molecular genetics to achieve this result,e.g., sperm labeled by transgenesis, or identifiable through other genetic manipulations of the genome (San Andrés et al. 2007; Scolari et al. 2008; Zimowska et al. 2009). These methods, however, run the risk that the system of tagging sperm affects their behavior or viability in a fashion that affects paternity outcomes. Also, these methods of tagging are strain specific, thus only applicable to the individuals that are altered. Thus, these methods of tagging sperm have typically been used to identify the presence or absence of sperm from a given strain of males that is stored in fe-

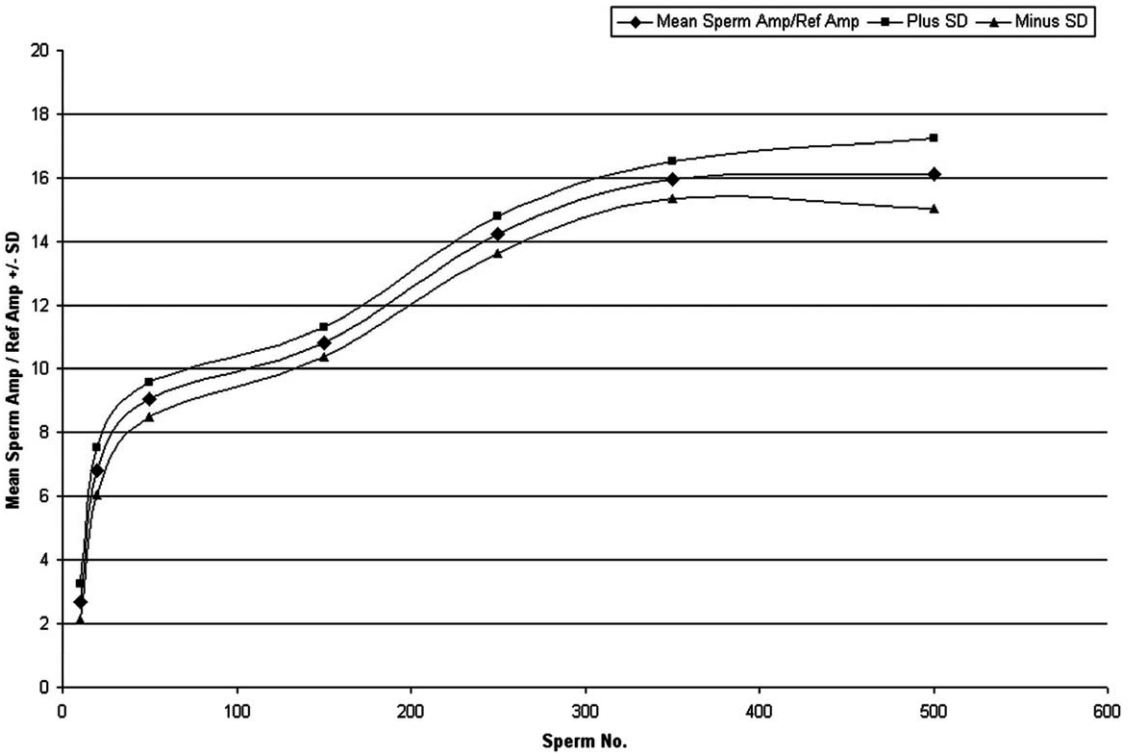

Fig. 3 Standard curve generated by plotting the mean (± SD) peak area ratios of the reference standard to the peak area of amplified sperm DNA of different quantities.

males. Alternatively, we have described protocols that are not strain-specific, do not rely on tagging sperm and, therefore, can be used to study mating behaviors, sperm storage patterns, and paternity outcomes in wild-caught females. Furthermore, our protocols allow for the isolation of sperm DNA from very small sperm storage organs in females without contamination with female DNA and without a reduction in the copy number of sperm. And, finally, our protocols allow for the relative or absolute quantification of sperm stored in females that have mated with 1 or more males.

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