CYTOLOGICAL ATTRIBUTES OF SPERM BUNDLES UNIQUE TO F_1 PROGENY OF IRRADIATED MALE LEPIDOPTERA: RELEVANCE TO STERILE INSECT TECHNIQUE PROGRAMS

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ABSTRACT

The unique genetic phenomena responsible for inherited F_1 sterility in Lepidoptera and some other arthropods provide advantages for the use of inherited sterility in a sterile insect technique (SIT) program. Lepidopteran females generally can be completely sterilized at a dose of radiation that only partially sterilizes males of the same species. When these partially sterile males mate with fertile females, many of the radiation-induced deleterious effects are inherited by the F_1 generation. At the appropriate dose of radiation, egg hatch of females mated with irradiated males is reduced and the resulting (F_1) offspring are both highly sterile and predominantly male. Lower doses of radiation used to induce F_1 sterility increase the quality and competitiveness of the released insects. However, during a SIT program it is possible that traps used to monitor wild moth populations and over-flooding ratios (marked released males vs unmarked wild males) may capture unmarked F_1 sterile males that cannot be distinguished from wild fertile males. In this study we developed a cytological technique with orcein and Giemsa stains to distinguish adult F_1 progeny of irradiated males and fertile males. Our observations on 6 pest species in 5 families of Lepidoptera indicate that F_1 males (sterile) from irradiated fathers can be distinguished from fertile males by the nuclei cluster in the eupyrene sperm bundles. The nuclei cluster in the fertile males exhibited a regular and organized arrangement of the sperm and was homogeneously stained, whereas in F_1 males the nuclei cluster of sperm was disorganized, irregular and unevenly stained.
Supplementary Material: color illustrations are available online at http://www.fcla.edu/FlaEnt/fe921.htm

Key Words: SIT, inherited sterility, Lepidoptera, eupyrene sperm

RESUMEN

El fenómeno genético único responsable para la esterilidad heredada en estado F_1 de los lepidópteros y otros artrópodos provee una ventaja para el uso de la esterilidad heredada en un programa de técnica de insecto estéril (TIE). Las hembras de lepidópteros generalmente pueden ser completamente esterilizadas con una dosis de radiación que solamente esteriliza parcialmente los machos de la misma especie. Cuando estos machos esterilizados parcialmente se aparean con hembras fértiles, muchos de los efectos delectivos inducidos por la radiación son heredados por la generación F_1. A la dosis de radiación apropiada, la eclosión de los huevos de las hembras apareadas con machos irradiados es reducida y las progenies que resultan son altamente estériles y predominantemente del sexo macho. Las dosis mas bajas de radiación usada para inducir la esterilidad de F_1 aumentan la calidad y la competividad de los insectos liberados. Sin embargo, durante el programa de TIE es posible que las trampas usadas para monitorear poblaciones de polillas salvajes y la tasa de sobre liberación (machos liberados marcados versus los machos salvajes no marcados) pueden capturar machos estériles de F_1 no marcados que no pueden ser distinguidos de machos salvajes fértiles. En este estudio nosotros desarrollamos una técnica citológica usando cepas de orcein y Giemsa, lo cual distingue la progenie F_1 de los adultos machos irradiados y machos fértiles. Nuestras observaciones sobre especies de 6 plagas en 5 familias del orden Lepidóptero indican que los machos F_1 (estériles) que provienen de machos irradiados pueden ser distinguidos de los que provienen de machos fértiles por un grupo de núcleos en bultos de esperma eupirene. El grupo de núcleos en los machos fértiles presentaron un arreglo de esperma re-

gular y organizado y fue homogéneamente teñido, mientras que en los machos de F_1 el grupo de los núcleos de esperma fue desorganizado, irregular y teñido de una manera dispareja.

Established and invasive lepidopteran pests representing numerous taxonomic families are a major threat to agriculture, forestry, and natural areas throughout the world (Peters 1987; Bloem & Carpenter 2001; Bloem et al. 2005). In response to this threat, industry, communities, and pest managers continually seek to develop pest control programs that are economically, environmentally, and socially acceptable. A common challenge in this process is the desire to identify strategies that are species-specific, broad-based, and portable across several taxonomic families. These apparently divergent features often have been bridged by the use of the sterile insect technique (SIT) in area-wide, integrated pest management programs with goals including pest suppression, eradication, containment, and prevention (Hendrichs et al. 2005). The SIT involves mass rearing, sterilization, and release of sterile pest insects to mate with feral insects, and is therefore species specific. SIT is a broad-based tactic because it is currently, or recently has been, used in programs targeting tortricid, gelechiid, lymantriid, and pyralid pests of row crops, orchards, forests and natural areas, respectively (Bloem et al. 2007; Carpenter et al. 2007; Suckling et al. 2007; Henneberry 2007).

The current method of choice in SIT programs for rendering insects reproductively sterile is exposure to ionizing radiation (Bakri et al. 2005). Selection of the most appropriate dose of radiation is important because quality and performance of the released insect often are reduced as the dose of radiation increases (Calkins & Parker 2005). In Lepidoptera, females can be completely sterilized at a dose of radiation that only partially sterilizes males of the same species. However, when these partially sterile males mate with fertile females, many of the radiation-induced deleterious effects are inherited by the F_1 generation. At the appropriate dose of radiation, egg hatch of the females mated with the irradiated males is reduced and the resulting (F_1) offspring are both sterile and predominantly male (Carpenter et al. 2005). The unique attributes of F_1 sterility (also referred to as inherited sterility) have been reported for all lepidopteran species examined (LaChance 1985).

Reproductively sterile moths released during a SIT program often are marked with an internal or external dye so that they may be distinguished from wild moths. Marking techniques are used in field monitoring of sterile to wild ratios, measuring performance of released moths, and determining the sensitivity of trapping grids used to confirm eradication (Kean & Suckling 2005). Reliable methods to distinguish released moths from wild moths mitigate the chance that a released moth may be misidentified as a wild moth, possibly leading to huge financial consequences for an area-wide IPM eradication program (Parker 2005). Because the appropriate dose of radiation for maintaining high quality moths for release permits the development of unmarked F_1 sterile moths in the field, it is possible that both unmarked F_1 sterile males and wild fertile males may be captured in traps. Concerns about the use of F_1 sterility include the erroneous view that the production of sterile F_1 larvae will cause economic damage to crops, especially in high-value crops such as fruit, and that F_1 sterile moths will be misidentified as wild moths and lead to expensive, yet unnecessary, interventions. Use of F_1 sterility makes it difficult to accurately estimate the wild population and the over-flooding ratio with traps because both wild males and sterile F_1 males are unmarked. A suitable method for distinguishing wild males and sterile F_1 males would avoid these concerns and would provide a positive feedback that irradiated males released in the field were mating with wild females.

Tothová & Marec (2001) demonstrated that various types of radiation-induced translocations are responsible for the production of genetically unbalanced gametes in F_1 progeny of irradiated moths and represent the main chromosomal mechanism of F_1 sterility. Several authors have reported the effects of radiation on the incidence of visible chromosomal aberrations in F_1 male larvae that were detected with light microscopy (Carpenter et al. 2005), and North & Snow (1978) and Carpenter & Gross (1993) used the incidence of chromosomal aberrations in field-collected larvae as verification that irradiated/released males had mated with wild females. These chromosome translocations make successful meiotic divisions impossible, resulting in genetically unbalanced gametes (Tothová & Marec 2001; Carpenter et al. 2005). Because the primary spermatocytes in a cyst undergo two meiotic divisions leading to the formation of a sperm bundle containing four sperm for each spermatocyte, we surmised there would be an unbalanced amount of chromatin material among the sperm nuclei within eupyrene sperm bundles from an F_1 sterile male moth. We examined the homogeneity of chromatin material in the nuclei cluster of eupyrene

sperm bundles to determine if there were differences between fertile and F_1 sterile males. Our goal was to identify a characteristic that can be used to accurately categorize wild males and sterile F_1 males captured in traps, regardless of age, mating status, and taxonomic family.

Materials and Methods

Sources of Insects

Six lepidopteran species examined in this study included codling moth (*Cydia pomonella* L., Tortricidae), obtained from the Okanagan Kootenay-Sterile Insect Release rearing facility in British Columbia, Canada; South American cactus moth (*Cactoblastis cactorum* (Berg), Pyralidae), obtained from the colony reared at the USDA, ARS, Crop Protection and Management Research Unit (CPMRU) insectary, Tifton, GA; diamondback moth (*Plutella xylostella* L., Plutellidae), obtained from the CPMRU colony; painted apple moth (*Teia anartoides* Walker, Lymantriidae), examined by authors DMS and SLW in New Zealand because of quarantine restrictions on transport of this Australian species to the US; corn earworm (*Helicoverpa zea* (Boddie), Noctuidae), obtained from the CRMRU colony, and fall armyworm (*Spodoptera frugiperda* J. E. Smith, Noctuidae), from the CPMRU colony.

Irradiation

In each species, newly emerged males less than 24 h old were exposed to 150 Gy of gamma radiation from a ^{60}Co source except for painted apple moth, which was exposed to 100 Gy at the late pupal stage. Similar males were not irradiated and their progeny served as controls. Irradiated and control males were caged individually with unirradiated conspecific females, eggs were collected from the mated pairs, and the larvae were reared on the appropriate diet in the laboratory. F_1 males were dissected and smears of sperm bundles were prepared from the seminal vesicles and testes.

Dissection of Moths

Moths were killed by immersion in 70-95% ethanol for a few seconds, rinsed and dissected in tap water in a small Petri dish. Insect saline solution may be used for the dissection instead of water, but we found no advantage in using it. In addition, saline forms crystals on the slide, interfering with staining and requiring an extra step for removal.

The posterior abdomen just above the claspers of the freshly killed moth was pulled with a pair of fine forceps until most of the simplex was exposed. This procedure will pull the duplex, *vasa deferentia*, seminal vesicles, and testes to the posterior abdomen, where they can be more easily dissected free from adhering fat body and tracheal tubules. Because there is a daily rhythm in the descent of mature sperm bundles from the testes to the seminal vesicles and then to the duplex for lepidopteran species (Riemann et al. 1974, Seth et al. 2002), the relative abundance of sperm bundles through out the reproductive tract varies by species and the photoperiod at the time of dissection or male death. Therefore, it is advisable to examine the duplex, *vasa deferentia*, seminal vesicles, and testes in the development of the best protocol for each species.

Seminal vesicles, duplex, and testes can be smeared separately on the same slide. The 2 testes are enclosed within a single membrane and can be treated as a single structure. Each part of the reproductive tract can be opened with help of fine forceps and the contents agitated in a very small drop of water on the slide until the sperm bundles are freed from the surrounding secretions. They are readily visible on a stereoscope under low magnification (30×). Smears may be allowed to dry for several hours, or overnight, or for a few minutes on a hot plate (~60°C).

Staining of Slides

For tissue fixation, slides containing smears were placed on a staining rack over a sink and flooded with absolute methanol for 5 min. The slides were then drained of methanol and allowed to dry before staining with aceto-orcein stain for 5 min. The stain was prepared by adding 4.0 g of synthetic orcein to 80 mL of glacial acetic acid in 80 mL of distilled water. The solution was boiled for 5 min, filtered, allowed to stand overnight, and filtered again. Then 40 mL of acetic acid were added drop-wise while stirring to dissolve any precipitate. Finally, 20 drops of glycerol were added to the solution (White 1973). The stained slides were rinsed in running tap water for 10 s and allowed to dry. The dried slides were stained with freshly prepared Giemsa stain. The Giemsa stain was prepared by mixing Giemsa stock with phosphate buffer (pH 7.41) at 1:1 (v/v) ratio. Slides may be stained from 30 min to 2 h to obtain good results. Slides were then rinsed in running tap water for 30 s, allowed to dry completely, rinsed for 5 s in absolute ethanol, and allowed to dry again before being mounted with Permount or other mounting medium.

Sperm nuclei in intact bundles of some species, particularly *H. zea*, were frequently non-respon-

sive to the staining procedure described above. In this case, before staining the slides with aceto-orcein, they were hydrolyzed with 1 M HCl for 2-5 min at 60°C on a hot plate and then stained satisfactorily. However, the intensity of subsequent Giemsa staining is progressively fainter after more than 5 min of hydrolysis. After treatment with HCl, the slides were rinsed in running tap water for 5 s and dried before staining with the aceto-orcein and Giemsa stains, as described above.

Each of the steps above can be varied depending on species and the procedures should be adapted to produce satisfactory staining results. The proportions of stain: buffer can be varied from 1:1 to 1:9, with consistently good, but variable results, as can the pH of the buffer, ranging from pH 4.0 to 10.0. In general, variations in the proportions of the stain:buffer and/or the pH of the buffer will produce variations in color of the stained tissues, usually from predominantly red to predominantly blue. Omitting the orcein step produced slides that were too blue, and omitting the Giemsa step produced slides that were too red. Using both stains produced slides with the best contrast and color; therefore, we recommend the use of both stains. Stained slides of control and irradiated F_1 individuals were studied and compared at low and high magnification (200× and 400×) on a Nikon Optiphot compound microscope.

Results and Discussion

In the testes and seminal vesicles, consistent and obvious differences were observed in individual sperm nuclei of eupyrene sperm bundles between the control (fertile) and F_1 (sterile) males. In control males of all species we examined, the sperm nuclei in the eupyrene sperm bundles are usually clustered and arranged in an orderly manner with the anterior ends of individual sperm usually equidistant from the anterior end of the sperm bundle (Fig. 1, A-D and Fig. 2, A-C). Even in poorly stained slides, the consistent orderly arrangement of individual sperm nuclei at the anterior end of the bundle is an obvious indicator that the preparation was from a fertile moth. The nuclei cluster of the eupyrene sperm bundles of normal males was stained homogeneously, indicating that equal amounts of chromatin material were present in each sperm.

In contrast, individual sperm nuclei in eupyrene sperm bundles from F_1 sterile moths were irregularly arranged and irregularly stained patterns were consistently observed, often with only a few or a low fraction of sperm nuclei in the bundle being stained (Fig. 1, E-H and Fig. 2, D-H). This heterogeneously stained nuclei cluster revealed that the amount of chromatin material in each sperm nucleus of F_1 sterile males was highly variable and disorganized compared with controls of the same species. Variations in the nuclear staining patterns were often great because some sperm within a bundle had no visible evidence of chromatin material while other sperm within the same bundle exhibited abnormally high levels of chromatin material. These cytological characteristics, observed in all males examined, are consistent with the findings of Tothová & Marec (2001) who showed that various types of chromosomal translocations produce genetically unbalanced gametes in F_1 progeny of irradiated parents.

In well-stained preparations, the distinction between the 2 treatments is often immediately apparent. However, in the process of developing the staining procedures in this study, inadequately stained slides were frequently produced. For evaluation purpose, it was necessary to distinguish between a poorly-stained smear from a normal moth and a preparation from an F_1 sterile moth. In this case, familiarity with the characteristics of the sperm bundles of the species under investigation, the application of a satisfactory staining procedure, and a thorough scan of each slide should allow a rapid and consistently accurate determination of the status of each moth examined. As a precaution, extra smears from the same individual could be prepared on separate slides in case the first stained slide cannot be confidently scored. In addition, a set of slides for each species should be produced for future reference, if necessary. Because all the sperm bundles within a male moth will be either normal (i.e., from a fertile male) or abnormal (i.e., from F_1 sterile male) and thus fall into a simple 'all-or-none' category, there is no need for a statistical analysis for status determination of the moths tested.

The best slides were those smears obtained from freshly killed moths. Unsatisfactory results were produced with frozen or ethanol-preserved moths. However, moths that appeared to be dead but still fully hydrated could have eupyrene sperm bundles that are responsive to the staining procedures (Wee et al. unpublished data). Intensity of staining and the overall quality of a smear preparation in certain species can be improved by acid hydrolysis (Fig. 1C). Unstained slides, previously fixed with absolute methanol, can be stored for at least 1 month for later staining (Wee et al. unpublished data). The species used in this investigation representing 5 lepidopteran families varied in size from the small diamondback moth, *P. xylostella* (~1.2 cm wingspan) to the larger corn earworm, *H. zea* (~4.0 cm wingspan). Similarly, the mature sperm bundles of these species varied in shape, length, and staining characteristics. Nevertheless, this technique worked well for all species examined.

As an overall conclusion, our observations indicate that F_1 males (sterile) from irradiated fathers can be distinguished from fertile males based on the homogeneity of staining in the nuclei

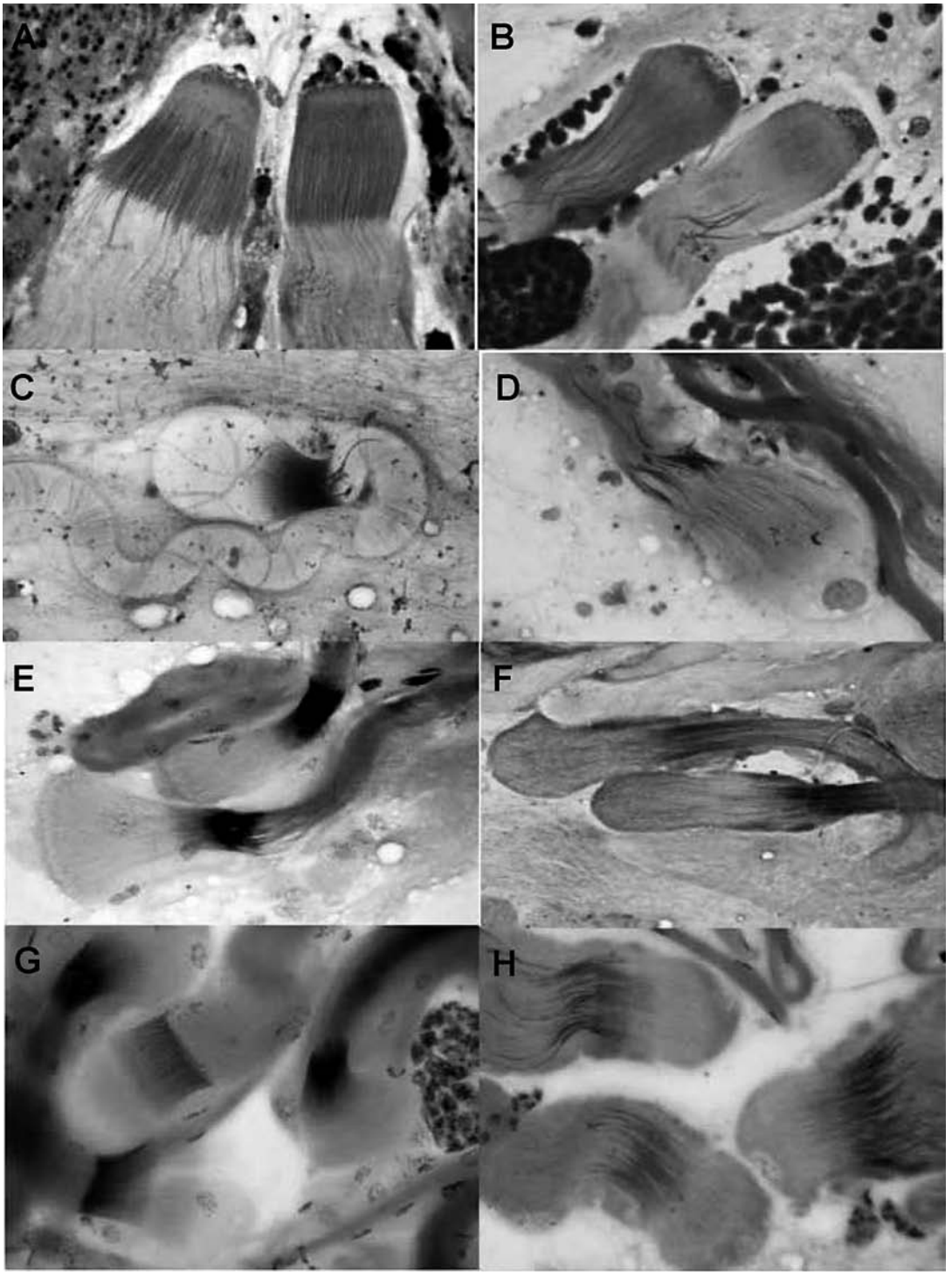


Fig. 1. A-D: Codling moth, *Cydia pomonella*. A and C: sperm bundles from testis smears of normal males, B and D: sperm bundles from testis smears of sterile F_1 males (progeny of irradiated males mated with normal females). E-F: Fall armyworm, *Spodoptera frugiperda*, E: normal male, sperm bundles from testis smear hydrolyzed in 1 M HCl, F: sperm bundles from sterile F_1 male. G-H: Corn earworm, *Helicoverpa zea*, G: sperm bundles from testis smear of normal male, H: sperm bundles from testis smear of sterile F_1 male. In all normal males, nuclei clusters of the eupyrene sperm bundles were homogenously stained but were heterogeneously stained in the sterile F_1 males.

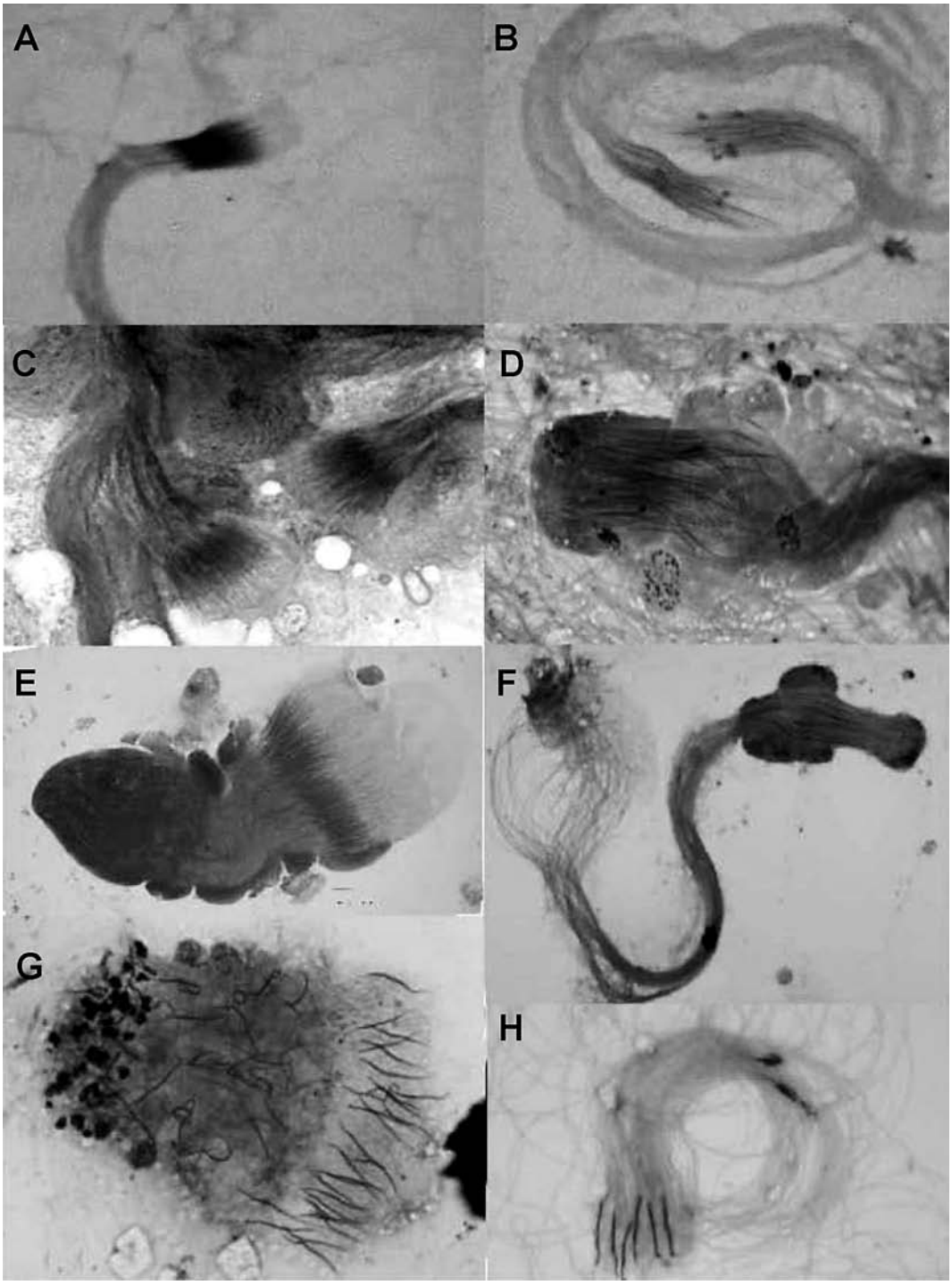


Fig. 2. A-B: Painted apple moth, *Teia anartoides*, A: sperm bundles from seminal vesicle smear of normal male, B: sperm bundles from seminal vesicle smear of sterile F_1 male (progeny of irradiated male mated with normal female). C-D and F: cactus moth, *Cactoblastis cactorum*, C: sperm bundles from testis smear of normal male, D and F: sperm bundles from testis smear of sterile F_1 males. E and G-H: Diamondback moth, *Plutella xylostella*, E: sperm bundle from testis smear of normal male, G-H: sperm bundles from testis smears of sterile F_1 males. In all normal males, nuclei clusters of the eupyrene sperm bundles were homogenously stained but were heterogeneously stained in the sterile F_1 males.

cluster of the eupyrene sperm bundles. Because this technique uses cytological attributes of the nuclei of sperm bundles in the testes, it cannot be affected by the age nor mating status of the male specimen to be evaluated. Although this technique has limited use on dead moths, it is the only method that could directly determine the origin/history of the trapped moth in a SIT sterile moth release program. This technique will be a great asset and potentially be adaptable for all lepidopteran pests involved in future SIT programs.

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