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# Panning for sperm gold: Isolation and purification of apyrene and eupyrene sperm from lepidopterans

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ABSTRACT

We describe a simple and straightforward procedure for the purification and separation of apyrene and eupyrene forms of lepidopteran sperm. The procedure is generally applicable to both butterfly and moth species with results varying according to the relative amounts of sperm produced and size of sperm storage organs. The technique relies upon inherent differences between eupyene sperm bundles and free apyrene sperm morphology. These differences allow for separation of the sperm morphs by repeated "panning" of sperm bundles into the center of a plastic dish. The purified eupyrene sperm bundles can then be removed and apyrene sperm collected from the supernatant by centrifugation. Efficacy of the purification process was confirmed by light microscopy and gel electrophoresis of the resulting fractions. Both one- and two-dimensional gel electrophoresis identified significant protein differences between the fractions further suggesting that the panning procedure effectively separated eurpyrene from apyrene sperm. The panning procedure should provide a convenient and accessible technique for further studies of sperm biology in lepidopterans.



## 1. Introduction

Sperm have long been the focus of physiologists, morphologists and cell biologists interested in cellular function and evolution. A typical sperm cell consists of an axoneme based sperm tail, a mitochondria (or mitochondrial derivative) energy source, and a head structure containing a highly condensed haploid content of DNA. This 'typical' sperm has been considered a premier example and model system for the study of cellular development, specialization and differentiation. Historically, sperm have also served a central role in the development of evolutionary theory, particularly as it relates to sexual selection, adaptation and sperm competition (Birkhead, 2009).

The spermatozoa has also been held up as a paradigm for "form denotes function" as sperm morphology clearly informs on sperm function and vice versa. However, the sperm biology of the Lepidopterans presents a unique challenge to this prevailing paradigm because nearly every species in this order of insects produces two distinct types of sperm within a common testis. This phenomenon termed, "dichtomous spermatogenesis" (Meves, 1903) results in a nucleated sperm containing a haploid content of DNA (eupyrene) and another type (apyrene) that completely lacks a nucleus and nuclear DNA. Characteristic differences in developmental pathways are typically observed during the early stages of spermatogenesis and produce the two distinct groups of encased sperm bundles containing the apyrene and eupyrene sperm. The sheath cells surrounding the apyrene sperm bundles break down in the testis and enter the seminal vesicles as individualized sperm whereas eupyrene sperm bundles remain intact (Friedländer et al., 2005).

The production of sperm with varying morphological characteristics has also been termed "sperm heteromorphism"; this term is used to describe the phenomenon for a wide variety of other taxa including insects, rotifers, sea urchins and nematodes (Swallow and Wilkinson, 2002; Friedländer et al., 2005). However, sperm heteromorphism in taxa other than Lepidoptera usually refers to differences in sperm length or size and not in the presence/absence of nuclear DNA. As previously reviewed (Snook, 1997), sperm heteromorphism (polymegaly) in members of the *Drosophila obscura* group is characterized by nucleated sperm of distinct length categories (Beatty and Burgoyne, 1971; Bressac and Hauschteck-Jungen, 1996). However, only the longer sperm morph is used in fertilization and therefore the two sperm morphs are not functionally equivalent (Snook et al., 1994; Snook and Karr, 1998). Other morphometric parameters such as sperm size in the nematode

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worm *Caenorhabditis elegans* are involved in the determinants of reproductive success (LaMunyon and Ward, 1998) and some of these determinants are under selective pressure (LaMunyon and Ward, 1999). Indeed, sperm morphological traits have been observed to effect reproduction at all levels of analysis including, e.g., factors such as motility, sperm quality and quantity, fertilizability and storage (Pitnick, 2009). Thus it is clear that sperm form and function can vary across diverse taxa and Lepidopterans represent a particularly extreme example of this variation.

In all of its various forms, sperm heteromorphism presents an evolutionary conundrum-how and why has this type of gametogenesis evolved? What are the function(s) for the observed variation in sperm morphs in fertilization and/or reproduction in general? The perplexing nature of this phenomenon is most clearly apparent in Lepidopterans that produce an entire class of a nucleated sperm incapable of participating, directly, in the all-important act of fertilization, karyogamy and re-establishment of the diploid state. The majority of empirical data on lepidopteran sperm heteromorphism has historically come from morphological studies of spermatogenesis based on light and electron microscopy (Friedländer et al., 2005). A number of functional and evolutionary hypotheses have been proposed to explain the existence of sperm heteromorphy. Due to the obvious fact that apyrene are devoid of a nucleus and lack genetic material to contribute to the zygote, these hypotheses typically propose functions for apyrene sperm that are peripheral to the primary reproductive role played by eupyrene sperm. Major ideas put forward include a role in sperm competition (Cook and Wedell, 1999), facilitation of eusperm (Holman and Snook, 2008; Sahara and Takemura, 2003), provisioning or nutritive functions (Boggs and Gilbert, 1979) and mediation of post-copulatory sexual selection (LaMunyon and Eisner, 1994). These, and other scenarios for the evolution of sperm heteromorphy have been comprehensively reviewed (Friedländer et al., 2005; Swallow and Wilkinson, 2002). However, beyond the obvious impact that fertilizing sperm have on organismal fitness, the genetic and cellular processes involved in origin and maintenance of sperm heteromorphism remain poorly understood.

Systems level analyses of sperm (e.g., RNA profiling and proteomics) have provided a wealth of new knowledge on the fundamental building blocks of this remarkable cell (Dorus et al., 2006; Fischer et al., 2012; Wasbrough et al., 2010; Zareie et al., 2013). Application of these modern "omics" technologies is providing new and exciting avenues to study the developmental biology of spermatogenesis and the molecular evolutionary basis of sperm competition and sperm evolution [detailed in several recent reviews (Amaral et al., 2014; Baker et al., 2012; Dacheux and Dacheux, 2014; Dorus et al., 2008, 2012)]. These technical advances offer great promise for insight into the mysteries posed by sperm heteromorphism, but have not yet been so employed. One major limitation in this effort has been the lack of high quality whole genome sequences necessary for comprehensive proteomic analyses. However, genome sequences for several Lepidopteran taxa are now publicly available, (Heliconius Genome Consortium, 2012; You et al., 2013; International Silkworm Genome Consortium, 2008), with several additional species expected in the near future. Thus the door is now open for detailed molecular genetic and functional genomic studies of this diverse group of insects.

To date, the absence of an efficient and accessible method for the separation of apyrene and eupyrene sperm suitable for biochemical, proteomic or functional analyses stands as one of the largest barriers to a deeper understanding of the cellular and molecular basis of sperm heteromorphy in Lepidoptera and other taxa. A prior published study described the successful separation of apyrene and eupyrene sperm from *Bombyx mori* using a discontinuous density gradient sedimentation technique (Osanai et al., 1989). While successful, the approach involves rather sophisticated centrifugation methods and analyses not commonly available to standard entomology laboratories and has therefore not found wide application and usage.

Here we describe a simple and straightforward procedure, applicable to both butterflies and moths, for the separation and purification of apyrene and eupyrene forms of lepidopteran sperm using only simple tools and reagents, a dissecting microscope and table top centrifuge. Recently, high throughput discovery proteomics have been successfully applied to the *Manduca sexta* sperm proteomes (Whittington et al., 2015). This detailed analysis of the total "A + E" sperm proteomes clearly paves the way for future in depth functional and genomic analyses of this important lepidopteran. Future studies of *M. sexta* sperm proteomes and other lepidopterans should benefit by use of the panning techniques described in this report.

## 2. Materials and methods

### 2.1. Lepidopterans used in this study

Two butterfly species, the Monarch (*Danaus plexippus*, kind gift of Monarchwatch.org, Lawrence, KS) and the American Painted Lady (*Vanessa virginiensis*, obtained from Monarchs Forever, Bixby, OK) and a moth, the tobacco horned-worm (*Manduca sexta* from Carolina Biological, Burlington, NC) were used in this study.

### 2.2. Bulk sperm isolation

5–10 day old adult males were euthanized and the reproductive tract, associated organs and connective tissue dissected from abdomens using small surgical scissors and fine tipped forceps. To facilitate sperm removal, the testis, *ductus deferens* and the vesicula seminalis (seminal vesicles) were removed from the surrounding connective tissue and placed into 100 mm plastic petri dishes containing phosphate buffered saline (PBS). Sperm and seminal fluids were removed by making a small incision near the mid-to-distal section which released the contents due to residual pressure within the seminal vesicle. Both apyrene and eupyrene sperm were commingled within an envelope of highly viscous fluid which remained relatively intact as it exited the seminal vesicle thus facilitating its easy removal to the petri dish using a pipettor fitted with P200 tip (the tip was cut back approximately 5 mm to enlarge the opening). In addition, for bulk purification of mixed samples of apyrene and eupyrene sperm, seminal vesicle contents were deposited into 1.5 ml microcentrifuge tubes and the contents spun down in a Sorvall benchtop centrifuge 2 min at 15 K rpm. Following supernatant removal, resulting pellets were resuspended in 1 ml PBS. This process was repeated 2X resulting in a purified mixture of apyrene and eupyrene sperm (termed, "AE" sperm).

### 2.3. Panning and purification of eupyrene sperm

All stages of the panning process are performed using a stereo dissecting microscope. Seminal vesicle contents containing AE sperm are used to prepare purified apyrene ("A"-) and eupyrene ("E"-) sperm using a novel panning technique (this technique is described in detail below and in the Supplemental Movie). To begin the process, seminal vesicle contents were transferred to a 100 mm plastic petri dish containing approximately 10 ml of PBS and dispersed by gentle mixing using a P1000 pipettor. (N.B. Note-experience suggests the use of plastic petri dishes yield optimal results over glass petri dishes). The dispersed contents are allowed to settle for approximately 2–3 min and the manual panning process begins with gentle circular rotation of the dish for