2nd Edition: Techniques for Semen Evaluation, Semen Storage, and Fertility Determination

Edited by

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PREFACE

Four years after the publication of the second edition, we hosted another hands-on workshop at the 2014 *Midwest Poultry Federation* meeting and, as a result, added two addenda to the workbook providing updates on turkey semen cryopreservation (Chapter VI: Section 2) and alternative methods for isolating the perivitelline layer for the sperm penetration assay (Chapter VII: Section 4). As in the first edition of this workbook published in 1997, the second edition provides the user with the "why's and how's" of various semen evaluation and fertility determination techniques and procedures. Given the success of the first edition and the intervening time, it became a matter of when to begin a second edition rather than if one is warranted. To our knowledge, there are no other contemporary comprehensive texts describing these or similar evaluation procedures dedicated to poultry.

Several of the techniques and procedures described in the first edition have remained relatively unchanged over the years and others have become obsolete and or are simply rarely used. The latter were deleted. Not falling into either of these categories are those techniques that have been further refined and developed over the past decade and have subsequently become important tools in the analysis of semen quality and the understanding of reproductive biology. These chapters have been revised to bring them up-to-date. Commercially available instruments and staining kits dedicated to the analysis of sperm motility characteristics, as well as the concentration and viability of sperm are increasingly available. While it was beyond the scope of this workbook to address all these new technologies, several such 'newer-generation' systems are included. Notwithstanding, we feel that the selected information described **in this second edition of the workbook** can be applied successfully on the breeder farm/hatchery or in the research laboratory.

As a note of caution, several of the chemicals listed in this workbook are hazardous and possibly carcinogenic. We remind you to have your Material Data Safety Sheets up to date and available, use extreme caution when handling these chemicals, wear appropriate body protection, and dispose of hazardous wastes according to in-house or government guidelines. We also remind you to follow applicable animal care guidelines when handling all poultry and other bird species.

Finally, throughout the chapters of this workbook, mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or a warranty and does not imply its approval to the exclusion of other suitable products. We would like to thank the *Midwest Poultry Federation* (Buffalo, MN), particularly Lara Durben, for all their support, the *Poultry Science Association* for their generosity, and all the authors and others in our laboratory for their contributions.

Murray R. Bakst and Julie A. Long

March 18, 2014

Chapter I. Semen Collection and Dilution

Vern L. Christensen, Lynn Bagley and Julie A. Long

PURPOSE

The artificial insemination (AI) of poultry is a two-step procedure. First, the semen is collected and second, the semen is inseminated. What transpires between these two-steps is a function of management goals and the staff's technical abilities. This chapter will review important aspects of semen handling for AI including semen collection and its initial evaluation, and semen dilution and short term storage.

SEMEN COLLECTION

Preparation

All utensils used in semen collection and dilution need to be clean, dry, and at ambient temperature at the time of use. If a collection chair is used, the pneumatic devices, air filters, and light sources should be checked regularly.

Semen Collection and Initial Evaluation

With minor variation, the process of manual semen collection from either chickens or turkeys is basically the same. The male is placed on his breast and his legs are gently restrained. The semen collector firmly "massages" the male's abdomen with one hand while concurrently stroking firmly the back and tail feathers with the other hand. (The duration and "feel" of these hand actions vary from male to male.) Within a few seconds, the phallus should enlarge slightly (phallic tumescence) and the semen collector shifts hands to straddle the cloaca. The thumb and forefinger of the hand situated just above the cloaca squeeze gently downward and inward while simultaneously the thumb and forefinger of the hand situated just below the cloaca squeeze upward and inward. This action, referred to as a "cloacal stroke", results in a partial ejaculation. It is repeated a second time to complete the ejaculation process. It is advisable to use no more that two cloacal strokes when collecting semen, as further strokes may injure the cloaca (Cecil and Bakst, 1985).

The semen collector does the initial evaluation of semen quality by collecting only semen that is thick and pearly-white or cream colored. Yellowish semen and semen contaminated with blood, urates, feces, or other debris should not be used, as yellow or contaminated semen will lower the fertilizing potential of the entire semen pool.

Helpful Hints

1. To minimize possible contamination and evaporation, keep the opening of the collection vial covered but not sealed air-tight. **Do not allow semen to contact water.**

2. If debris or contaminates are observed in the pooled semen sample, carefully aspirate contaminates (or remove with cotton-tipped applicator stick) from the sample **before** mixing diluent with the semen.

3. Vials containing turkey semen should not be tightly capped and should have an equal volume of fluid and air. Turkey sperm require constant exposure to oxygen to remain viable.

4. Turkey semen should be diluted with semen extender within 30 min after semen collection.

SEMEN DILUTION

Semen extender is a chemical solution designed to keep sperm alive after collection and to dilute the initial concentration of sperm to a desired level for AI. Diluting the semen increases the number of hens that can be inseminated per unit volume of semen. It is generally recommended to dilute turkey semen 1:1 (e.g. 1 mL semen + 1 mL extender), while chicken semen can be diluted 1:2 or 1:3 without affecting fertility.

There are several commercial extenders available. Before using, always check the bottled extender and make sure the liquid is clear. Any cloudiness or stringy material means the extender is either contaminated or its chemical components are coming out of solution, and these bottles should not be used. Never use diluent that has been opened and left sitting at room temperature overnight or longer.

One milliliter (mL) of extender may be added to the semen collection vial before collecting an additional 4 mL of semen. The extender should be at ambient temperature at the time of collection. Never add cold (4 ^{o}C) extender to freshly collected or room temperature semen because the cold shock could damage the sperm. A gentle shaking of the semen collection vial after collecting from each male is sufficient to mix the semen and extender until the desired number of males has been collected. After collection, thoroughly mix the semen by:

1. rolling the tube between the hands;
2. partially tilting the semen vessel or swirling the semen several times;
3. pipetting the semen mixture up and down with a Pasteur pipette;
4. placing tube or flask on a rotary shaker (150 rpm) for 3 to 5 min to completely mix the semen and extender.

Helpful Hints

1. Place the diluted semen in a cooler or refrigerator (4 ºC) to cool down. If turkey semen is to be held longer than 1 h before insemination, place the flasks on a rotary shaker set to 150 rpm (see Figure 1). Before doing any evaluation procedure and before inseminating, mix semen as described above.

2. Because 1 mL of semen weighs 1 gram, precise volume estimates can be made using a balance. Pre-weigh all collecting vials, including initial volume of extender, and label the vial with the weight. The difference in weight between the vial containing semen and the pre-weighed vial is the semen volume in milliliters.

GENERALIZED STEPWISE PROCEDURES FOR SEMEN COLLECTION, DILUTION, AND STORAGE

1. Collect 4 mL of semen into a vial containing 1 mL of extender at ambient temperature.

2. Bring pooled semen into a clean preparation area (20 to 25 ºC), remove debris, mix thoroughly, and transfer into a storage flask, preferably a 25-mL Erlenmeyer flask, to maximize the air:semen surface area. Cover the flask loosely with aluminum foil.

3. If turkey semen is stored longer than 1 h, place the flasks in a beaker containing enough water so that the water rises to the level of the semen in the flask. Place the beaker containing the flask of semen on a rotary shaker (150 rpm) at 3 to 12 ºC.

4. Determine semen volume and sperm concentration (see Chapter II) and then calculate the volume of extender that should be added so that a known number of inseminations containing a known number of sperm can be inseminated using the following formulas:

Total sperm number = semen volume x sperm concentration

Total volume diluted semen = total sperm number ÷ desired concentration for AI

Amount of extender to add = total volume of diluted semen – actual semen volume

Example: The actual semen volume in the flask is 8.5 mL; the sperm concentration of the semen pool is 9.8 billion sperm/mL, and the desired AI dose is 200 million sperm/0.1 mL.

Total sperm number = (8.5 mL) x (9.8 x 10^9 sperm/mL) = **83.3 x 10^9 sperm**

Total volume diluted semen = (83.3 x 10^9 sperm) ÷ (200 x 10^6 sperm/0.1mL) = **41.65 mL**

Amount of extender to add = 41.65 mL – 8.5 mL = **33.15 mL to add**

Note: The total volume of 41.65 mL is equivalent to 416.5 doses of 0.1 mL each. To check that the final concentration is the desired concentration, divide the total number of sperm by the total concentration: (83.3 x 10^9) ÷ 41.65 = 2,000 x 10^6 sperm/mL or 200 x 10^6/0.l mL).

5. Gently but thoroughly mix the semen before insemination.

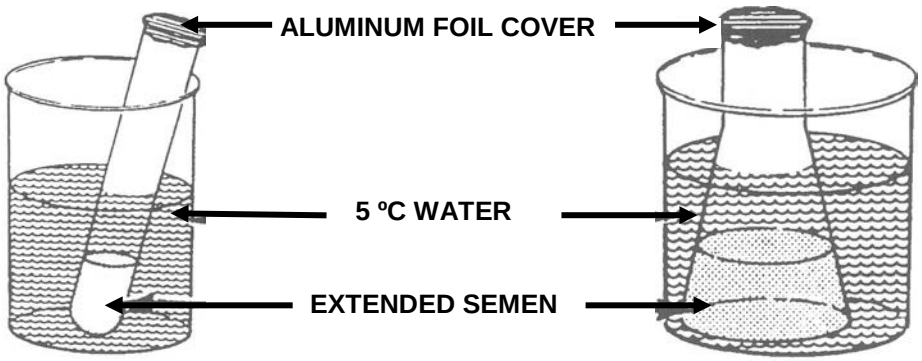


Figure 1. Diagram of proper placement of extended semen in water for short-term holding.

REFERENCES

Bakst, M. R., and J. P. Brillard, 1995. Mating and Fertility. Chapter 12 in: World Animal Science. Vol. C9. Poultry Production. P. Hunton, ed. Elsevier Science Publishers, Amsterdam, The Netherlands.

Cecil, H. C., and M. R. Bakst, 1985. Volume, sperm concentration, and fertilizing capacity of turkey ejaculates obtained from successive cloacal strokes during semen collection. Poult. Sci. 64:1219-1222.

Christensen, V. L., 1995. Diluents, dilution, and storage of poultry semen for six hours. Pages 90-106 in: Proceedings First International Symposium on the Artificial Insemination of Poultry. M. R. Bakst and G. J. Wishart, ed. The Poultry Science Association, Inc., Savoy, IL.

Sexton, T. J., 1982a. Beltsville poultry semen extender. 6. Holding turkey semen for 6 h at 15 C. Poult. Sci. 61:1202-1208.

Sexton, T. J., 1982b. Short-term holding of turkey semen. Pages 110-113 in: Proceedings, Symposium on Turkey Reproduction. Raleigh, NC.

Sexton, T. J., 1984. Effect of temperature and method of semen collection on the viability of turkey spermaztozoa. Poult. Sci. 63:844-846.

Thurston, R. J., 1995. Storage of poultry semen above freezing for twenty-four to forty-eight hours. Pages 107-122 in: Proceedings First International Symposium on the Artificial Insemination of Poultry. M. R. Bakst and G. J. Wishart, ed. The Poultry Science Association, Inc., Savoy, IL.

Chapter II. Determination of Sperm Concentration

Section 1. Hemocytometer Procedure

Murray R. Bakst

PURPOSE

The hemocytometer is a specialized slide with a finely etched micro-grid on its surface that is used to directly count blood cells, sperm and other single cells. When carefully added, highly diluted poultry semen disperses by capillary action between the hemocytometer and its overlying cover-glass thus forming a 10µL thick mono-layer of scattered sperm. Sperm within designated squares of the micro-grid are counted and then multiplied by conversion (based on size of grid counted) and dilution factors to derive the number of sperm per unit volume in the original semen sample. Because of the time required and eye fatigue, routine hemocytometer use for determining sperm concentration is not recommended. However, hemocytometer counts should be used in the preparation of standard curves (see Chapter II.2) to correlate the indirect readings of sperm concentrations obtained from instruments such as spectrophotometers, colorimeters, and packed cell volumes (spermatocrits) with actual numbers of sperm.

MATERIALS AND METHODS

Materials

Hemocytometer slide
Hemocytometer cover glass
Micropipettor (positive displacement to deliver 10 µL)
Laboratory tissues
Parafilm
Clean test tubes, 10-mL capacity
Volumetric flask, 1-L capacity
Hand-held tally counter
Volumetric flasks, 10-mL capacity (optional)

Equipment

Digital balance
Microscope with phase contrast optics and 40x objective

Chemicals

Sodium chloride (NaCI) reagent grade; M.W. 58.44

Distilled water

Reagents

3% NaCl solution preparation:

1. Weigh out 30 g of NaCI into a 1-L volumetric flask.
2. Add distilled water to the flask, filling to the 1-L mark.
3. Mix thoroughly until the NaCI is completely dissolved.
4. 3% NaCl can be stored at room temperature for several months if tightly sealed. Discard and make up a new solution if particles are observed.

SEMEN SAMPLE PREPARATION

Semen Sample A: Dilute neat (undiluted) semen, 1:1, or 1:2 with poultry semen extender.

Semen Sample B: Preparation of 1:1000 dilution for counting.

1. Fill test tube precisely with 10-mL of 3% NaCI.
2. Pipette 10 µL Semen Sample B into test tube containing 10 mL 3% NaCl. (Make sure semen carried on the outside of the pipette tip is wiped off.)

3. Prepare duplicate tubes of each semen sample. Cover each tube with parafilm and gently disperse (inverting the tube four or five times) the semen without foaming the solution. Sperm diluted in 3% NaCI are stable for 1h before sperm begin to lyse.

(NOTE: If a *volumetric flask is used, first pipette the semen into the flask then add the NaCl to bring the volume* to *the 10-mL line, seal top with Parafilm, and mix the diluted sample by inverting the flask four or five times.)*

PROCEDURE FOR COUNTING SPERM WITH A HEMOCYTOMETER

1. Place cover glass over the grid surface on a clean, dry hemocytometer.

2. With a micropipette, aspirate 10 µL of Semen Sample B, carefully wipe off the outside of the pipette tip to remove any adhering semen, and "charge" (fill) one chamber of the hemocytometer. This is done by placing the tip of the pipette against the underside of the cover glass overlying the small beveled region leading to the grid chamber. Slowly discharge the semen and make sure the semen disperses uniformly throughout the chamber. If clumps are observed, clean and dry the hemocytometer and start over.

3. Repeat Step 2 to charge the other chamber and gently secure the hemocytometer on the microscope stage.

4. Let the hemocytometer sit undisturbed for 3 min to allow the sperm to settle.

5. Using a phase contrast objective, bring one chamber's grid into focus. Off the 25 squares in the large central grid (Figure 1, red border), only count the sperm overlying and clearly within the four corner squares and the center square (yellow border) boundaries

NOTE: Sperm heads will straddle the outside boundaries of the individual small squares to be counted (Figure 2). Standardize your counting technique by only counting those sperm that straddle the top and left border of these squares (Figure 2).

a. Use counter to accumulate sperm counts for the five squares (four corner squares and the center square (yellow border) boundaries within the red border square (Figure 1).
b. Record total counts of the five squares for the chamber. Reset counter to zero.
c. Carefully move the microscope stage so that second chamber is in focus.
d. Repeat Steps 5a to 5d for the second chamber.
e. After counting, remove cover slip and rinse it and the hemocytometer with distilled water and wipe dry with a laboratory tissue.

6. Repeat above steps with duplicate semen sample.

CALCULATIONS

Calculate the average number of sperm counted on both grids (5 squares per grid):

Example: Total sperm = 250, and semen sample A was diluted 1:1

1. Divide total sperm by the number of squares counted 250/10 = 25 (average count/square)
2. Dilution factor (A) = Neat semen diluted either 1:1 (dilution factor = 2) or 1:2 (dilution factor = 3)
3. Dilution factor (B) = 3% NaCl, 10μL: 9990mL (dilution factor = 1000)
4. Cells per mL = average count per square x dilution factor x 10^4

   $25 \times 2 \times 1000 \times 10^4 = 5.0 \times 10^8$ cells/mL

NOTE: Hemocytometer chamber measures: 0.1 mm depth (this is the depth of the semen layer between the underside of the cover glass and the grid surface); each large square is 1mm x 1mm for a volume of 0.1 mm^3. Each of the 400 small squares has a volume of 0.00025 mm^3.

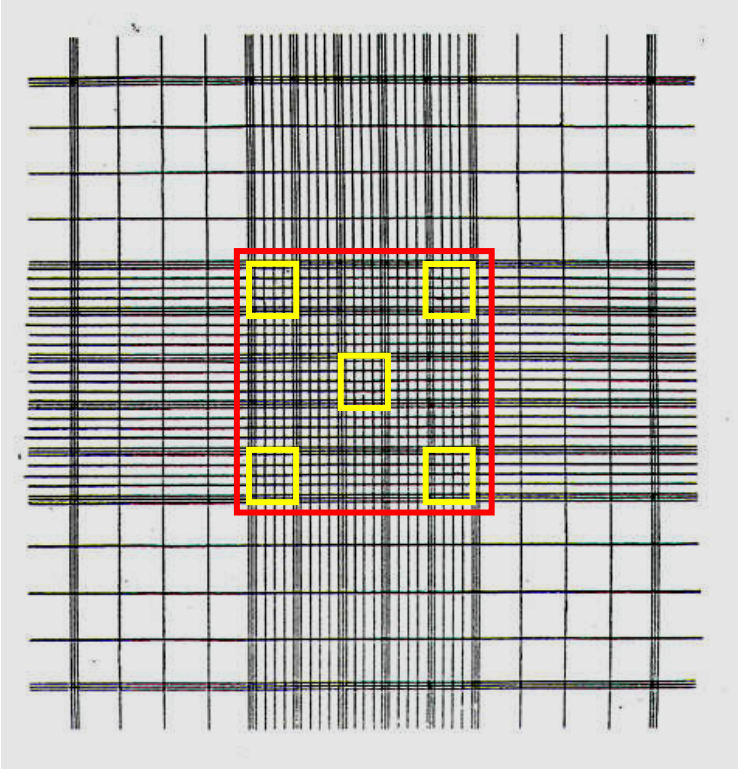


Figure 1. Observed is a single standard hemocytometer grid when viewed through a microscope at low magnification. The red border highlights the central square that is subdivided further into 25 smaller squares. Of the 25 smaller squares, only the center and the four corner squares are used to count poultry sperm (yellow border). Figure 2 depicts one of these squares.

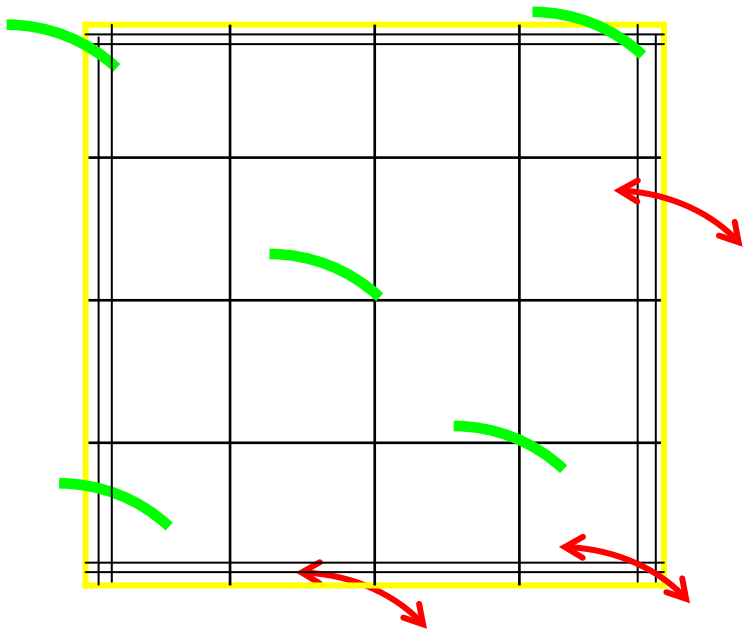


Figure 2. Enlargement of one of the 5 squares used to count poultry sperm in Figure 1 (yellow borders) showing sperm distributed over the grid. Sperm clearly within the yellow boundary are counted as well as the sperm straddling the left and upper boundary (green sperm heads). Sperm straddling the right and bottom boundary lines are not counted (red sperm heads).

Chapter II. Determination of Sperm Concentration

Section 2. Establishing a Standard Curve

Murray R. Bakst

Purpose

Several different methods are used to determine the concentration of sperm in a semen sample. The hemocytometer (see Chapter II.1) is the simplest method by which sperm are *directly* counted. *Indirect* methods of determining sperm numbers, such as packed cell volume (PCV), spectrophotometry, colorimetry, and turbidity, require a conversion factor that converts the actual instrument reading to sperm number or concentration in the sample. This conversion factor is derived by establishing a regression equation and standard curve, which provides the mathematical relationship between the reading provided by the instrument or technique and the actual number of sperm.

To derive a regression equation and standard curve, several semen dilutions are prepared with sperm concentrations above and below the range observed after semen is diluted 1:1. The sperm concentration is determined from each sample dilution using both a hemocytometer and the instrument or method that requires the standard curve. This is repeated at least five or more times using different semen samples.

To maintain accurate and reproducible sperm counts on all instruments, it is prudent to determine the regression equation and standard curve for *each* instrument each year. Internal calibrations and light emitted and detected for each instrument may vary and will change slightly with time and extended use. Therefore, a single regression equation and standard curve for and conversion factor derived for one instrument may not be accurate for other similar instruments.

MATERIALS AND METHODS

Materials

4 mL fresh, undiluted semen
6 small vials or tubes (10-mL capacity)
6 pairs of test tubes (10-mL capacity)
Hemocytometer and cover glass
Microcapillary tubes, bulb, and sealant

Pipettor to deliver 1 mL

Micropipettor (positive displacement) to deliver 10 µL

Ice bath (crushed ice in shallow pan)

Parafilm

Kimwipes

Equipment

Digital balance

Computer with statistical programs

Chemicals

Sodium chloride (NaCl) reagent grade, M.W. 58.44

Distilled water

Reagents

3% NaCl solution preparation:

1. Weigh out 30 g of NaCI into a 1-L volumetric flask.
2. Add distilled water to the flask, filling to the 1-L mark.
3. Mix thoroughly until the NaCI is completely dissolved.
4. 3% NaCl can be stored at room temperature for several months if tightly sealed. Discard and make up a new solution if particles are observed.

SEMEN SAMPLES – STOCK DILUTIONS

1. Pooled, neat (undiluted) semen is diluted to give the *Stock Semen Dilutions* (see Table 1). These *Stock Semen Dilutions* are used to develop standard curves for sperm concentration.

    a. The standard curve should be constructed from the six points distributed between approximately 1 billion (most highly diluted semen) and 10 billion (neat semen) sperm per mL.

    b. Each point should be counted twice (duplicate readings). This procedure should be repeated with at least five (but preferably more) different semen samples to get an accurate standard curve.

2. The suggested volumes (mL) in Table 1 are for making duplicate readings on a single dilution. Therefore, approximately 4 mL of neat semen is required.

Table 1. Serial dilution of a neat turkey semen sample for an estimated range of sperm concentrations between 1 billion and 10 billion sperm per milliliter

Stock Semen Dilutions			
Vial label	Semen : Diluent	Semen : Diluent (mL)	Total Volume (mL)
Neat	1 : 0	0.75 : 0.0	0.75
0.5	1 : 0.5	0.5 : 0.25	0.75
1	1:1	0.5:0.5	1.00
1.5	1 :1.5	0.5:0.75	1.25
2	1:2	0.5:1.0	1.50
3	1:3	0.5:1.5	2.00

PREPARATION OF STOCK SEMEN DILUTIONS (TABLE 1)

1. Gently but thoroughly mix the stock neat semen thoroughly, using a pipettor remove 0.75 mL of stock neat semen (using a Kimwipe, wipe the outside of the pipette tip) to the first vial labeled "neat".

    a. *NOTE: If a 1-mL pipettor is not available, use a digital balance to determine semen and diluent volume. Place the small vial on the digital balance, tare to zero, and then add the semen or diluent to the precise weight which is equivalent to the volume desired.*

2. Gently but thoroughly mix the stock neat semen thoroughly, using a pipettor remove 0.5 mL of stock neat semen (using a Kimwipe, wipe the outside of the pipette tip) and add to each of the remaining vials.

3. Using the same precautions described in Steps 1 and 2 above, pipette 0.25 mL of diluent to the second small vial and label the vial "0.5". This dilution is 1 part semen to 0.5 parts diluent, or a 1 to 0.5 (1:0.5) dilution.

4. To the third small vial of semen, add 0.5 mL of diluent, mix thoroughly, and label the vial "1". This is a 1 to 1 (1:1) dilution.

5. To the fourth small vial of semen, add 0.75 mL of diluent, mix thoroughly, and label the vial "1.5". This is a 1 to 1.5 (1:1.5) dilution.

6. To the fifth small vial of semen, add 1 mL of diluent, mix thoroughly, and label the vial "2". This is a 1 to 2 (1:2) dilution.

7. To the sixth small vial of semen, add 1.5 mL of diluent, mix thoroughly, and label the vial "3". This is a 1 to 3 (1:3) dilution of semen.

8. Keep all Stock Semen Dilutions vials in beakers containing enough water to reach the level of the semen. Place beakers in a 5 ºC refrigerator as described in Chapter I.

STANDARD CURVES

Packed Cell Volume (PCV) vs Hemocytometer Counts

1. Determine the PCV (in duplicate) of the six *Stock Semen Dilutions* without any further dilution. Follow the PCV procedure described in Chapter II.4.

2. Use the same six *Stock Semen Dilutions* to do the corresponding hemocytometer counts according to the steps outlined below.

3. Record readings for the PCV and the hemocytometer counts. Calculate the mean value for each duplicate. (see Table 2).

Table 2. This is an example of a worksheet that can be used for the correlation of hemocytometer counts to PCV readings. These data reflect instrumentation in the author's lab.

		Hemocytometer			PCV		
Neat Semen Samples	Dilution	Cell Count	sperm per mL(10^9)	Mean	% PCV	mean	
1	neat	225	11.25	11.4	23.5	24.30	
1	neat	230	11.5		25		
1	1 :0.5	155	6.75	6.83	16.5	16.20	
1	1 :0.5	158	6.9		15.9		
1	1 :1	111	5.55	5.79	9.8	10.10	
1	1 :1	121	6.03		10.4		
1	1 :1.5	103	5.15	5.08	8.7	8.80	
1	1 :1.5	100	5		8.8		
1	1 :2	88	4.38	4.26	6.1	6.20	
1	1 :2	83	4.15		6.2		
1	1 :3	41	2.05	2.13	4.5	4.80	
1	1 :3	44	2.2		5.1		
2	neat	207	10.35	10.9	24.9	24.20	
2	neat	230	11.5		23.4		
2	1 :0.5	173	8.65	8.39	15	15.00	
2	1 :0.5	163	8.13		15		
2	1 :1	98	4.9	5.44	9.3	9.20	
2	1 :1	120	5.98		9.1		
2	1 :1.5	81	4.05	4.36	7.5	7.50	
2	1 :1.5	94	4.68		7.4		
2	1 :2	77	3.83	3.73	5.5	5.50	
2	1 :2	73	3.63		5.5		

2	1 :3	56	2.8	2.65	6.1	5.60	
2	1 :3	50	2.5		5.1		
3	neat	213	10.65	11	26.2	26.10	
3	neat	226	11.3		26		
3	1 :0.5	184	9.2	9.4	16.8	16.50	
3	1 :0.5	192	9.6		16.2		
3	1 :1	148	7.38	6.68	11.3	11.40	
3	1 :1	120	5.98		11.5		
3	1 :1.5	104	5.18	5.5	7.4	7.60	
3	1 :1.5	117	5.83		7.7		
3	1 :2	66	3.28	3.34	5.5	5.50	
3	1 :2	68	3.4		0		
3	1 :3	56	2.8	2.9	4.4	4.60	
3	1 :3	60	3		4.8		
4	neat	261	13.03	14.5	26	26.00	
4	neat	321	16.03		26		
4	1 :0.5	178	8.88	9.64	16	15.80	
4	1 :0.5	208	10.4		15.5		
4	1 :1	120	6	5.5	9.5	9.30	
4	1 :1	100	5		9		
4	1 :1.5	79	3.93	4.28	8.2	8.30	
4	1 :1.5	93	4.63		8.3		
4	1 :2	80	4	3.98	6.8	6.50	
4	1 :2	79	3.95		6.2		
4	1 :3	50	2.5	2.7	4.7	4.60	
4	1 :3	58	2.9		4.5		
5	neat	239	11.93	13.5	28	28.00	
5	neat	302	15.08		28		
5	1 :0.5	173	8.63	8.54	14.8	15.10	
5	1 :0.5	169	8.45		15.5		
5	1 :1	134	6.7	5.9	10.8	10.90	
5	1 :1	102	5.1		11		
5	1 :1.5	100	5	5.35	8.5	8.50	
5	1 :1.5	114	5.7		8.5		
5	1 :2	83	4.15	4.08	7.3	7.40	
5	1 :2	80	4		7.5		
5	1 :3	63	3.73	3.36	5.3	5.30	
5	1 :3	60	3		5.3		
6	neat	120	6	6.5	12.1	12.20	
6	neat	140	7		12.2		
6	1 :0.5	92	4.6	4.3	7.2	7.10	
6	1 :0.5	80	4		7		
6	1 :1	60	3	3.3	4.9	4.90	
6	1 :1	72	3.6		4.8		
6	1 :1.5	53	2.65	2.75	3.7	3.80	
6	1 :1.5	57	2.85		3.8		
6	1 :2	47	2.35	2.18	2.4	2.40	
6	1 :2	40	2		2.4		

CALCULATIONS

Hemocytometer

Calculate the average number of cells counted and determine sperm concentration (see Chapter II.1).

Regression analysis

Table 3. This is an example of a regression analysis for the data in Table 2 derived from a computer software program and used to plot the best fit line in Figure 1.

Packed Cell Volume (PCV) vs hemocytometer:

Regression Output:	
Constant	1.0904
SE of Y Est	0.7514
R2	0.949
n	35
df	33
X Coefficient	0.447
SE of Coefficient	0.018

DRAWING THE STANDARD CURVE

On separate sheets of graph paper plot the points for the following:

1. y axis - Sperm Concentration (hemocytometer) as 10^9sperm/mL.

2. x axis - Percentage PCV

3. Use regression analysis to determine the best fit straight line for the points and according to the equation for the straight line, y = ax + b, where x = the slope of the line and b = the intercept on the y axis. Computer software programs are available for this calculation. (see example, Table 3).

4. Draw the best fit straight line as determined in Step 2. An example of the Hemocytometer vs PCV graph is shown in Figure 1. If regression analysis is not possible, the best fit line can be drawn by eye.

   NOTE: the best fit line does not have to pass through the % coordinates, but is a *straight line that passes closest to the maximum number of points.*

HOW TO USE THE GRAPH AND EQUATION TO DETERMINE SPERM CONCENTRATION

Two methods are available:

1. Read the concentration directly from the best fit line on the graph:

   Example: If the PCV was 20%, find 20% on the x-axis and follow it to it intersects with the plotted regression line. At that intersection follow it to the Y-axis where the concentration reads 10x10^9 sperm/mL, the concentration of sperm in your original sample.

2. Substitute the value for x in the equation and solve the equation for y:

   Example: The regression equation to derive sperm concentrations from PCV readings using this laboratory's instrumentation is Y = .447X + 1.090, where X is the %PCV and Y is the sperm concentration x 10^9.

   If the %PVC reading was 20% then solve for Y:

   Y = .447(20) + 1.090

   Y = 10.03 x10^9 sperm (concentration)

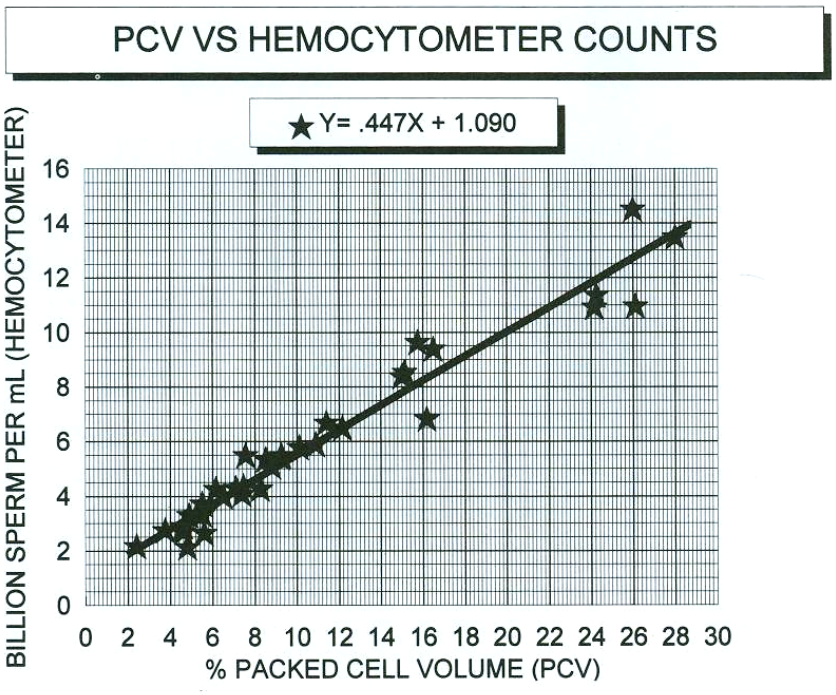


Figure 1. Worksheet example of standard curve for packed cell volume. Plot of data (Table 2) for standard curve to compare percentage packed cell volume to sperm concentration determined by hemocytometer counts. The best fit line (y = 0.447x + 1.090) was determined by regression analyses (Table 3).

Chapter II. Determination of Sperm Concentration

Section 3. Photometer Procedure

Julie A. Long

PURPOSE

A photometer provides an indirect estimate of the sperm concentration by measuring the optical density of a highly diluted semen sample. The optical density is directly proportional to the concentration of sperm in the sample. Before reading any samples, it is necessary to zero the machine using a *"blank sample",* a tube filled only with 3% sodium citrate). The number of sperm in the semen sample is determined by comparing the sample's optical density to a standard curve or using the line equation supplied by the manufacturer for the photometer.

MATERIALS AND METHODS

Equipment and Supplies

Digital balance
Photometer
Volumetric flask, one liter size
Spatula
Bottles with caps, 100 mL size
Pipettor, 1 or 2 mL delivery, and corresponding pipette tips
Pipettor, 0.01 mL delivery (10 µL), and corresponding pipette tips
Disposable pasteur pipettes, 1 for each semen sample
Cuvettes or other tubes to fit the photometer, minimum 3 mL capacity
Rack to hold cuvettes

Chemicals

Sodium citrate
Distilled water

PREPARATION of 3% SODIUM CITRATE SOLUTION

1. Place the volumetric flask on the balance and 'tare' or zero the balance.

2. Weigh 30 g sodium citrate into the volumetric flask.
3. Remove the flask from the balance.

4. Add distilled water to the flask, filling to the 1L mark.

5. Mix thoroughly until the sodium citrate is completely dissolved.

6. Separate the liquid into 10 bottles containing 100 mL each.

7. Label each bottle with "3% NaCl", the date, and your initials.

8. 3% NaCl can be stored at room temperature for several months.
   NOTE: If particles are present at any time after storage, discard and make up a new solution of 3% sodium citrate.

SEMEN SAMPLE PREPARATION

The best results are obtained when neat (undiluted semen) is used to determine sperm concentration. Cuvettes must be clean. To avoid fingerprints, only handle the top of the cuvette.

1. Label each cuvette with the sample number.

2. Precisely fill each cuvette with 1.99 mL of 3% sodium citrate. Fill one extra cuvette to be used as the *blank sample.*

3. Pipette 10 µL of semen into the corresponding labeled cuvette. Use a different pipette tip for each semen sample. It is very important to be consistent with your pipetting technique. It is highly recommended that the semen be expelled in one complete motion followed by two rinses of the pipette. Rinsing more than twice will artificially inflate the sperm concentration reading. The semen should appear as a thin white ribbon in the cuvette, while the subsequent two pipette tip rinses should expel a barely noticeable amount of semen.

4. Using a different Pasteur pipette for each cuvette, gently mix the semen and sodium citrate to a uniform consistency. Avoid introducing air bubbles into the cuvette.

5. Semen samples can be mixed with the 3% sodium citrate prior to zeroing the photometer (See Step 1, below). Semen diluted in 3% sodium citrate is stable for 1 h, but should be covered to minimize evaporation. After 1 h, sperm numbers may decrease as sperm lyse.

PROCEDURE FOR READING OPTICAL DENSITY WITH A PHOTOMETER

1. Zero the photometer using the *blank sample*.

2. Check each cuvette before reading to ensure there are no fingerprints or liquid outside the tube, as this could generate a faulty reading.

3. Place the first sample cuvette in the photometer and press the read button.

4. Record the optical density (usually a number between 0.5 and 1.0).

DETERMINING SPERM CONCENTRATION FROM OPTICAL DENISTY

1. To determine a line equation using Microsoft Excel, first plot the data with optical density on the X axis and sperm concentration on the Y axis using the Chart function to create a line graph.

2. Right click on the resulting line and select “add trend line”.

3. Under the “type” tab, select “linear”; then under the “options” tab, select “display equation on chart”. The line equation will then appear on the graph.

4. The line equation format is “y = m(x) + b”, where y = the dependent variable (sperm concentration), x = the independent variable (optical density), m = the slope of the line, and b = the y-intercept. For example, an optical density of 0.865 plugged into the line equation y = 11.83(x) + 0.24 would yield a sperm concentration of 10.47 x 10^9 sperm/mL.

5. Most photometers are pre-set in the factory with a line equation. It is helpful to prepare a sheet for use with each photometer for easier determination of sperm concentration. Table1 shows a sample sperm concentration table follows based on the line equation y = 11.34(x) + 0.03.

Table 1. Relative sperm concentrations based on a photometer-specific line equation.

OD Reading	Sperm Conc (x10^9/mL)
0.18 - 0.21	2.0
0.22 - 0.26	2.5
0.27 - 0.30	3.0
0.31 - 0.35	3.5
0.36 - 0.38	4.0
0.39 - 0.43	4.5
0.44 - 0.47	5.0
0.48 - 0.52	5.5
0.53 - 0.56	6.0
0.57 - 0.61	6.5
0.62 - 0.65	7.0
0.66 - 0.70	7.5
0.71 - 0.74	8.0
0.75 - 0.79	8.5

OD Reading	Sperm Conc (x10^9/mL)
0.80 - 0.83	9.0
0.84 - .087	9.5
0.88 - 0.92	10.0
0.93 - 0.96	10.5
0.97 - 1. 00	11.0
1.01 - 1.05	11.5
1.06 - 1.09	12.0
1.10 - 1.13	12.5
1.14 - 1.17	13.0
1.18 - 1.22	13.5
1.23 - 1.26	14.0
1.27 - 1.31	14.5
1.32 - 1.35	15.0
1.36 - 1.39	15.5
1.40 - 1.44	16.0

Chapter II. Determination of Sperm Concentration

Section 4. Packed Cell Volume (Spermatocrit) Procedure

Murray R. Bakst

PURPOSE

Based on the same principle as determining blood hematocrits, the estimated sperm concentration can be determined by the high speed centrifugation of a sample of poultry semen until all the sperm are tightly packed. Immediately after centrifugation, the percentage of tightly packed sperm relative to the total volume of the semen sample is determined. This 'packed cell volume' (PCV) or spermatocrit is then converted to sperm concentration using either a conversion factor or standard curve, both of which were previously derived by comparing and graphically plotting hemocytometer counts to corresponding PCV (see Chapter II.2).

MATERIALS AND METHODS

Materials

Microhematocrit capillary tubes (plain)

Microhematocrit capillary tube bulbs

Capillary tube sealant

Standard curve or conversion factor (see Chapter II.2)

Equipment

Microcentrifuge (for hematocrits) with rotor and reader for microhematocrit tubes

Microcapillary tube reader

Venier caliper

PROCEDURE

1. Gently but thoroughly mix neat or diluted semen sample.
2. Prepare two tubes (duplicate samples) for each semen sample.
3. Attach bulb to capillary tube and aspirate semen into the capillary tube until it is at least 75% filled.
4. Quickly push one end of the capillary tube into capillary tube sealant to plug.

5. Place the tubes on the centrifuge bed with the plugged end facing out.

6. Place cover on centrifuge head, close the centrifuge and set timer for 10 min at 10,000 to 12,000 rpm.

7. After centrifugation, determine the percentage of sperm in each tube using the microcapillary reader.

8. If a microcapillary reader is not available, the percentage can be determined using a venier caliper to measure the length of the capillary tube with the packed sperm and the length of the capillary tube containing the packed sperm and the remaining fluid composing the semen sample.

9. Calculate the percentage PCV as follows:

$$\% \text{ PCV} = (\text{length of packed sperm}) \div (\text{length of packed sperm} + \text{seminal plasma}) \times 100$$

CALCULATIONS

1. Average the two PVC percentages for the duplicate samples.

2. Refer to the standard curve or equation for the standard curve. Determine the concentration of the sample by reading from the best fit line, or solving for y in the equation for the best fit (see Chapter II.2).

3. If the original semen sample was diluted add the appropriate dilution fact for the final sperm concentration reading.

Chapter III. Sperm Viability

Section 1. Nigrosin/Eosin Stain for Determining Live/Dead and Abnormal Sperm Counts

Murray R. Bakst

PURPOSE

The nigrosin/eosin (N/E) staining procedure is based on the principle that live, viable sperm have intact cell membranes capable of excluding the eosin stain. Conversely, dead (non-viable) sperm possess permeable cell membranes that permit the eosin to stain the sperm. The nigrosin component of this procedure provides a blue background in the smear to enhance the contrast of the ivory white, unstained "live" sperm, as well as the pinkish or magenta, stained "dead" sperm. Based on the N/E staining procedure, a good fresh semen sample from most breeder toms or broilers will have more than 90% live and morphologically normal sperm.

MATERIALS AND METHODS

Materials

Volumetric flasks, 1-L capacity

Beaker (10-mL capacity), small tube, or vial bottles (100-mL capacity)

Micropipettors to deliver 10, 200, and 250 µL

Pasteur pipettes (1-mL volume)

Slides and cover-slips (37.5 x 22 mm)

Hand counter with totalizer key

Semen diluent

Immersion oil for microscope

Gloves

Equipment

Balance

pH meter and buffers to measure pH 7.0

Microscope (bright field or phase contrast)

Chemicals

Sodium glutamate [(L-(+)-glutamic acid, monosodium salt, monohydrate] (M.W. 187.13)

Potassium citrate (citric acid, tripotassium salt) (M.W. 306.4)

Sodium acetate, trihydrate, crystal (M.W. 136.08)

Magnesium chloride, hexahydrate (M.W. 203.31)

Distilled water

nigrosin (water soluble crystals)

eosin Y (90+% purity)

Reagents

Buffer (pH 7.0) for N/E stain

1. Weigh out the following and place in a 1-L volumetric flask:
   17.35 g sodium glutamate;
   1.28 g potassium citrate;
   8.51 g sodium acetate;
   0.68 g magnesium chloride.

2. Fill to 1 L with distilled water.

3. Mix intermittently until all components are dissolved.

4. Measure pH and adjust to pH 7.0.

5. Keep buffer refrigerated in a tightly sealed bottle. Discard if any particulate matter is observed.

N/E Stain:

1. In bottle or flask add 5 g nigrosin and 1 g eosin.

2. Add 100 mL buffer.

3. Mix thoroughly and let the stain stand a minimum of 24 h before use. Keep the stain in a tightly sealed bottle in a refrigerator. The stain is good indefinitely but should be checked a day before use. Discard the stain if any bacteria or particulates are observed when viewing in the microscope.

4. Filter before use.

SPERM EVALUATION

1. Dilute neat semen 1:1 with semen diluent.

2. Pipette 250 µL of N/E Stain and, using a clean pipette tip, pipette 200 µL of the diluted semen into a small tube or vial.

3. Mix thoroughly by gentle inversion at 30-s intervals for precisely 2 min (ambient temperature).

4. Using a clean pipette tip, place 10 µL of sperm-stain mixture near the short edge of a microscope slide (first slide, lying flat on bench-top).

5. Bring the short edge of a second slide to the edge of the inside edge of the 10 µL droplet (that side facing the longer end of the first slide) and position second slide at about a 30° angle. Allow the sperm-stain mixture to disperse along the outside edge of the second slide and then *pull* the sperm-stain mixture along the first slide to form a smear.

6. Let the smear air dry or gently blow dry. Slides may be covered with a cover-slip.

7. Smears should be read as soon as possible to minimize staining artifacts. However, if the slides are stored in a cool dry place the stains are stable for 24 h.

8. Count 200 to 300 sperm (hand counter with totalizer is helpful) using a bright field (for N/E) or phase contrast (for sperm morphology) objectives at 40x or with an oil immersion lens.

CATEGORIES OF SPERM MORPHOLOGY AND THEIR VIABILITY AFTER N/E STAINING

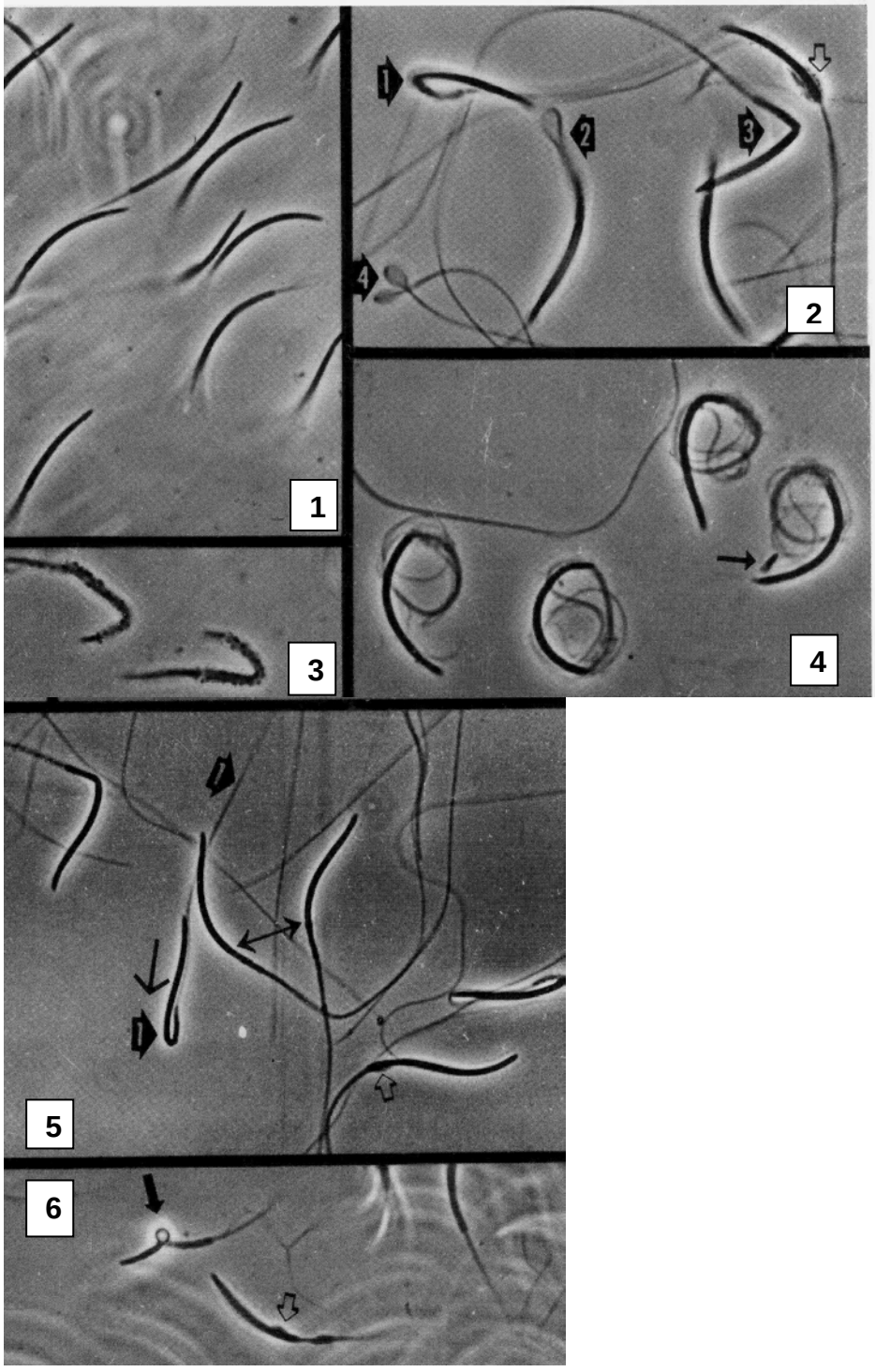
The following 4 categories of sperm can be differentiated (see Figures 1 to 11):

Normal viable sperm: Morphologically normal sperm that are unstained (white under bright field and black under phase contrast microscopy)

Abnormal, viable sperm: Morphologically abnormal sperm that are unstained (white under bright field and black under phase contrast microscopy)

Normal, non-viable sperm: Morphologically normal sperm with any pink discoloration of the head region (use bright field microscopy)

Abnormal, non-viable sperm: Morphologically abnormal sperm with any pink discoloration of the head region (use bright field microscopy)



Figures 1-6 are images of turkey sperm observed using an oil immersion with a 100x phase contrast objective.

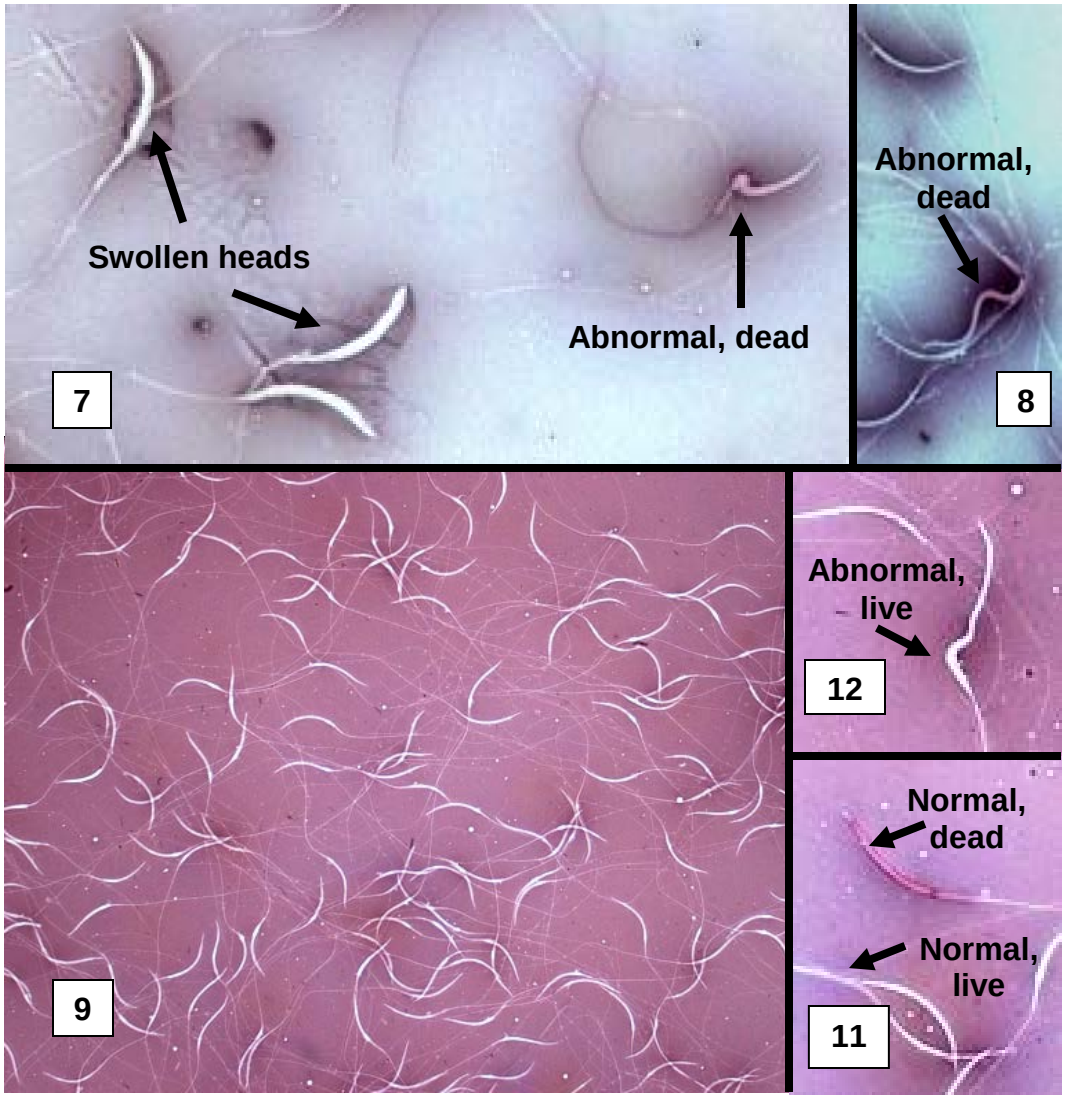
Figure 1. At this magnification morphologically normal turkey sperm are characterized by their long tapered shape. The head region is thicker than the tail and has a comma-like curvature. A conical-shaped acrosome is located at the tip of the sperm nucleus (see Figure 4). The midpiece, the location of the sperm's mitochondria, is the most anterior portion of the sperm tail and is slightly less thick than the head. In normal sperm it has no distinguishable features.

Figure 2. Common sperm anomalies include: arrow-1, bent sperm, with the acute bend at the midpiece; arrow-2, bent sperm, with the bend (or looping) of the tail just the below the head region; arrow-3, a sperm which exhibits an acute bend around the neck region (the neck region is the juncture of the nucleus and midpiece); arrow-4, slight "coiling" of the distal end of the tail is observed; and, the hollow arrow, which highlights swollen, detached mitochondria associated with the distal end of the sperm nucleus. These anomalies are common after semen is stored for 18 h or longer, but can be observed in fresh diluted semen as well.

Figure 3. Sperm nuclear decondensation is rarely observed. When compared to normal sperm, the decondensing nucleus is generally less dense, but thicker and characterized by the discrete chromatin granules. Also see Figure 7.

Figure 4. The highly coiled appearance of these four sperm is due to semen dilution with water. This is an extreme example of the effects of a hypotonic environment on sperm morphology. The arrow indicates a broken acrosome. This is a sperm organelle containing a hydrolytic enzyme that digests a path for through the perivitelline layer (PL) thus permitting sperm entry into the ovum at the time of fertilization. These patches (holes) created by sperm through the PL are the "sperm-holes" that are counted during the PL-sperm hole counting procedure (see Chapter VII.4).
.
Figure 5. The double-headed arrow is pointing to two normal sperm. The hollow arrow highlights a swollen mid-piece of a sperm. The remaining sperm are bent. The lower arrow-1 points to a bent sperm with the bend at the mid-piece. Although the head is flexed back and aligned parallel with the sperm tail (indicated by the upper arrow-1), this sperm was probably highly motile progressing in the direction of the long arrow.

Figure 6. The arrow highlights a "knotted" sperm head. This is probably a genetic defect. It is first observed during spermatid formation during spermatogenesis (Bakst, unpublished observations). The hollow arrow highlights a swollen mid-piece with displaced mitochondria.



With the exception of Figure 9, which is 40x, Figures 7-11 are images of chicken sperm observed using an oil immersion with a 100x bright field objective.

Figure 7. Three abnormal but viable sperm with swollen heads (possibly indicative of senescence) and one abnormal, non-viable sperm are observed. The latter sperm is pink, indicative of eosin staining, and has a shortened sperm head with a knot-like anomaly (see Fig.6).

Figure 8. Observed are normal, viable sperm surrounding an abnormal, non-viable sperm with a wavy head.

Figure 9. A survey micrograph showing a good distribution of normal, viable sperm on a N/E smear.

Figure 10. A portion of a normal, viable sperm is adjacent to an abnormal, non-viable sperm with an elongated head and possibly swelling along the midpiece.

Figure 11. A normal, nonviable sperm is stained pink with the eosin. The darker pink band in this sperm is most likely the neck region which the juncture between the nucleus and midpiece. Also note the acrosome in this sperm is unstained.

REFERENCES

Dott, H. M., and G. C. Foster, 1972. A technique for studying the morphology of mammalian spermatozoa which are eosinophilic in a differential 'live/dead' stain. J. Reprod. Fertil. 29:443-445.

Ernst, R. A., and F. X. Ogasawara, 1970. A live-dead stain to test poultry semen quality. One Sheet Answers #193 (9/70), University of California Agricultural Extension Service, University of California, Davis, CA.

Chapter III. Sperm Viability

Section 2. Ethidium Bromide Exclusion and Stress Test Procedures

Murray R. Bakst

PURPOSE

Ethidium bromide (EtBr) is a nuclear fluorescent stain that is excluded from cells with an intact cell membrane (viable, alive) but readily stains the nuclei of cells with a permeable cell membrane (nonviable, dead cells). Thus lies the basis for the 'EtBr exclusion procedure' (Bilgili and Renden, 1984) used to differentiate live from dead poultry sperm and determine the percentage of non-viable sperm in a semen sample For example, when a semen sample is initially stained with EtBr, only nonviable sperm will fluoresce and be detected by a fluorometer. This is the 'initial' reading. Digitonin is then added to the same test sample and disrupts all sperm cell membranes. All sperm nuclei are stained and the 'final' reading is made with the fluorometer. The percentage of non-viable sperm in the original semen sample is calculated by dividing the 'initial' by the 'final' reading and multiplying by 100.

The "stress test" (Bakst et al., 1991), which is best used after semen is stored for 12 h or longer, determines the percentage of viable but "weak" sperm plus dead sperm. The procedure is nearly identical to the EtBr exclusion procedure. However, in the stress test, the semen sample is mixed in a hypotonic solution containing EtBr. The low osmolarity causes sperm to take up water and swell. Dead sperm and more labile, 'weak' sperm swell and burst and their nuclei are subsequently stained. In contrast, the viable sperm swell but remain intact and unstained. Using this 'hypotonic stress test', a more labile subpopulation of sperm not detected using the EtBr exclusion procedure becomes evident.

MATERIALS AND METHODS

Materials

Semen sample(s)

Glass tubes (borosilicate tubes) 10 mm x 75 mm for use in fluorometer pipettes (2 mL)

Micropipettor (10 µL and 20 µL□)

Volumetric flasks (1-L and 2-L)

Bottle, 100-mL capacity

Gloves (latex)

Kimwipes

Equipment

Balance

Osmometer

pH meter

Fluorometer, equipped with 365 nm interference filter and 570 nm emission filter

Chemicals

(NOTE: Ethidium bromide is hazardous. Handle this and other chemicals and solutions carefully; wear appropriate protective clothing and discard according to hazardous waste guidelines.)

Ethidium bromide, M.W. 394.32 Caution: mutagenic substance

Digitonin anhydrous, M.W. 1229.31

Sodium chloride (NaCI), reagent grade or USP grade, M.W. 58.44

Sodium phosphate monobasic monohydrate ($NaH_2P04 \cdot 1\ H_20$), M.W. 137.99

Sodium phosphate dibasic (Na_2HP0_4), M.W. 141.96

Absolute ethanol

Distilled water

Reagents

Solution A: Buffered EtBr

1. Weigh out the following into a 2-L flask:

   17.0 g NaCI
   0.524 g $NaH_2P0_4 \cdot 1\ H_20$
   2.302 g Na_2HP0_4
   0.025 g EtBr

2. Dilute to 2-L with distilled water. Cover flask and mix until all chemicals are dissolved.

3. Label as Solution A plus date. Osmolarity should be 290 ± 10 mOsm and pH 7.2.

Solution B: EtBr in Water

1. Weigh out 0.0125 g EtBr into a 1-L volumetric flask and dilute to 1-L with distilled water.

2. Cover flask and invert several times to mix thoroughly.

Solution C: Stress Solution

(NOTE: Omit this step if evaluating semen stored for 12 h or less.)

1. Transfer 200 mL Solution A and 800 mL Solution B into a 1-L volumetric flask.

2. Cover flask and invert several times to mix thoroughly. Osmolarity should be 60 ± 5 mOsm.

Solution D: Digitonin Solution

1. Weigh out 0.5 g digitonin into 100-mL flask.

2. Add 100 mL absolute ethanol.

3. Store in stoppered bottle.

ETHIDIUM BROMIDE EXCLUSION PROCEDURE

(NOTE: The exact procedure will vary with the make of the fluorometer.)

1. Turn on fluorometer a minimum of 10 min before use.

2. Label clean 10 x 75 mm clean borosilicate tubes (two tubes per sample) with the sample number. (*NOTE: Hold tubes at the top to keep from getting fingerprints on the lower portion of the tube.*)

3. Put on gloves. Pipette 2 mL of Solution A into each tube.

4. Make sure the fluorometer is in the off position before removing the cap covering tube port.

5. Insert tube containing Solution A, recap opening of tube port in fluorometer, and slide the lever to "on". Adjust to zero using the fine adjust knob, turn the lever to "off", remove cap, remove tube from the fluorometer. Fluorometer is now "zeroed" for this tube.

6. Mix semen sample and pipette 10 uL of the semen into the tube that has been zeroed. Cover the top of the tube with Parafilm, and mix by holding finger on top of Parafilm while inverting the tube four or five times.

7. Wipe the outside of the tube quickly with a laboratory tissue, insert the tube into the tube port, replace cap, and slide the lever to "on". Immediately start the timer.

8. At the end of 60 s, record the "initial reading", slide lever to "off", and remove the tube (Table 1).

9. Pipette 20 uL of Solution D (digitonin solution) into the tube. Cover tube with Parafilm, and mix by inverting the tube four or five times.

10. Wipe the outside of the tube quickly with a Kimwipe, insert the tube into the tube port, replace cap, and slide the lever to "on". Immediately start the timer.

11. At the end of 60 s, record the "final reading", slide lever to "off", remove the tube (Table 1).

12. Repeat Steps 4 to 10 for each tube.

13. After completion of the assays, pour waste solutions into a flask labeled "EtBr - Digitonin Waste", and discard following hazardous waste disposal guidelines.

CALCULATION OF PERCENTAGE OF DAMAGED SPERM

The ratio of the initial/final reading multiplied by 100 gives the percentage of damaged sperm in the original semen sample.

% of damaged sperm = (initial reading) ÷ (final reading) x 100

Table 1. Work sheet (example)

			Readings			
Semen sample number/type	EtBr solution type	Tube/duplicate label	Initial (Step 8)	Final (Step 11)	% Damaged sperm (Initial/Final) x 100	Av % Damaged (Tubes 1 & 2)
#504 Fresh	EtBr	504F-1 EtBr	2.9	13.1	22	20.5
#504 Fresh	EtBr	504F-2 EtBr	2.8	14.6	19	
#504 Fresh	Stress	504F-1 Stress	3.8	18.7	20	20
#504 Fresh	Stress	504F-2 Stress	3.6	18.2	20	
#504 Stored	EtBr	504S-1 EtBr	3.7	13.3	28	27.5
#504 Stored	EtBr	504S-2 EtBr	3.5	12.9	27	
#504 Stored	Stress	504S-1 Stress	9.2	19.4	47	49
#504 Stored	Stress	504S-2 Stress	8.3	16.1	51	

REFERENCES

Bakst, M. R., H. C. Cecil, and T. J. Sexton, 1991. Modification of the ethidium bromide exclusion procedure for evaluation of turkey semen. Poult. Sci. 70:366-370.

Bilgili, S. F., and J. A. Renden. 1984. Fluorometric determination of avian spermatozoa viability and concentration. Poult. Sci. 63:2275-2277.

Bilgili, S. F., K. J. Sexton, and J. A. Renden. 1987. Fluorometry of poultry semen: Influence of dilution and storage on chicken spermatozoal viability and fertility. Poult. Sci. 66:2032-2035.

Chapter III. Sperm Viability

Section 3. Determination of Sperm Viability Using Fluorescence Microscopy

Ann M. Donoghue and Juan Manuel Blanco

PURPOSE

To determine the percentage of viable sperm in a semen sample using stains that differentiates viable (live) sperm from nonviable (dead) sperm. Viable sperm are detected by SYBR-14, which stains the sperm nuclei green. Nonviable sperm are detected by propidium iodide (PI), which stains the sperm red. The PI is not incorporated into live sperm with intact membranes.

MATERIALS AND METHODS

Materials

Eppendorf centrifuge tubes with caps, 1.5-mL capacity

Pipettor, 1-20 µL delivery and corresponding pipette tips

Pipettor, 1-5 mL delivery and corresponding pipette tips

Microscope slides and cover slips

Vials, lightproof

Nalgene filters, 0.45 µm size

Chemicals

NOTE: SYBR-14 and propidium iodide solutions are available as a kit; alternatively, stains can be ordered individually and stock solutions prepared. These stains are hazardous. Handle these and other chemicals and solutions carefully; wear appropriate protective clothing, and discard according to hazardous waste guidelines.

Semen diluent

Dimethysulfoxide, anhydrous (DMSO)

Live/Dead Sperm Viability Kit; Molecular Probes, #L-7011

or

SYBR® Green I nucleic acid gel stain; Sigma-Aldrich, #S-9430 (10,000X in DMSO)

Propidium iodide (PI)

Phosphate buffered saline (PBS)

Sterile distilled H_2O

Equipment

Balance (if preparing stock solutions)

Fluorescent microscope with fluorescein isothiocyanate (FITC) or triple band filter set

PREPARATION OF STAIN SOLUTIONS

1. For the Live/Dead Sperm Viability Kit from Molecular Probes:

    a. Dilute the SYBR 14 as recommended in the product insert (50-fold dilution of the SYBR stock solution using DMSO). Store the working solution of SYBR 14 in small aliquots (50-100 µL) in light-proof tubes in the freezer. Working aliquots can be thawed and re-frozen up to 6 times without affecting the SYBR 14 function.

    b. Propidium iodide in the kit does not require further dilution and can be stored at 4 ºC in the original vial. We find that for poultry sperm a higher concentration works better. Our recommendation is to double the amount of stain recommended in the product insert (see below for amount).

2. To prepare Sigma stock solutions:

    a. Add 2 µL of SYBR Green I (10,000xsolution) to 1 mL of filtered sterile PBS or physiological saline.

    b. Weigh and add 10 mg of PI to 2 mL of sterile water and filter through a 0.45 Nalgene filter.

    c. After preparation, store both stains in light-proof vials at 4ºC. Solutions are stable if kept in the dark and refrigerated.

PROCEDURE

1. Dilute semen to approximately 30 x 10^6 sperm/mL. Samples should be run in duplicate.

2. For the Live/Dead Sperm Viability Kit from Molecular Probes:

    a. Thaw an aliquot of SYBR 14.

    b. Add 0.5 mL of diluted semen for each sample to be measured into 1.5 mL Eppendorf centrifuge tubes.

    c. Pipette 2.5 µL SYBR and 5 µl of PI into each tube.

    d. Invert tubes to mix and incubate at room temperature for 15 min.

3. For the Sigma stock solutions:

    a. Place 20 µL of diluted semen for each sample to be measured into 1.5 mL Eppendorf centrifuge tubes.

b. Pipette 10 µL SYBR and 20 µl of PI into each tube.

c. Invert tubes to mix and incubate at room temperature for 15 min.

d. After 15 min, pipette 5-10 µL of stained sperm onto microscope slide, cover with cover slip, and examine with fluorescent microscope using a FITC or triple band filter set. Important: Stain will bleach under the fluorescent light so count immediately.

e. In several microscope fields, count all the red-stained (dead) and all the green stained (live) sperm in the field (Figure 1). Count a minimum of 100 sperm). The percentage of viable sperm is calculated as follows:

% viability = # green cells ÷ (# green cells + # red cells) x 100

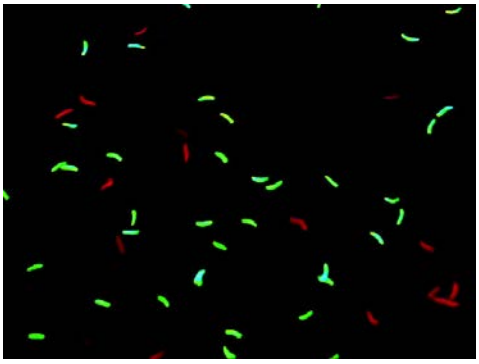


Figure 1. Poultry sperm demonstrating SYBR/PI live/dead stain results. Sperm nuclei of viable sperm are green; dead sperm stain red.

NOTE: For very low semen volumes such as some of the non-domestic species, place a 2 µL drop of PI solution on a microscope slide followed by the addition of a 1 µL drop of SYBR-green stock solution. A 2 µL drop of semen is then added to the drop of stains and gently mixed by pipetting in and out. After 2 min at room temperature, a cover-slip is placed on top and the sample immediately evaluated.

REFERENCES

Donoghue, A. M., D. L. Garner, D. J. Donoghue, and L. J. Johnson. 1995. Viability assessment of turkey sperm using fluorescent staining and flow cytometry. Poultry Sci. 74:1191-1200.

Chapter III. Sperm Viability

Section 4. Determination of Membrane Integrity of Sperm Using Fluorescent Stains and Hypo-Osmotic Stress

Ann M. Donoghue

PURPOSE

The integrity of the sperm plasma membrane before and/or after semen storage is an indicator of the viability of sperm. Sperm are placed in a hypo-osmotic solution containing fluorescent viability stains. Viable sperm with intact membranes swell due to the uptake of water and stain green with Calcein-AM, an esterase substrate that reacts with intracellular, nonspecific esterases. Conversely, sperm that are dead or have damaged membranes fail to swell, or burst after swelling and are stained red with propidium iodide (PI). The PI is not incorporated into viable sperm with intact membranes.

MATERIALS AND METHODS

Materials

Eppendorf centrifuge tubes with caps, 1 .5-mL capacity

Heat block, 37 °C

Pipettors, to deliver 0.2 µL to 1 mL and corresponding tips

Slides and cover-slips

Equipment

Balance

Fluorescence microscope with fluorescein isothiocyanate (FITC) or triple band filter set

Chemicals

(NOTE: Calcein-AM and propidium iodide are hazardous. Handle these and other chemicals and solutions carefully; wear appropriate protective clothing and discard according to hazardous waste guidelines.)

Calcein-AM

Propidium iodide (PI)

Dimethysulfoxide, anhydrous (DMSO)

PBS or semen extender

Deionized water (H_2O)

Reagents

Solution A: Calcein-AM Stock Solution (1 mg Calcein-AM/mL DMSO)

1. Weigh out 1 mg Calcein and place into Eppendorf centrifuge tube.
2. Add 1 mL of DMSO, cap and mix thoroughly by inverting tube several times.
3. Solution should be kept refrigerated and in the dark.

Solution B: Calcein-AM Working Solution (100 pg Calcein-AM/mL DMSO)

1. Place 90 µL DMSO into Eppendorph tube.
2. Add 10 µL of the Calcein stock solution, cap and mix thoroughly by inverting tube several times.
3. Solution is stable if kept in dark and refrigerated.
4. When pipetting working solution into semen, make sure to use clean tips so that the stain solution does not get contaminated.

Solution C: PI Stock Solution (4 mg PI/mL diluent)

1. Weigh out 4 mg of PI and add to 1 mL of semen diluent, cap, and mix thoroughly by inverting tube several times.
2. Solution is stable if kept dark and refrigerated.
3. When pipetting into semen, make sure to use clean tips so that the stain solution does not get contaminated.

PROCEDURE

1. Dilute turkey semen 1:1 in semen extender or PBS.
2. With a micropipettor, pipette 5 µL of diluted turkey semen into two tubes.
3. Add 1 mL PBS to one tube and 1 mL H_2O to the other to get 30 million sperm/mL. Samples should be run in duplicate.
4. Place 0.5 mL of diluted turkey semen (from Step 2) into four 1.5-mL Eppendorf centrifuge tubes for each treatment, two tubes for H_2O, and two tubes for PBS.
5. With a micropipettor, pipette 0.27 µL Calcein AM (Solution B) and 8.0 µL PI (Solution C) into each tube. When pipetting stains into semen, make sure to use clean tips so that the stain solution does not get contaminated.

6. Invert tubes to mix and incubate in heat block at 37 °C for 15 min.

7. Immediately after the 15-min incubation period, add 1 mL PBS to all tubes (both PBS and H_2O tubes).

8. Pipette 10 µL of sperm onto microscope slide, cover with cover slip, and examine with fluorescent microscope using a FITC or triple band filter set.

9. To determine viability: In PBS treatments, examine several microscope fields and count all the red-stained (dead) and all the green-stained (live) sperm (count at least 100 sperm). The percentage of viable sperm is calculated as follows:

   % viable sperm = # green cells ÷ (# green cells + # red cells) x 100

10. To determine membrane integrity: In H_2O treatments, examine several microscope fields and count all the red-stained (unswollen) and all the green-stained (swollen) sperm (count at least 100 sperm). The percentage of sperm with intact membranes is calculated as follows:

    % membrane intact sperm = # green cells ÷ (# green cells + # red cells) x 100

REFERENCES

Bakst, M. R., H. C. Cecil, and T. J. Sexton, 1991. Modification of the ethidium bromide exclusion procedure for evaluation of turkey semen. Poult. Sci. 70:366-370.

Donoghue, A. M., D. L. Garner, D. J. Donoghue, and L. J. Johnson, 1996. Flow cytometry assessment of the membrane integrity of fresh and stored turkey sperm using a combination of hypo-osmotic stress and fluorescent staining. Theriogenology 46:153-163.

Chapter IV. Sperm Motility and Metabolism

Section 1. Visual Scoring of Motility Using the Hanging Drop Method

Graham Wishart and Murray R. Bakst

PURPOSE

The hanging-drop method is a subjective method of estimating the motility of sperm in a semen sample. It is simple and fast and requires a minimal amount of equipment. However, it only can provide a crude estimate of sperm motility. The hanging-drop method is generally used with neat semen or semen diluted 1:1 with a semen diluent. When viewing a slide preparation at 20x or 40x magnification, one observes numerous clusters of swirling sperm. It is the vigor, or lack thereof, of "swirling" movement that one uses to 'score' sperm motility. In experienced hands it can be a useful monitor of sperm quality. However, unless individuals within and between laboratories are trained together with their scoring criteria there will be significant variation in the scoring data. In addition, statistical interpretation of such a scoring system is awkward. It is very important to standardize the conditions and timing for the whole procedure, as different temperatures, diluents (if used), and age of semen sample (time from collection) will affect the result (see Chapter IV.3).

MATERIALS AND METHODS

Materials

Standard microscope slides (76 x 26 mm) cover-slips (22 x 22 mm). Alternatively, use a well/depression slide with accompanying cover glass large enough to fully extend over the sides of the well.

Vaseline or similar material

Micropipettor (50-µL capacity)

Equipment

Microscope with 20x or 40x phase contrast objectives

PROCEDURE

1. Place a ring of Vaseline, about 15 mm diameter and 2 to 3 mm high, in the center of a microscope slide.

a. If using a well/depression slide, encircle the well's outside perimeter (on the slide surface) with a thin layer (~1mm) of Vaseline.

2. Make sure the semen sample is gently mixed before placing a 50-µL drop of semen in the middle of a cover-slip.

3. Carefully invert the cover-slip with the semen sample and place it on the Vaseline ring such that the semen drop is centered. Take care not to smudge the semen sample.

4. Place the slide preparation on the microscope stage and focus on the semen sample at the desired magnification (it is recommended that the semen sample size and magnification used be consistent)

5. Examine the nature of the movement of the semen sample for at least 15 s. Score motility as follows:

   a. 5: Motion is vigorous throughout the whole sample
   b. 4: Motion occurs throughout the sample but is less vigorous at the outer edge
   c. 3: The outer edge of the sample is not moving
   d. 2: More than half of the sample is not moving
   e. 1: There is activity only in the middle of the sample
   f. 0: There is virtually no motion

REFERENCES

Wheeler, N. C., and F. N. Andrews, 1943. The influence of season on semen production in the domestic fowl. Poultry Sci. 22:361-367.

Chapter IV. Sperm Motility and Metabolism

Section 2. Sperm Motility Analysis Using the Sperm Quality Analyzer*

Graham J. Wishart and Yvonne I. Wilson

Editors Note: Since 1997 when this chapter was written there have been many technical developments and advances in the automated characterization of sperm viability, numbers per sample, and motility. It is beyond the scope of this workbook to review each of these techniques. Briefly, more contemporary methods of assessing the sperm characteristics listed above rely on two forms of technology, signal processing and image analysis. 'Signal processing' may be due to fluctuations from light passing through a sample or subtle differences in the emission of fluorescence from stained sperm. 'Image processing' relies on the analysis of object shape and the rapid integration of a succession of captured images to arrive at an estimate of sperm morphology, motility, and viability (with the assumption that non-motile sperm are non-viable). (The computer-assisted image analysis system, which is not addressed in this workbook, falls into this category). This chapter appears nearly unchanged from its original publication in the first edition of this workbook and should be viewed as a more generalized explanation of the operation of a 'signal processing' technique for sperm motility and viability. The procedures described reflect SQA instruments available around 1996. Newer generation SQAs with more efficient processing times and improved resolution are commercially available. For a review comparing automated versus manual semen analysis techniques of human semen see Agarwal and Sharma (2007).

PURPOSE

The Sperm Quality Analyzer (SQA) used in estimating sperm motility can be interpreted as a "mechanized" version of the hanging drop method described in Chapter IV.1. In the SQA, the fluctuations of a beam of light passing through a diluted semen sample held in a capillary tube are measured over time and integrated to provide a number, known as the "sperm motility index" (SMI). A highly motile sample will have a high SMI and a poor motility sample will produce a low SMI.

MATERIALS AND METHODS

Materials

Fresh semen sample

Nalgene conical flask, 25-mL capacity

Polycarbonate tubes (e.g., 12 x 75 mm)

Chemicals

Sodium chloride (NaCl)

([N-tris] hydroxymethyl) methyl-2-aminomethane sulfonic acid (TES)

Equipment

Sperm Quality Analyzer (SQA) (The procedures that follow were based on a SQA purchased from Merck Ltd UK.)

Capillary units for SQA

Shaker or shaking water bath at "room temperature" (~22 ºC)

Reagents

0.15 M NaCI 20 mM TES in distilled water; made to pH 7.4 with 1 M NaOH (NaCI-TES)

PROCEDURE

1. Dilute 1 part semen with 3 parts NaCI-TES, place 2 to 5 mL in a flask and shake for 10 min.

2. Switch on SQA and allow to self test (2 min). The machine will then be ready for use.

3. Dilute the semen a further 1 in 4 with NaCI-TES (i.e., a total dilution of 1 in 16).

4. Load the sperm sample into a disposable capillary, ensuring that the sample passes the circular window.

5. Insert loaded capillary into the SQA and depress the red button. After approximately 40 sec the SMI will be displayed.

INTERPRETATION

The SMI units provide an arbitrary measure of sperm motility.

FURTHER COMMENTS

The method is simple to perform taking 2 to 3 min per sample. The SQA is relatively inexpensive, although the capillaries in their customized holder are costly if used only once, as recommended by the manufacturers. We have found that some recycling of these is possible. The SQA can be used with turkey sperm.

REFERENCES

Agarwal, A. and R. K. Sharma. 2007. Automation is the key to standardized semen analysis using the automated SQA-V quality analyzer. Fert. Steril. 87:156-162.

Bartov, B., A. Mayevsky, M. Sneider, L. Yogen, and A. Lightman, 1991. Sperm motility index: a new parameter for human sperm evaluation. Fertil. Steril. 56:108-112.

Chapter IV. Sperm Motility and Metabolism

Section 3. Sperm Mobility Determination – The Laboratory Method

David P. Froman

PURPOSE

Poultry sperm mobility is simply defined as the net movement of a sperm population against resistance at body temperature. While mobile sperm must be motile, not all motile sperm are mobile. This is the key distinction that renders sperm from a highly mobile population more likely to fertilize a succession of ova than sperm from a population exhibiting relatively poor mobility. It is suggested that to obtain maximum fertility, those males producing semen with the highest mobility ratings should be preferentially used in one's artificial insemination program. The sperm mobility assay can be performed with two similar but different approaches. The first and most simple to use is the commercial version described in the Appendix. Described below is a laboratory version, which also can be used for on-farm work as well. Adaptability is perhaps the only advantage with the laboratory version. For example, the Accudenz concentration can be adjusted, as has been done in some work with turkeys. However, there is potential for three unwelcome outcomes should such an adjustment be made. First, the overlaid sperm suspension must not mix with the Accudenz solution as this generates a false positive. Loading becomes progressively more difficult as Accudenz concentration is reduced. Second, if the incubation interval is too long relative to Accudenz concentration, then a larger number of sperm may enter the Accudenz layer even if the overlay is properly applied. Such an outcome can compromise stringency as the test subjects may appear better than they are. In other words, the power to discriminate among test subjects can be compromised by changing assay conditions, and there is no point in performing the assay if one cannot discriminate among test subjects. However, either version will yield reliable results if reagents are prepared properly and attention is given to detail while the assay is performed.

MATERIALS AND METHODS

Equipment

Rainin P-1000 Pipetman

Rainin P-100 Pipetman

Rainin MP-50 Pipetman

Rainin MP-250 Pipetman
Digital thermometer with thermocouple
Analytical balance
Osmometer
pH meter
Water bath
Stir plate with stir bar
Submersible cuvette rack
Repipet II dispenser
Spectrophotometer
Autoclave
Bunsen burner
Refrigerator
Calculator or lap-top computer
Bench-top timer

Materials

Pipet tips
Pipet pistons and sleeves for positive displacement pipets
Weigh boats
Stainless steel micro spoons
Kimwipes
Nalgene graduated cylinders
Nalgene or glass beakers
Osmometer standards
pH standards
Standard polystyrene
Semi-micro polystyrene cuvette
Parafilm
12 x 75 mm polypropylene culture tubes
Culture tube rack
Disposable 50-mL syringes
0.2 µm Acrodisc filters
8-mL screw-cap amber bottles

Chemicals

Deionized water

Accudenz (Accurate Chemical and Scientific Corporation, Westbury, NY)

N-tris-[hydroxymethyl]methyl-2-amino-ethanesulfonic acid (TES)

Sodium chloride (NaCl)

Potassium chloride (KCl)

Sodium hydroxide (NaOH)

Calcium chloride ($CaCl_2$)

Reagents

3% (wt/vol) NaCl

5 mM TES, pH 7.4, containing 3 mM KCl

30% (wt/vol) Accudenz prepared with 5 mM TES, pH 7.4, containing 3 mM KCl

50 mM TES, pH 7.4, containing 128 mM NaCl and 2 mM Ca^{2+} (TES-buffered saline)

Dilute TES-buffered saline (93% strength at 290 mOsm)

6% (wt/vol) Accudenz prepared with dilute TES-buffered saline

NOTE: A solution of 5 M NaOH is added drop-wise to adjust pH when buffered media are prepared. Do NOT prepare the 30% Accudenz stock solution by heating. The Accudenz powder should be added to a volume of buffered 3 mM KCl equivalent to approximately 40% of the final dissolved volume in a graduated cylinder. Thereafter, the solution is brought to volume with buffered 3 mM KCl. The Accudenz will dissolve in response to stirring. Another approach that works is sealing the cylinder with Parafilm and mixing the contents by repetitive inversion and righting the sealed cylinder. The osmolarity of the TES-buffered saline and the 6% (wt/vol) Accudenz should be between 300 and 315 mOsm. Both media can be cold-filtered into autoclaved screw-cap amber vials. If the rim of the vial is passed through a flame and the vial sealed with an autoclaved cap, then media can be stored in a refrigerator for an interval of weeks to months.

PROCEDURE

1. Warm reagents to 41º C in the water bath. One or more 8-mL vials can be submerged as needed. Check reagent temperature with a thermocouple and digital thermometer.

2. Place a row of empty polypropylene culture tubes and a row of empty semi-micro cuvettes with submersible rack in water bath (maximum = 5 vessels per row).

3. Once reagents are at body temperature, pipet 610 µL 6% Accudenz into each semi-micro cuvette. Lightly cover cuvette top with a small square of Parafilm.

4. Collect semen samples. If assay is to be done batch-wise in groups of 4, then collect 4 ejaculates.

5. Determine sperm concentration for each ejaculate. In each case, dispense a 2-mL volume of 3% NaCl into a standard polystyrene cuvette. Remove a 10-µL semen sample with a positive displacement pipette and extrude contents into saline by reflux action.

Push piston in and touch to Kimwipe (there is no need to use a new piston and sleeve for each ejaculate). Cover cuvette with Parafilm square, mix contents vigorously, tap cuvette on table top to clear air bubbles from the sperm suspension, and place cuvette into a spectrophotometer well. This instrument should have the wavelength set to 550 nm in advance and blanked with 3% NaCl (which is interchangeable with 6% Accudenz). Measure the absorbance, and estimate sperm concentration (x 10^9 per mL) by solving for *x* in the equation below.

observed absorbance = 0.0558 + (0.1212)(x)

NOTE: This standard curve was prepared by the author by the mixture of concentrated fowl sperm and fowl seminal plasma in different proportions in order to procure sperm concentrations ranging from 0.87 to 8.70 x 10^9 sperm per mL. (see Chapter II.2)

6. Estimate volume of TES-buffered saline required as follows:

   (observed conc)(0.050 mL) = (0.5 x 10^9 sperm per mL)(suspension volume);

   suspension volume – 0.050 mL semen sample = volume TES-buffered saline

7. Place calculated volume of pre-warmed TES-buffered saline into a 12 x 75 mm polypropylene culture tube, add the 0.050-mL volume of semen, and mix by finger vortexing.

8. Overlay a 60 µL volume of sperm suspension upon the pre-warmed Accudenz solution

9. Start timer. Add remaining samples at 1-min intervals. With practice, samples can be loaded on the minute if the sperm suspension is prepared about 30 seconds in advance and then overlaid upon the Accudenz solution.

10. Remove the first cuvette within a series after 5 min of incubation. Use a Kimwipe to gently remove water from the cuvette's exterior, place the dry cuvette in the spectrophometer well, and close the chamber. Read absorbance 1 minute after removing the cuvette from the water bath.

NOTE: In this fashion, a series of assays can be performed precisely. This approach greatly increases the number of assays that can be performed per hour. That said, attention must be paid to the ambient temperature because if it is either too cold or too warm, then semen quality can be compromised as ejaculates are held prior to performing the assay. In the author's experience, the semen quality of individual chicken ejaculates held in conical 15-ml polypropylene tubes (the vessels into which the author collects rooster ejaculates) will not be compromised at room temperature over a period ≤ 20 min. In general, it is best to make holding time negligible.

INTERPRETATION

As explained for the commercial assay (see Appendix 1), one's technique can be assessed by mixing the contents after a measurement is made and then taking a second reading. If there is negligible experimental error associated with determination of sperm concentration, preparing a sperm suspension containing 0.5 x 10^9 sperm per mL, and overlaying a constant volume of this suspension upon the Accudenz solution, then the coefficient of variation should be reasonably small. For example, consider the data in the table below from the author's experimental lines of

New Hampshire chickens. The CV for sperm mobility in this sample of ten males is enormous due to *biological* differences among males. In contrast, the CV for the second reading is only 6%. This value indicates that loading was reasonably precise. A second means by which the loading technique can be evaluated is as follows. Heat a pooled semen sample to 56º C for 10 minutes in order to render sperm immotile. Then perform a set of replicate assays. In this case, the Accudenz layer should appear clear and each reading should be near zero absorbance units after a 5-min incubation at body temperature. Otherwise, mixing may have occurred upon loading or the spectrophotometer's light beam is too close to the media interface within the cuvette.

Even though heat-treated sperm would be incapable of fertilization, note that a reading near zero with freshly ejaculated sperm does not necessarily mean that a male is infertile. It does mean, however, that such a male would be subfertile to males with higher mobility scores. For example, the low line male with an absorbance of 0.157 units in the table above would most likely be superior to all other low line males represented in the table. However, this same male would be subfertile to all high line males represented in the table.

In conclusion, the meaning of any given sperm mobility measurement depends upon several things. For example, one must ask if the assay was performed properly? Poor loading yields false positives. Such cuvettes will contain three zones after incubation: the overlay, an intermediate layer, and clear Accudenz solution near the bottom of the cuvette. In contrast, the Accudenz layer of a properly loaded cuvette, regardless of the number of mobile sperm therein, should appear homogeneous. Recall that straight line velocity is temperature-dependent and that the sperm mobility assay affords a context in which the *consequences* of variation in sperm velocity become evident in time. Consequently, false negatives can be generated if reagent temperature is not controlled.

A second question to consider is how an observation compares to those of other males within a flock? In this regard, the author has used the terms low, average, and high in a statistical sense when applied to random bred flocks. For example, *low* denotes those males whose values are less than one standard deviation from the population mean. It is noteworthy that population means vary among breeds and lines of poultry. In fact, it is likely that differences in male fertility among breeds of chickens are due to sperm mobility phenotype.

Line	Sperm Concentration (x 10^9 per mL)	Sperm Mobility (A_{550})	2nd Reading (A_{550})	Proportion of Mobile Sperm (%)
High	5.44	0.530	0.954	55.6
	3.98	0.434	0.906	47.9
	4.85	0.550	0.983	55.9
	4.87	0.376	0.888	42.3
	4.31	0.531	0.971	54.7
Low	5.16	0.003	0.935	0.3
	4.56	0.157	0.814	19.3
	5.26	0.070	0.925	7.6
	6.63	0.006	0.859	0.7
	5.72	0.024	0.976	2.5
		Mean = 0.268 SD = 0.2372 CV = 88.5%	Mean = 0.921 SD = 0.0551 CV = 6%	

SELECTED REFERENCES *

Donoghue AM, Holsberger DR, Evenson DP, Froman DP. 1998. Semen donor selection by in vitro sperm mobility increases fertility and sperm storage in the turkey hen. J. Androl. 19:295-301.

Froman DP, 2006. Application of the sperm mobility assay to primary broiler breeder stock. J. Applied Poult. Res. 15:280-286.

Froman DP, Feltmann AJ, 1998. Sperm mobility: A quantitative trait of the domestic fowl (*Gallus domesticus*). Biol. Reprod. 58:379-384.

Froman DP, McLean DJ, 1996. Objective measurement of sperm motility based upon sperm penetration of Accudenz®. Poult. Sci. 75:776-784.

* See Appendix 1 for a more comprehensive reference list.

Chapter V. Evaluation of Semen from Non-domestic Birds

George Gee, Juan Manuel Blanco and Ursula Höfle

PURPOSE

Aspects of poultry AI technology are applicable to non-domestic birds. However, modifications in the methods of semen collection, evaluation, and insemination are often necessary to accommodate the bird's size, semen volume, sperm numbers, or female anatomy. This section provides a brief overview of procedures used to evaluate semen from non-domestic birds. Unless otherwise stated, materials, reagents, etc., are identical to those used in evaluating poultry semen (see appropriate chapters).

SEMEN VOLUME

Small semen volumes and field conditions often make semen evaluation more difficult with non-domestic birds than with poultry. Operators should delay taking semen measurements until the birds respond to almost every collection and exhibit cooperative behavior. The small semen samples from non-domestic birds can be measured with a variety of pipettes and syringes. Calibrated disposable glass capillary tubes (20 to 100 µL) with suction control and microliter syringes (50 to 1,000 µL) attached to disposable capillary tubes may be used for measuring small (10 to 500 µL) semen samples. Blood cell diluting pipettes attached to microliter syringes work well when measuring semen volume and have the added advantage that the pooled samples can be held in the bulb part of the pipette. A "cut-off" barrel from a 1-mL syringe attached to a suction device works well when pooling ejaculates of 100 to 500 µL. The tip of any collecting device should be narrow to minimize dehydration and semen loss from the surface of the tube. A small tomcat catheter (126 mm or 5 in) attached to the Luer end of a 1-mL syringe works well to reduce surface tension and semen loss.

From all the choices listed above, we strongly recommend the use of 63 µL glass capillary tubes for semen collection from most non-domestic species, except very large species such as cranes. Because a microliter corresponds to a mm on the capillary tube, semen volume can be calculated simply by measuring with a caliper, preventing the need to transfer semen and incurring potential loss of valuable sperm through residual volume. In addition, some males ejaculate small amounts of semen with each cloacal stroke. These can be collected in different capillary tubes, which facilitate evaluation and separation of urate-contaminated samples from clean samples.

For larger species 0.25 mL French straws can be used in a similar manner. The main problem encountered with determining accurate semen volume is urate contamination, especially clear urine, which can artificially increase the collected volume. Use of multiple capillary tubes or French straws can help to alleviate this problem, so a ready supply of the collecting devices should be close at hand. Finally, each capillary can be quickly evaluated under the microscope so that contaminated samples can be excluded for AI purposes. Tubes should be always sterile to avoid bacterial or fungal contamination. During evaluation, capillary tubes need to be held in the horizontal position by resting on any semi-adhesive material that prevents accidents. Immediately after evaluation, semen from each capillary can be blown into a sterile Eppendorf tube using air from a sterile insulin syringe with a 0.25 – 0.45 µm filter attached.

SHORT-TERM SEMEN STORAGE

Sperm metabolic requirements vary between species with some being predominantly aerobic. Therefore it is preferable to store sperm in uncapped containers with some form of agitation. For small-volume pooled samples (e.g. 0.25 mL-0.5 mL) sterile Eppendorf tubes work well. For larger semen volumes, we use 0.5 mL blunt-end centrifugation tubes which provide a larger surface for gaseous exchange. When removing semen from the tubes, use the appropriate sterile pipette tips corresponding to final volume and gentle pippetting to ensure minimal loss through residual volumes. Do not use insulin syringes because of possible toxicity associated from contact with the rubber plunger tip.

Clean semen that is to be stored for a short period of time should be immediately diluted no more than 1:1 with the species-specific diluent (e.g. optimized for pH, osmolality, nutrients) containing the antibiotic and antifungal combination of choice and held at 4ºC to avoid bacterial growth. Avian sperm does not suffer the cold shock experienced by mammalian sperm but, in order to minimize lipid phase transition changes, the temperature can be progressively decreased by simply placing the Eppendorf in a refrigerator at 4ºC. Remember to save an appropriate volume of fresh undiluted semen at room temperature for further evaluation procedures, as outlined below.

ESTIMATING SPERM CONCENTRATION AND MOTILITY

There are three basic methods that can be used to determine sperm concentration. The choice of method depends upon the semen volume and available equipment. Use of a hemocytometer yields the most an accurate measure of sperm concentration and does not require a large volume of semen. For non-domestic species, sperm can be diluted 1:200 by mixing 2 µL semen with 400 µL of buffered 1% formalin solution. Thereafter, use the

hemoctyometer procedure described in Chapter II.1

A second option that allows for determination of both sperm concentration and motility is to use a plain (e.g. non-heparinized) microhematocrit or capillary tube. Collect a small quantity of undiluted semen in the tube by making a quick stab into an uncontaminated part of the semen in the collection device. Gently tap the end opposite the sample to move the semen to the middle of the capillary. Seal both ends of the tube with Critoseal (or other capillary tube sealant) and follow these steps:

1. Score sperm concentration as follows: 0 = no sperm; 1 = few sperm with large empty spaces; 2 = many sperm with moderate spacing; 3 = numerous sperm with little space between them; 4 =packed sperm, hard to detect individual sperm.

2. If a sample has been held at 4 ºC, allow sample to warm before scoring motility, because sperm are less active at lower temperatures. Using bright field microscopy at 10x, observe the sperm at the semen to air interface.

3. Score sperm progressive motility (percentage of sperm moving in a *forward* direction) as follows: 0 = no motile sperm; 1 = less than 25% motile; 2 = 25 to 49% motile; 3 = 50 to 74% motile; 4 = more than 75% motile.

4. Photomicrographs depicting concentration "scores" from species that are regularly evaluated will help standardize the evaluation procedure. Although these procedures are adequate for routine AI programs, better means of estimating sperm concentration and motility are necessary for research purposes.

It is best to inseminate with more than the recommended minimum number of sperm which, for example, in cranes is around 16 million sperm (Gee et *al.,* 1985). Generally, good fertility is possible with an average sperm concentration and motility score of 3. In some species, scores of 2 or less are acceptable if normal for the species (Gee et *al.,* 1993) and the entire ejaculate is used for AI.

The third procedure involves the use of a hematocrit centrifuge to estimate concentration through packed cell volume, as described in Chapter II.4. Unfortunately, semen volume and sperm concentration in non-domestic birds are generally too low to effectively use the spermatocrit procedure.

If the hemocytometer or spermatocrit procedures are used to determine sperm concentration, then the hanging drop technique can be used to evaluate sperm motility, as described in Chapter IV.1. For non-domestic species, pipette a 4 µL drop of fresh undiluted sperm onto a cover-slip and place it upside down over a depression-well slide at room temperature. Evaluation should be done immediately in the central area of the drop using bright field or phase contrast microscopy at 20x. For species like passerines with extremely high sperm concentrations, samples may need to be diluted from 1:1 to 1:3 to make evaluation possible.

Avian sperm motility is a parameter affected by many known and unknown factors making accurate evaluation a real challenge. For many species, simple refrigeration or dilution procedures render sperm temporarily non-motile; while these procedures are beneficial for sperm motility in other species. Therefore, the motility evaluation may need to be repeated after leaving the slide at 4ºC for 15 min for some species, or warming the microscope stage up to 40ºC for others. The percentage of sperm showing a progressive motility is a parameter of crucial interest. Highly sensitive motility evaluations can be conducted using computerized-assisted sperm tracking equipment, but these procedures may need to be standardized for each species. For example, the chamber size can adversely impact sperm motility from some species due to the limited oxygen availability.

SPERM MORPHOLOGY AND VIABILITY

Avian sperm morphology and viability can be evaluated simultaneously using the eosin-nigrosin stain. For scarce highly-valuable samples from endangered species, sperm morphology and concentration can be evaluated at the same time using the hemocytometer. The best viability stains are methylene blue or fluorescent DAPI nuclear staining procedures (Barna and Wishart, 2003) that allow clear nuclear definition and permanent smears for future re-evaluation. The live/dead fluorescence stain SYBR-PI also is reliable and compares favorably with the results of the nigrosin-eosin procedure; although, the former should be read immediately.

For most species, the percentage of viable sperm can be easily determined using the nigrosin-eosin stain which has the advantage of working with fixed smears so evaluation can be delayed. The extent of eosin uptake is not uniform for sperm of all non-domestic species (e.g. the budgerigard). To accommodate the extremely small semen volumes obtained from non-domestic birds, the following procedure is used to estimate sperm viability and morphology:

1. Place 1 drop (15 µL) of 5% eosin in upper right corner of a clean slide.
2. Place 3 drops of 10% nigrosin directly below the eosin.
3. Place 1 drop of semen to the left of the eosin.
4. Mix eosin and semen and allow to stand for 15 to 20 sec.
5. Mix eosin-semen with nigrosin for 15 to 20 sec.
6. Make a smear by dragging the edge of a second dry slide back from the left to the right to approach the "stained" semen drop. Hold the slide at a 30° angle to the semen. When the slide makes contact with the "stained" semen, the drop spreads across the edge of the slide. Quickly and uniformly smear the "stained" semen over the slide by pushing the slide to the left. In this manner, the "stained" semen drop is dragged behind the slide edge, thereby avoiding damage to the sperm.

7. Air dry the smear quickly to avoid artifacts. For uniformity, use a hair drier set on warm to speed the drying process. This can be especially important if the semen contains contaminants or cryoprotectants.

8. When dry, apply a mounting medium and mount a cover-slip over the smear to protect the sample. Once properly dried and mounted, the slides will remain unchanged for 2 wk or longer.

9. View the slide using an oil immersion objective and determine the live:dead ratio (unstained/ whitish sperm were alive when stained; while the stained, purple or red sperm were dead when stained).

The percentage of morphologically normal or abnormal sperm can also be estimated at the same time. The morphology evaluation should include the percentage of primary and secondary abnormalities, as well as defining and quantifying sperm populations based on nuclear size. The percentage of abnormal sperm, particularly secondary abnormalities, varies during the reproductive season. In particular, certain abnormalities such as the percentage of spermatogonia and spermatids are higher either early or late in the season, depending on age, health and pathological conditions. Photomicrographs of methylene blue-stained smears also are useful for sperm measurements, including acrosome, head, midpiece and tail length. Calculations can be easily carried out using digital image analyzers. Ideally, flow cytometry is the optimal method to evaluate and distinguish sperm populations. However, the typically small samples from endangered avian species become a limiting factor for this technique to be used on a regular basis.

Before determining what is normal or abnormal it is essential to study and establish reference values for each species. For example, falcons and kestrels normally show variable percentages of spermatogonia in their ejaculates, with levels usually higher both early and late into the season, and more often in young versus adult birds. While considered normal for these species, the presence of spermatogonia typically indicates failure to resume meiosis in other species. As another example, presence of cytoplasmic droplets in the ejaculate is considered very rare and abnormal in birds, only observed in senile or infertile males indicating the inability to resume spermatogenesis. Small percentages of certain abnormalities like micro-heads or giant sperm are considered normal in many species. In contrast, abnormalities like double heads, heads showing aberrant Y forms, or quadruple flagella are classified as very abnormal and often are related to high inbreeding coefficients. Before doing evaluations, one should be familiar with intra- and inter-specific species variation in sperm morphology.

SEMEN pH AND OSMOLALITY

For small semen volumes, pH can be determined with either pH paper or micro pH probes. Nowadays, there are field reliable pH meters in the market able to work with a 5 µL sperm sample which avoids the loss of valuable ejaculates when working with critically endangered species. For each species, semen pH falls into a normal narrow range. For example, semen pH is more alkaline for Psittacine species compared to raptors. Also, the pH of falcon semen is more acidic compared to eagles. Moreover, there is a species -specific wider pH range out of which 90% sperm remains immotile. This information is crucial when developing and optimizing diluents for each species, as well as for understanding how pH changes can influence motility. Most of the changes are commonly due to contamination mostly with urine that, due to its acidic nature, tend to decrease semen pH and damages sperm.

Avian semen has osmolalities ranging from 270 to 350 mOsm. Similarly to what is described for pH, there is a species-specific range where sperm gradually become non-motile. For osmolarity, a vapor point osmometer has been found to work best for accurate determinations. Theses devices require only 10 µL. Another technique is based on the cryogenic point but, although the equipment is cheaper, a larger sample is required. Avian sperm is, in general, more resistant to changes in osmolality compared to mammalian species. Knowing the normal semen osmolality is helpful in the development of specific diluents.

OTHER USEFUL ASSAYS

Evaluating membrane integrity is relatively easy to accomplish and can be done using different methods including the hypo-osmotic swelling test (See Chapter III.2). The principle of the test is identical to what used for mammals, but it requires further modifications. The main one is due to the fact that, with some exceptions, avian sperm can resist very low osmolality. It is essential to bring sperm to osmolalities as low as 50 mOsm. This cannot be obtained by just mixing sperm with a 50 mOsm hypotonic solution as semen osmolality increases the final osmolality. In order to avoid this, sperm need to be washed twice and held for an additional 30 min.

A description of species-specific mitochondria location and evaluation of mitochondrial activity can be added to the studies on sperm motility in a given sample. Two fluorescent probes: Rhodamin 123 and Mitotrack® can be successfully used in many species. Care should be taken as both location and staining patterns may show some variation between species.

Acrosome integrity can be effectively evaluated using PNA (peanut agglutinin), the more

reliable fluorescence probe for this purpose in avian sperm. Using this lectin, acrosome -intact sperm will not bind the lectin and thus can be distinguished from acrosome-reacted sperm which will bind the lectin. Finally, the use of the chicken inner perivitelline layer (IPL) assay (Chapter VIII.1) has been explored for some endangered avian species, but major modifications are required, and our experience indicates that some avian species unrelated to the *Galliformes* are unable to penetrate chicken IPL.

REFERENCES

Barna J. and G. J. Wishart. 2003. Excess nuclear DNA in spermatozoa of guinea fowl. Theriogenology. 59:1685-1691.

Blanco J.M. G. Gee, D. E. Wildt, and A. M. Donoghue. 2000. Species variation in osmotic, cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon spermatozoa. Biol Reprod. 63:1164-1171.

Blanco JM, J. A. Long, G. Gee, A. M. Donoghue, D. E. Wildt. 2008. Osmotic tolerance of avian spermatozoa: influence of time, temperature, cryoprotectant and membrane ion pump function on sperm viability. Cryobiology. 56:8-14.

Gee, G. F., 1995. Artificial insemination and cryopreservation of semen from nondomestic birds. Pages 262-279 in: Proceedings First International Symposium on the Artificial Insemination of Poultry. M. R. Bakst and G. J. Wishart, ed. The Poultry Science Association, Inc., Savoy, IL.

Gee, G. F. and S. A. Temple, 1978. Artificial insemination for breeding nondomestic birds. Symp. Zool. Soc. Lond. 43:51-72.

Gee, G. F., M. R. Bakst, and T. J. Sexton, 1985. Cryogenic preservation of semen from the greater sandhill crane. J. Wildl. Mgmt. 49:480-484.

Gee, G. F., C. A. Morell, J. C. Franson, and O. H. Pattee, 1993. Cryopreservation of American Kestrel semen with dimethylsulfoxide. J. Raptor Res. 27:21-25.

Chapter VI. Semen Cryopreservation

Section 1. Cryopreservation of Poultry Sperm Using Glycerol

Julie A. Long

PURPOSE

This is a general protocol for cryopreservation of poultry sperm using glycerol as the cryoprotectant. Although glycerol is considered to be contraceptive and must either be removed or the concentration reduced prior to insemination, this cryoprotectant is still considered the best option for freezing poultry sperm when straw packaging is desired. Both manual and programmable freezer options are presented in this chapter.

MATERIALS AND METHODS

Equipment and Supplies

Pipettor, 1 or 2 mL delivery, and corresponding pipette tips

Pipettor, 200 µL delivery, and corresponding pipette tips

Cryopreservation straws, 0.25 mL size

Sealing beads to fit 0.25 mL straws

Conical tubes, 12-15 mL size

Rack to hold conical tubes

Long-handled forceps, 2 pair

Cryogloves

Goggles

Rack to hold cryopreservation straws (horizontally)

Rubber tray suitable to hold 5-6” depth of liquid nitrogen

Canes or goblets for straw storage in nitrogen tank

Styrofoam box with lid (2 boxes if manual freeze)

2 Wire racks (7-10 cm tall) to fit in Styrofoam box (manual freeze only)

Chemicals

Lake’s pre-freeze diluent (see Appendix 2 for components)

Lake’s diluent with 33% glycerol (See Appendix 3 for components)

Liquid nitrogen

PROCEDURES

Semen Preparation and Cooling

1. Visually inspect all semen and only pool good quality samples for freezing. Do not use semen containing blood, fecal material or other contaminants.
2. Dilute pooled semen 1:1 with Lake's pre-freeze diluent (room temperature) and mix gently.
3. Place diluted semen in 4 ºC walk-in cold room or refrigerator and equilibrate for 10 min.
4. Add a volume of cold (4 ºC) Lake's diluent with 33% glycerol to yield a final concentration of 11% glycerol. For example, for an original semen volume of 1.5 mL, an equal volume of Lake's pre-freeze diluent (1.5 mL) will be used for the initial dilution followed by an addition of 1.5 mL of Lake's with 33% glycerol.

   <u>Important</u>: Add the Lake's with glycerol slowly and gently mix between each addition. A suggested rate for adding a total volume of 1.5 mL would be in 0.1 mL (or 100 µL) increments. After the final aliquot of cryodiluent is added, check to ensure that the sample is well-mixed. Often, the glycerol will sink to the bottom and not equilibrate properly.
5. Allow the semen/cryodiluent mixture to equilibrate at 4 ºC for 15 min before filling cold (4 ºC) straws.
6. Fill straws with 200 µL of semen using a pipettor, ensuring that the semen touches the cotton plug and that no air bubbles are introduced into the straw.
7. Seal each straw with a bead and place on horizontal rack.
8. Steps 4-7 must be conducted at 4 ºC. If a refrigerator is used, work inside as far as possible. Use a pre-cooled Styrofoam box to transport the racks holding semen straws for manual or programmable freezing.

Manual Semen Freezing*

1. Calibrate a Styrofoam box with cover by marking walls at 10 and 20 cm from the bottom of the box.
2. Place one rack in the box and fill with liquid nitrogen to the 10 cm mark. Place the second rack on top of the first, place the Styrofoam cover on, and allow 10 min to come to thermal equilibrium (temperature gradient ranges from ambient temperature at cover to -196 ºC at the liquid nitrogen surface).
3. After thermal equilibration, remove the cover from Styrofoam box and check that the level of liquid is still 10 cm from the bottom.
4. Place the filled cryopreservation straws on the top wire rack, and replace cover. (Goal is to freeze straws in the vapor at 10 cm above the liquid, or 20 cm.)
5. Leave straws on rack for 20 min and then plunge into liquid nitrogen.

6. Keep straws under liquid nitrogen all times while packing into canes or goblets for storage in the nitrogen tank. At most, straws can be out of the liquid for no more than 6 sec without adversely raising the temperature.

   **NOTE: See Chapter VI.3 for thawing methodology specific to glycerol-based cryoprotectants.*

Programmable Semen Freezing*

1. Pre-cool the freezer to 5 ºC.

2. Set the program for 2-stage freezing rates. The first rate will be to cool from +5 to -37 ºC at a rate of 7 ºC/min. The second rate will be to cool from -37 to -140 ºC at a rate of 20 ºC/min.

3. After the freezer has reached -140 ºC, open the door and quickly plunge the straw rack into liquid nitrogen.

4. Follow the guidelines in Step 6 above for manipulating straws.

   **NOTE: See Chapter VI.3 for thawing methodology specific to glycerol-based cryoprotectants.*

REFERENCES

Blesbois, E., F. Seigneurin, I. Grasseau, C, Limouzin, J. Besnard, D. Gourichon, G. Coquerelle, P. Rault and M. Tixier-Boichard. 2007. Semen cryopreservation for ex situ management of genetic diversity in chicken: creation of the French avian cryobank. Poult. Sci. 86:555-564.

Donoghue, A. M., and G. J. Wishart. 2000. Storage of poultry semen. Anim. Reprod. Sci. 62: 213-232.

Long, J. A., D. C. Bongalhardo, J. Pelaéz, S. Saxena, P. Settar, N. P. O'Sullivan, and J. E. Fulton. 2010. Rooster semen cryopreservation: Effect of pedigree line and male age on post-thaw sperm function. Poult. Sci, In Press.

Chapter VI. Semen Cryopreservation

Section 2. Cryopreservation of Rooster Sperm Using DMA

Julie A. Long

PURPOSE

This is a general protocol for cryopreservation of rooster sperm using dimethylacetamide (DMA) as the cryoprotectant. One advantage of using a DMA-based diluent is that the cryoprotectant does not have to be removed from thawed semen prior to insemination. Dimethylacetamide is most effective at very fast freezing rates achieved with the pellet method. Use of straw packaging in conjunction with DMA is still being investigated.

MATERIALS AND METHODS

Equipment and Supplies

Pipettor, 1 or 2 mL delivery, and corresponding pipette tips

Pipettor, 100-200 µL delivery, and corresponding pipette tips

Conical tubes, 12-15 mL size

Rack to hold conical tubes

Cryovials, 1 mL size

Long-handled forceps, 2 pair

Cryogloves

Goggles

Black rubber tray or bucket suitable to hold 12-15 cm depth of liquid nitrogen

Canes or goblets for cryovial storage in nitrogen tank

Water bath or beaker on hotplate

Chemicals

Lake's pre-freeze diluent (see Appendix 2 for components)

DMA

Liquid nitrogen

PROCEDURES

Semen Preparation and Cooling

1. Visually inspect all semen and only pool good quality samples for freezing. Do not use semen containing blood, fecal material or other contaminants.

2. Dilute pooled semen 1:1 with room temperature Lake's pre-freeze diluent and mix gently.

3. Place diluted semen in 4 ºC walk-in cold room or refrigerator and equilibrate for 10 min.

4. Put on a pair of latex gloves for the remaining steps of the freezing protocol. DMA is easily absorbed through the skin.

5. Add a volume of cold (4 ºC) DMA to yield a final concentration of 6% DMA. For example, for an original semen volume of 1.0 mL, an equal volume of Lake's pre-freeze diluent (1.0 mL) will be used for the initial dilution followed by an addition of 0.12 mL DMA.

   <u>Important</u>: DMA is slightly toxic to sperm cells; therefore do not add the DMA until the freezing apparatus is set up.

6. Allow the semen/cryodiluent mixture to equilibrate at 4 ºC for no longer than 1 min.

7. Diluted semen should not be allowed to re-warm to room temperature. If a refrigerator is used, work inside as far as possible. Set the freezing apparatus next to the refrigerator and work with one tube of diluted semen at a time.

Semen Freezing

1. Set up the freezing apparatus by filling the black rubber tray halfway with liquid nitrogen.

2. Set a 100-200 µL pipettor to deliver a 25 µL volume.

3. Keeping the pipette tip above the vapor, drop 25-µL aliquots directly into the liquid nitrogen. The semen will form a pellet and sink to the bottom. If the tip inadvertently freezes, discard the tip before retrieving another aliquot from the diluted semen pool.

4. After the semen has been pelleted, immerse cryovials into the liquid nitrogen.

5. Using forceps, pack 20-25 pellets per cryovial. Be sure to keep the bottom portion of the cryovial filled with liquid nitrogen so that there is liquid in the cryovial when capped.

6. The cryovials will tend to float after being capped and can be snapped onto canes or loaded into goblets for storage in the liquid nitrogen tank.

Semen Thawing

1. Semen can be thawed using either a water bath set to 60 ºC or a beaker on a hotplate set to the same temperature.

2. To thaw an entire vial of pellets, remove the cryovial from the storage tank and immediately plunge the vial into the water, vigorously moving the cryovial to ensure even thawing. This procedure will take about 1 min.

3. To thaw a few pellets at a time, place a rack in the water bath filled with the appropriate number of tubes and a volume of semen extender equivalent to a 1:1 dilution. For example, to thaw 5 pellets/tube, fill each tube with 125 µL of extender (25 µL/pellet x 5 pellets). Allow the tubes to equilibrate to 60 ºC.

4. Meanwhile, open the cryovial under liquid nitrogen and pour out the desired number of pellets. It is best to use a plastic spoon to scoop all the pellets up with some liquid nitrogen, and then very quickly add each pellet to the tube using forceps. The pellets will make a sizzling sound when dropped into the warm extender.

5. Semen should be inseminated within 15-30 min of thawing.

References

Tselutin K., F. Seigneurin and E. Blesbois. 1999. Comparison of cryoprotectants and methods of cryopreservation of fowl spermatozoa. Poult. Sci. 78:586-590.

Chapter VI. Semen Cryopreservation

Section 3. Glycerol Removal from Thawed Semen

Julie A. Long

PURPOSE

This is a protocol for removing glycerol from thawed poultry semen prior to insemination. The advantages of this method versus serial dilution include fewer dilutions and centrifugations and a more complete reduction in glycerol content. However, this protocol does require the use of a refrigerated centrifuge to maintain temperature during the long centrifugation. The following protocol is based on the original publication from McLean et al., (1998).

MATERIALS AND METHODS

Materials

Conical tubes, 12-15 mL size

Pipettor, 1-5 mL delivery, and corresponding pipette tips

Pipettor, 200 µL delivery, and corresponding pipette tips

Rack to hold conical tubes

Centrifuge, capable of 4 ºC temperature setting

Tubes for recovered sperm fraction

Chemicals

TES (2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid)

Potassium Chloride (KCl)

Sodium chloride (NaCl)

Accudenz (Accurate Chemical & Scientific Corporation; Westbury, NY)

Sodium hydroxide (NaOH)

PROCEDURES

Solution Preparation:

1. TES-KCL (5 mM TES; 3 mM KCl)

    a. Weigh 1.146 g TES
    b. Weigh 0.224 g KCL
    c. Add both to 1 L distilled H_20
    d. Adjust pH to 7.4 (w/10 N NaOH)

2. 30% Accudenz (wt/vol), using TES-KCl as solvent

    a. Weigh 3 g Accudenz
    b. Add to 10 mL TES-KCL = 30%
    c. Accudenz will clump when wet but will dissolve on a stir-plate.

3. TES Buffer (50 mM TES; 130 mM NaCl) (315 mmol/kg)

    a. Weigh 11.46 g TES
    b. Weigh 7.597 g NaCl
    c. Add both to 1 L distilled H_20
    d. Adjust pH to 7.4 (w/10 N NaOH)

4. 12% Accudenz, using TES buffer to dilute 30% Accudenz stock

    a. *Dilute TES buffer to 275 mmol/kg osmolarity* with distilled H_2O prior to preparing 12% solution.
    b. 12% = 2.5 dilution of 30% accudenz stock
    Examples:
        1 mL 30% + 1.5 mL TES buffer = 2.5 mL 12% Accudenz
        10 mL 30% + 15 mL TES buffer = 25 mL 12% Accudenz
        20 mL 30% + 30 mL TES buffer = 50 mL 12% Accudenz

Thawing Semen

1. Prepare ice-water slurry at 4 ºC.

2. Immerse straws completely under the surface of the ice-water bath for 5 min.

3. Straws will be encased in an ice-sheath when first removed from the bath.

    Important: Use a dry paper towel to wipe the ice-sheath and any water on the straw before cutting the straw. Direct contact with water will damage sperm cells.

4. Hold the straw over the collection tube and cut the beaded end. Most of the thawed semen will flow into the tube. Cut the cotton plug end to release any semen remaining within the straw.

Gradient Preparation

1. Bring all solutions to room temperature.

2. Place 5 mL of the 12% Accudenz gradient in a 12 mL conical tube.

3. Layer 0.5 mL of the 30% Accudenz gradient *underneath* the 12% layer.

4. Maintain gradient tubes upright once set up.

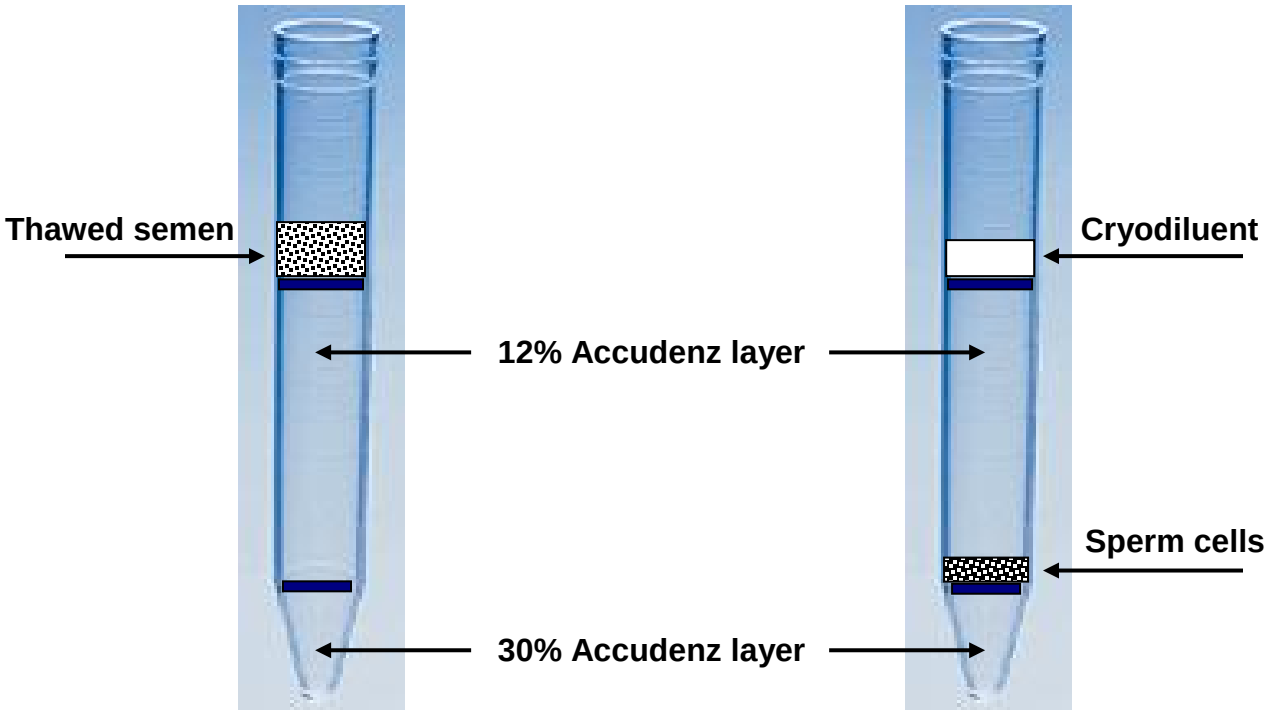
5. Layer 1-2 mL of thawed semen on top of the 12% layer (volume greater than 2 mL will exceed the separation capacity of the gradient).

Gradient Centrifugation

1. Pre-chill refrigerated centrifuge to 4 ºC.
2. Place gradients in swinging bucket rotor.
3. Centrifuge at 1,250 x g for 25 min.
4. *Do not use the brake feature.*

Sperm recovery

1. Sperm will be located at the interface between the 12% and 30% layers; sperm cells are unable to penetrate the 30% layer (see figure below).
2. Remove the cryodiluent layer and discard.
3. Remove the 12% layer and discard.
4. Remove the 30% layer underneath the sperm layer.
5. Transfer the sperm into a fresh tube and extend as needed for insemination.



Loaded Accudenz gradient before centrifugation.

Accudenz gradient after centrifugation.

REFERENCES

McLean D. J., A. J. Feltmann and D. P. Froman. 1998. Transfer of sperm into a chemically defined environment by centrifugation through 12% (wt/vol) accudenz. Poult. Sci. 77:163-168.

Hammerstedt, R. H., and J. K. Graham. 1992. Cryopreservation of poultry sperm: the enigma of glycerol. Cryobiology 29:26-28.

Long, J. A., and G. Kulkarni. 2004. An effective method for improving the fertility of glycerol-exposed poultry semen. Poult. Sci. 83:1594-601.

Chapter VII. Predicting Fertility

Section 1.: Breakout Fertility

Jeanna L. Wilson

PURPOSE

Egg breakout is used to provide an estimate of flock fertility and hatchery efficiency. There are three types of egg breakout procedures:

1) Fresh, unincubated egg breakouts are performed when an estimate of true fertility needs to be determined as quickly as possible;

2) Breakouts of clear and early dead embryos removed during candling at 7 to 14 days of incubation gives the most accurate estimate of flock fertility;

3) Breakout and/or examination of hatch residues gives the most complete information as to when embryos died during incubation as well as indications of incubator equipment function.

PROCEDURES

Fresh Egg Breakout – Estimating True Fertility

1. Puncture the large end of the egg and remove enough of the egg shell and membranes to create a 1 to 1.5 inch (2.5 to 4.0 cm) diameter opening.

2. Discard a portion of the albumen and roll the yolk inside the shell or in the palm of the hand until the germinal disc (GD) is located. (The GD is also referred to as the blastodisc in unfertilized eggs and blastoderm in fertile eggs).

3. Determine if egg was fertilized by visual inspection. The unfertilized GD appear as a single small white mass on the surface of the yolk while the fertilized GD appears as a double ring and larger white disk on the surface of the egg yolk. (See Chapter VII.2 for more detailed descriptions of the fertilized and unfertilized GDs and pre-incubation early dead embryos.)

4. Record total number of eggs examined and number of fertilized eggs. To improve accuracy a 100 egg sample per flock should be used for fresh eggs estimates of fertility.

5. Calculate estimated flock true fertility as follows:

    a. estimated % flock true fertility = (# fertile eggs) ÷ (# eggs examined) x 100

Candled Egg Breakout

1. Candle chicken eggs after 7 to 12 days, turkey eggs after 10 to 14 days of incubation.

2. Apply a candling light or bright flash light to the blunt end of an egg in a dark room. If no embryo or blood vessels show through the shell, the egg is either infertile or has an early dead embryo. Remove from the incubation tray and place on a filler flat large end up. For each flock, candle 600-800 eggs and remove all suspected unfertilized GDs or early dead eggs.

3. Puncture the large end of all eggs removed during candling. Peel the shell open taking care not to disrupt the fragile embryonic mass and yolk membrane. If no blood or embryo is visible, the egg could contain a pre-blood early dead embryo or is infertile.

4. If the GD is not visible rotate the yolk until it is visible. The membrane around the yolk is easier to disrupt after incubation than when in the fresh state and must be carefully handled. Dead embryos often adhere to the shell when the egg is rotated.

5. Classify the stage of development as follows:

    a. Unfertilized: As with the fresh egg breakout, the GD of an unfertilized GD will be very distinct. In addition, the yolk of unfertilized eggs will often have the same bright yellow color that was observed in the unincubated egg.

    b. Early Dead embryos: The GD of early dead embryos will be larger and less distinct than that of an unfertilized GD. The yolk may have a mottled appearance and may appear paler compared to the yolk with an unfertilized GD. The first-week dead embryos will have a blood ring or a network of blood vessels in graded stages of development that are visible through the shell compared to live embryos which will be at a more advanced stage of development. Dead embryos and associated blood vessels will have a darker red color than the live embryos due to pigment oxidation.

6. On a tally sheet (example in Table 1) record the number of eggs candled, unfertilized GDs, early dead embryos, cracked eggs, and those set small-end-up.

7. Calculations:

    a. % "candling" fertility = (# eggs with viable embryos) ÷ (# eggs examined) x 100

    b. % early dead embryos = (# early deads) ÷ (# eggs examined) x 100

    c. % estimate fertility = (# fertile eggs + # early deads) ÷ (# eggs examined) x 100

Residual Egg Breakout

1. Once or twice monthly, a sample of hatch residue from every flock should be analyzed. Collect unhatched eggs (pipped and unpipped) and cull chicks/poults from at least four trays from each flock.

2. Record the number of cull chicks/poults and pipped eggs on a tally sheet (Table 2).

3. Open the large end of the egg and record the stage of development (unfertilized; early dead embryo, 1-7 d; middle dead embryo, 8-14 d in the chicken and 8-21 d in the poult; or late dead embryo, 15-21 d in the chicken and 22-28 d in the poult) See Table 3.

Unfertilized GD: The unfertilized GD observed during residual breakout will be similar in appearance to the unfertilized GD observed during candling. The GD will be distinct and intensely white, but have an unorganized appearance. The yolk will be bright yellow in color.

Early Dead Embryos: Early dead embryos (dead prior to 24 hr of incubation) are more difficult to distinguish from unfertilized GD after a full term of incubation than those identified at 10 to 15 days of incubation in a candle analysis. The best criteria to rely on are the appearance of the GD, which should always be evaluated, and yolk color. After 21 or 28 day of incubation, early dead embryos that died prior to blood formation are easily confused with unfertilized GDs.

Staging of Embryos: Morphological characteristics associated with embryonic development are shown in Table 3 for the chick and poult. Use this information to estimate the age of the embryo at death.

Contaminated Embryos: Contaminated embryos have a black or deep red color and a distinctive 'off-odor' when the egg is opened. Note that middle dead embryos lacking good feather cover, but having a dark black color should not be classified as "contaminated" unless there is a distinctive 'off-odor' when the egg is opened.

REFERENCES

Bauer, F., S. G. Tullett, and H. R. Wilson, 1990. Effects of setting eggs small end up on hatchability and posthatching performance of broilers. Brit. Poult. Sci. 31:715-724.

Brake, J. T., 1989. Hatchability of abnormal broiler breeder eggs. Poultry-Misset January, pages 24-25.

Cherms, F. L., 1980. Some causes of embryonic mortality in turkeys. Feedstuffs 52(12):45-47.

Krueger, K. K., 1993. Embryo mortality patterns in commercial crossbred parent stock turkeys. Proceedings of the Third International Symposium on Turkey Reproduction, pages 70-74.

Mauldin, J. M., and R. J. Buhr, 1990. Recognize problems in embryonic development. Poultry - Misset Dec 89/Jan 90, pages. 20-21.

Phillips, R. W., and C. S. Williams, 1944. External morphology of the turkey during the incubation period. Poult. Sci. 23:270-277.

Wilson, H. R., 1991. Crack your hatchability problems. International Hatchery Practice 6:29-39.

Table 1. 7-14 Day candling and break-out analysis

DATE **FLOCK #** **AGE (weeks)**							
BREED **(male)** **(female)** **# EGG** **SET** **SETTER #**							
Tray #	**# Eggs per tray**	**Unfertilized**	**Early Dead**	**Middle Dead**	**Farm Cracks**	**Small End Up**	**Cull Eggs**
TOTALS							
PERCENT							

Comments: Fertility

Table 2. Residue breakout-analysis

DATE			**FLOCK #**								**AGE (weeks)**
BREED											
(male)		**(female)**									**% EGG PRODUCTION**
# SET			**ACTUAL HATCH %**					**SETTER #**			**HATCHER #**
Eggs/Tray #	Infert	Dead Embryos			Pip	Cull Chick	Cracks		Cont	Cull Eggs	Small End Up
		1-7	8-14	15-21			Farm	Tran			
TOTALS											
PERCENT											
LIFE OF FLOCK GOALS	6-7	2.5-3.5	0.5	2.75-4.0	1	0.5-1.0	0.5	0.3	0.5	0.5-1.0	1

Hatchability Efficiency = Fertility - Hatchability = _____ - _____ = _____ Goal 9.0

Hatchery Efficiency = 100 - (embryonic deaths 15-21 days + pips + cull chicks + transfer cracks)

= 100 - (_____ + _____ + _____ + _____) = _____

Table 3. Embryo development[1] (Phillips *et al*., 1990)

DAY	CHICK	POULT
1	Primitive streak, somite	Primitive streak
2	Heart beat, blood circulation	Four somites
3	Amnion encircles embryo, embryo on left side	Blood islands visible, no heart beat or blood circulation embryo begins movement to left side
4	Eye pigment, leg but larger than wing	Amnion covers embryo, heart beat, blood circulation
5	Joints appear on legs and wings	Eye pigmentation, wing and leg buds present
6	Beak and toe formation	Wing appendages gain joint
7	Comb and egg tooth appear	Wing appendages slightly shorter than leg
8	Prominent feather tracts	Beak present, leg appendages twice the length of wings
9	Bird like appearance	Egg tooth present
10	Toe nails appear	Feather tracts visible on back
11	Comb serrated, tail feathers	Feather tracts on back, tail and thighs, separation of toes
12	Eye lids almost closed	Eyes partially closed, toe nails form
13	Overlapping scales on legs, down cover	Feathers on back, tail and thigh, snood appears
14	Embryo aligned with long axis	Feather growth, and more coverage
15	Small intestines taken into abdomen	Feather growth
16	Feather cover	Eyes closed, scales on legs
17	Head between legs	Complete feather cover
18	Head under right wing	Ear openings hidden by feathers
19	Amnionic fluid disappears, yolk sac half withdrawn	Nostrils are open
20	Yolk sac drawn into body, beak pips through membrane	Whitish deposits on feathers
21	Shell is pipped, chicks hatch	Whitish deposits leave feathers, attach to shell
22		Embryo appears as a newly hatched poult
23		Yolk begins to enter body
24		Half of yolk sac in body
25		Three-fourths of yolk sac in body, amnionic fluid is swallowed by embryo
26		Yolk sac in body, beak penetrates air cell membrane
27		Embryo pips shell
28		Poult hatches

[1] Used with permission from *Poultry Science*.

Table 4. Fertility and hatchability of broiler breeder eggs[1] (Brake, J. T., 1989)

TYPE OF EGG	FERTILITY (%)	HATCH (%)
Normal	92	74
Slightly misshaped	87	65
Slightly rounded	88	63
Small	83	62
White	89	49
Round	90	48
Very pimpled	83	19
Very wrinkled	78	13
Rough, dark top	87	8

[1] Used with permission from *Poultry Science*.

Chapter VII. Predicting Fertility

Section 2. Visual and Microscopic Approaches for Differentiating Unfertilized Germinal Discs and Early Dead Embryos from Pre-Incubated Blastoderms

Murray R. Bakst

PURPOSE

The ability to correctly differentiate fertilized from unfertilized germinal discs (GD) and fertilized but dead pre-incubation embryos could assist in determining the basis of decreased hatchability. This chapter will review the visual appearance and differentiating features of the fertilized and unfertilized GD, and the dead pre-incubation embryo. It will also describe how to isolate and stage poultry embryos from fresh or stored eggs prior to their incubation. 'Normal-tables' for different species provide a pictorial sequence of discrete, identifiable stages of embryo development that are used to provide objective, consistent and repeatable observations of embryo development within and between laboratories. With poultry, normal-tables are available for chicken, turkey, duck embryos, and quail embryos. While staging can be done by any trained individual, it is usually left to research laboratory personnel and not hatchery personnel primarily due to the substantial time investment in the preparation of each embryo. Notwithstanding, the ability to precisely identify a stage of embryo development extends to such activities as: defining normal versus abnormal embryo development; differentiating the fertilized from unfertilized GD and fertilized but dead pre-incubation embryos; evaluating the impact of egg handling and egg-storage conditions on pre-incubation development; evaluating the impact of hen age, strain, oviposition time, and shell quality relative to blastoderm development after oviposition; and, determining the stage-specific expression of genes and their products relative to embryo development and survival. Finally, to better appreciate the application of this chapter, refer to Chapter VII.1 in this text.

MATERIALS AND METHODS

Materials

Eggs

Filter paper, Whatman No.4 (used to make filter rings)

Paper-hole puncher

Fine forceps or fine rat's tooth forceps

Scissors (medium-sized)

Iris scissors (small)

Hair loop

(NOTE: To make a hair loop, start with a hair at about 5 cm long; secure one end of the hair against the tip of a wooden applicator stick; take the other end of the hair and bend it around 360^0 creating a small 'hair-loop' about 2 mm long extending above the tip of a wooden applicator stick; use tape or glue to stabilize the loop)

Petri dishes

Pasteur pipettes

Volumetric flask, 1-L capacity

Equipment

Balance

Stereomicroscope with transmitted light

Gooseneck fiber optic light source

Chemicals

Sodium chloride (NaCl), M.W. 58.44

Reagents

Physiological saline (0.9% NaCl)

1. Weigh out 9 g NaCl and transfer to a 1-L volumetric flask.
2. Add distilled water to volumetric flask to bring volume to 1 L.

PROCEDURES

Visually differentiating fertilized from unfertilized germinal discs (GD) and early dead embryos:

1. Preparation of the Egg
    a. Using medium scissors, hold the egg blunt end up and cut about 5mm above the equator (midline) of the egg. Care must be taken not to puncture the yolk (when cutting run the tip of the scissor just along the inside surface of the egg shell).

    b. Remove albumen by gently tipping the half of the shell containing the egg contents.

    c. Using medium scissors, cut-off more shell until it is at the same level as the top of the yolk.

    d. Drain off the remaining albumen.

        NOTE: Removal of as much albumen as possible is important for the filter ring to adhere strongly to the perivitelline layer (PL, an acellular investment surrounding the yolk). If a 'reflective' layer, rather than a more flat, muted layer of albumen remains overlying the GD, gently place a Kimwipe over the GD and slowly lift to remove the albumen.

e. Alternatively, follow steps 1 and 2 under Procedures - Fresh Egg Breakout described in Chapter VII.1.

2. Visual and Microscopic Anatomy of the GD from a Fresh Oviposited Egg

a. Using a gooseneck fiber optic light, adjust the light beam so that it is nearly parallel to the surface of the ovum (oblique illumination). For more details on the appearance of the blastoderm and unfertilized GD, see Bakst et al., (1998) and Sellier et al., (2006).

b. Descriptions are based on both observations with no added magnification and with a stereomicroscope:

i. The chicken blastoderm (Figure 1), which appears as a symmetrical, round disc about 3-4 mm in diameter (the chicken blastoderm is larger than the turkeys) possessing a uniformly lighter central region (*Area pellucida*) and a denser band around its perimeter (*Area opaca*) (See Sellier et al., 2006 for a comparison of blastoderms from different poultry species). Figure 3 is a stereomicrograph of a Stage XI chicken blastoderm isolated by the filter-ring technique described in the next section. **B**lastodermal cells are exposed in the *Area pellucida* (AP) but not in the *Area opaca* (AO) because of the yolk spheres tenaciously adhering to the overlying blastodermal cells.

ii. The unfertilized GD (blastodisc), when compared to the blastoderm, is smaller in diameter/size, circular or asymmetrical, more dense, and possesses no regional differentiation (Figure 2). Using a stereomicroscope with oblique illumination reveals a variable number of vacuoles surrounded by white yolk, a prominent feature of the unfertilized GD (Figure 4).

NOTE: Examining commercial table eggs and studying the appearance of the unfertilized GD will help in identifying the unfertilized GD during fresh egg break-outs.

iii. Dead embryos at the time of lay are difficult to distinguish and do require magnification for accurate identification. Using a stereomicroscope with oblique illumination, the fertilized GD that failed to develop in the oviduct may possess a cluster of cells surrounded by white yolk and vacuoles. Blastoderms that fail to develop after oviposition appear to have more organization than an unfertilized GD. They may have an irregular, round to oval appearance but lacks a definitive *Area opaca*.

iv. If differentiating an unfertilized GD from an early dead is critical, the following procedure can be used:

a. Aspirate the GD contents (mostly white yolk and possibly embryo cells) into a fine-tip disposable pipette containing about 30 µL of bisbenzimide (Hoechst – 33342, a nuclear fluorescence stain).

b. Express the contents into a small vial containing another 30 µL of the staining solution.

c. Pipette up-and-down gently for 20 seconds and let sit for another 30 seconds.

d. Transfer 15µL of the mixture to a glass slide, cover-slip, and view at 20x or 40x with a fluorescence microscope.

e. Bisbenzimide will intensely stain blastodermal cell nuclei (live or dead, See Figure 5) while imparting a light blue hue to the yolk granules (Figure 5).

Blastoderm Isolation for Staging or Manipulation

1. Preparation of the filter rings with a 5-6 mm inside diameter outside diameter of about 10mm.

    a. Punch about 20, 6 mm holes about 2 mm inside the edge of the filter paper with a space of about 20 mm between hole punches.

    b. Cut around each hole leaving a margin of about 2 mm to get filter rings of desired diameter.

    c. Store supply of filter rings in a clean container.

2. Record any other pertinent egg data (treatments, dates, etc.).

3. Preparation of the egg:

    a. Follow steps a. through c. listed under subheading *Visually differentiating fertilized from unfertilized germinal discs (GD) and early dead embryos*.

    b. Drain off the remaining albumen.

        NOTE: Removal of as much albumen as possible is important for the filter ring to adhere strongly to the perivitelline layer (PL, an acellular investment surrounding the yolk). If a 'reflective' layer (thicker albumen), rather than a more flat, muted layer of thinner albumen remains overlying the GD, gently place a Kimwipe over the GD and slowly lift to blot-off the extra albumen.

4. Placing the filter ring:

    a. Rotate yolk gently with your finger tips to bring the GD into view.

    b. Using the approach described above for the visual inspection of the GD, determine whether the GD is fertilized, unfertilized, or an early dead embryo.

    c. With fine forceps, grasp the edge of the filter ring and bring it near to the blastoderm

    d. Look for the blastoderm through the center of the filter ring and carefully place the ring so that the blastoderm is located in the center of the ring. (The filter ring must be placed correctly the first time, as it is very difficult to relocate the ring without disturbing the blastoderm).

5. Isolating the blastoderm:

    a. Fill about 30% of a Petri dish with physiological saline (any balanced salt solution or most cell culture media may be used).

b. Using fine a scissor cut the PL completely around the outer edge of the filter ring.
c. Using iris forceps clasp the filter ring on one side with the forceps and lift the ring gently as if making the ring stand vertical on the opposite edge. Some yolk will flow down.

NOTE: If the blastoderm does not adhere to the filter ring at this stage, most likely there is too much albumen adhered to the PL. If this problem persists, gently dab off the albumen overlying the blastoderm with the tip of a Kimwipe.

d. Submerge the filter ring with the adherent blastoderm (yolk surface up) by slowly sliding it into the saline solution.

6. Examination of the blastoderm:

a. Place the Petri dish on the stage of the stereomicroscope illuminated with a gooseneck fiber optic light positioned at a 30°angle to the stage.

b. Remove adhering yolk by pressing down on one side of the filter ring and blowing gentle streams of saline by a dropper or Pasteur pipette across the outer edge of the blastoderm (this is most effective with eggs stored les than 4-5 days).

c. Clean the ventral side (yolk side) of the blastoderm by using a fine hair loop to tease away the yolk until the blastodermal cells forming the *Area pellucida* and the denser *Area opaca* are visible (Figure 3).

d. Once completely exposed, the blastoderm can be staged and further manipulated.

7. If the blastoderm is to be cultured or dispersed, isolate it from the PL using a hair loop and transfer to another dish using a transfer pipette with its tip cut to an inside diameter of about 3mm. The isolated blastoderm may curl or fold.

8. If the blastoderm is to be used for microscopy, drop-wise add a fixative of choice directly over the blastoderm to minimize excessive curling and folding. After about 30 seconds, use the hair-loop to tease the blastoderm off the PL. With the minor fixation, the blastoderm should remain as a relatively flat disc.

REFERENCES

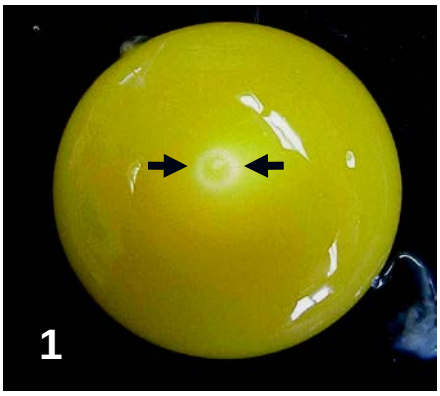
Bakst, M. R., S. K. Gupta, W. Potts, and V. A. Akuffo. 1998. Gross appearance of the turkey blastoderm at oviposition. Poult. Sci. 77:1228-1233.

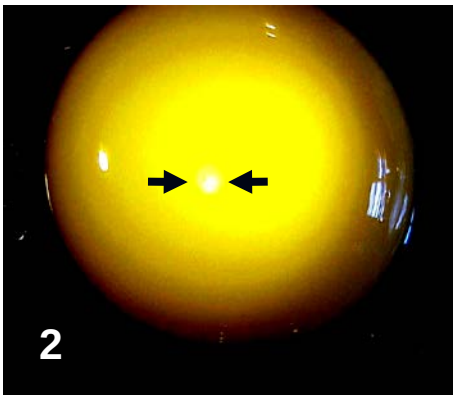
Sellier, N., J.-P. Brillard, V. Dupuy, M. R. Bakst. 2006. Comparative staging of embryo development in chicken, turkey, duck, goose, guinea fowl and Japanese quail assessed from five hours after fertilization through seventy-two hours of incubation. J. Appl. Poult. Res. 15:219-228.

Figure 1. This chicken blastoderm (between the arrows) has the characteristic appearance of Stage X embryo. The blastoderm consists of an outer whitish band, the *Area opaca* enveloping a thinner, less dense *Area pellucida*.

Figure 2. This unfertilized GD (between the arrows, chicken) is nearly indistinguishable from a fertilized but dead GD. It is smaller, more dense, and lacks any further differentiation when compared to a blastoderm. Occasionally vacuoles can be observed scattered in its white yolk.

Figure 3 A blastoderm from a chicken egg that was stored for 9 days was isolated by the filter-ring technique (FP, filter paper). The yolk underlying the blastoderm was cleared away using a hair-loop. The blastodermal cells are exposed in the *Area pellucida* (AP) but not in the *Area opaca* (AO) because of yolk spheres tenaciously adhering to the overlying blastodermal cells. The diameter of the blastoderm (arrow to arrow) is 4 mm.





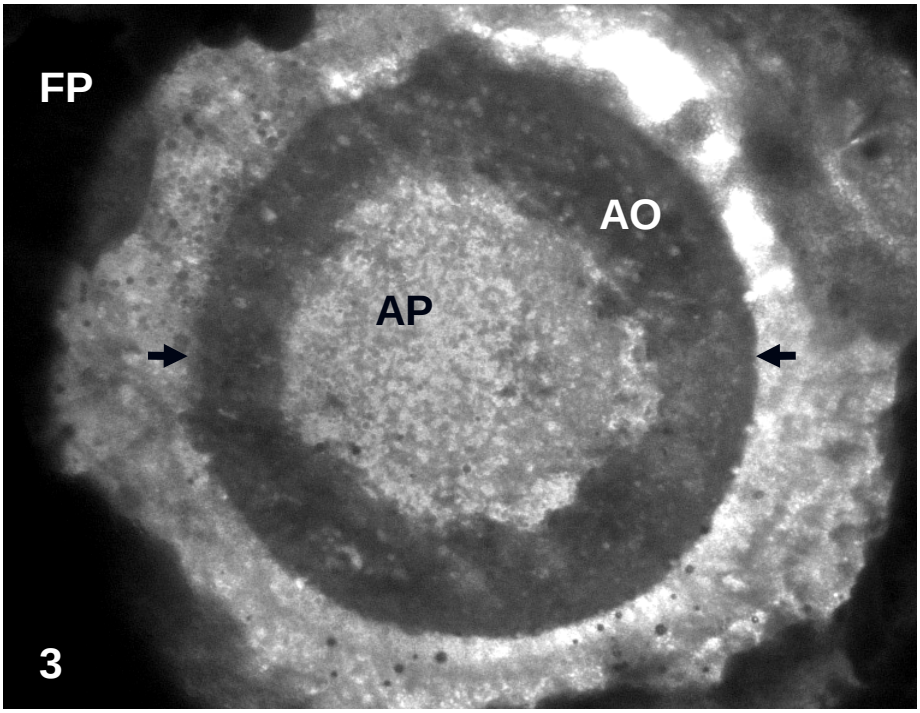


Figure 4. An unfertilized germinal disc (chicken) about 2.5 mm in diameter is characterized by numerous vacuoles that often can be observed without magnification using oblique illumination.

Figure 5. The early dead chicken embryo (either fresh or from a stored egg) is not easy to identify and can vary in appearance. This dead embryo is characterized by numerous vacuoles surrounding a cluster of relatively large cells (arrows). The egg was stored 9 days before breakout.

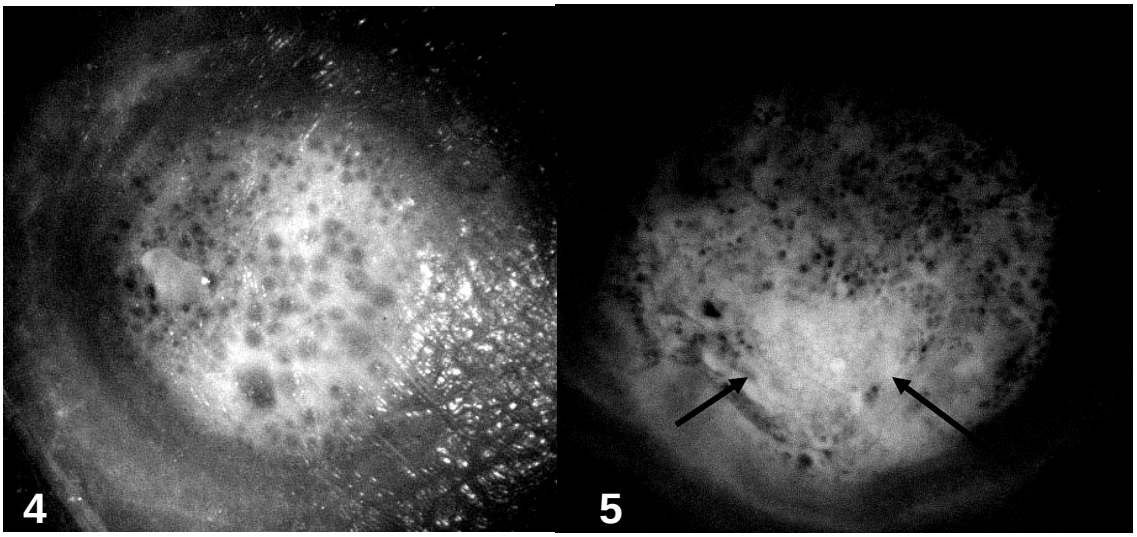
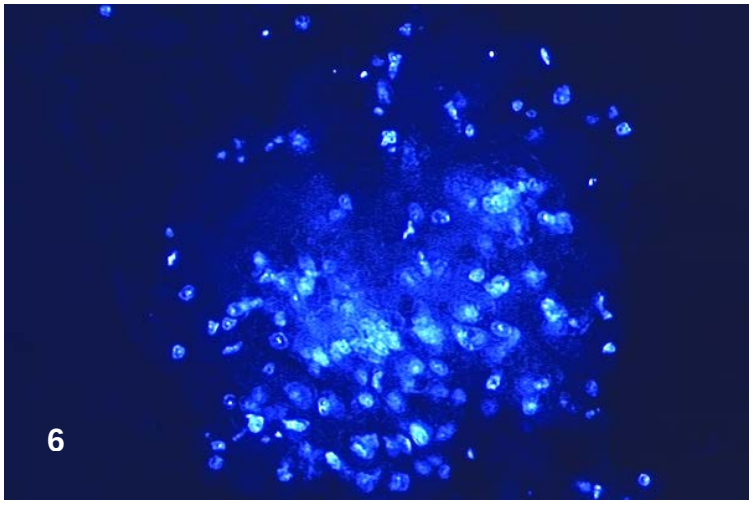


Figure 6: Blastodermal cell nuclei (pyknotic nuclei are condensed having an irregular appearance) are observed stained with bisbenzimide confirming an early-dead embryo. The light blue background surrounding the fluorescent nuclei is yolk or blastodermal cell contents.



Chapter VII. Predicting Fertility

Section 3. Predicting the Duration of Fertility by Counting Sperm in the Outer Perivitelline Layer of Laid Eggs

Graham J. Wishart and Murray R. Bakst

PURPOSE

This procedure is used to predict the duration of hen fertility and the numbers of sperm in the sperm storage tubules (SST), by determining the number of sperm per unit area in the outer perivitelline layer (PL) from laid eggs. Structurally and functionally different from the *inner* PL, the *outer* PL is a fibrous investment surrounding the ovum that is laid down by the upper end of the oviduct (infundibulum) shortly after fertilization. Those sperm that had not reached the inner PL become trapped within the fibrous mesh of the outer PL and can be visualized after staining with a fluorescent DNA-binding stain. Once the numbers of sperm per unit area of outer PL is determined, the total outer PL sperm per egg is calculated. With such data the duration of fertility can be predicted and the sperm population within the SST can be estimated.

MATERIALS AND METHODS

Materials

Eggs

Small, sharp-pointed scissors

Forceps, two pairs, fine-pointed

Small bowls to support the separated yolk

(NOTE: bowl size is about 5-cm high, 3.5cm diameter base, and 8-cm diameter lip, and should be able to support the whole yolk with minimal added fluid and also allow easy access to the PL for manipulation)

Petri dishes

Microscope slides and cover-slips

Latex gloves

Large beaker for waste

Paper towels

Chemicals

Diamidinophenylindole (DAPI) or Hoechst 33342 *(bis-benzimide)*

Phosphate buffered saline (PBS) in tablet form or see **Reagents** below

Equipment

Fluorescence microscope with 40x objective

Reagents

PBS (available commercially as tablets)

To make up 1-L PBS (x1):

1. Weigh the following:

   8.00g NaCl

   1.44g Na_2HP0_4

   0.20g KCl

   0.24g KH_2P0_4

2. Add 1 L of distilled water.

To make up DAPI or *bisbenzimide* (10μL/mL):

1. Stain stock solution: add 1 mg stain to 1-mL distilled water;
2. Dilute this to 10μL/mL in PBS (e.g., 50μL in 5mL PBS);
3. Freeze in working aliquots for future use.

   [NOTE: Fluorescent nuclear stains are potentially carcinogenic and accordingly all appropriate precautions must be taken when used. Refer to the Material Safety Data Sheets (MSDS) for proper handling and follow institutional/local hazardous waste disposal guidelines.]

PROCEDURE

1. Cover an area of work surface with towels and set out the liquid waste container and a bag for the eggshells; fill the small bowls and two Petri dishes about two-thirds full with PBS.

2. Over the waste container, crack an egg cleanly in half, retaining the yolk in one half-shell. Pass the yolk from one half to the other half of the shell (or pass yolk hand to hand) to eliminate as much albumen as possible. This is to be done carefully to avoid breaking the yolk. When completed, tip or place the whole yolk into the small dish.

3. If the total number of sperm nuclei per outer PL is to be calculated, measure the radius of the yolk when completely submerged in PBS.

4. Check whether or not the egg is fertile by looking for the presence of an intact blastoderm (refer to Chapters VII.1 and VII. 2). Then with the scissors, cut out a 15-

mm square of PL overlying the germinal disc. Remove this with the forceps, by holding one edge of the PL and "peeling" it away from the yolk. Discard the unwanted yolk and PBS and then rinse and refill the bowl.

5. Wash the adherent yolk away from the piece of PL by rinsing it in PBS contained within a Petri dish (the PL is quite strong and you can do this vigorously). When clear of yolk, rinse the PL again in the second dish of PBS. Change the PBS every five to six samples. Sometimes, thick albumen remains stuck to the PL. To remove, hold the PL with fine forceps and dab or drag it along a paper towel. The "trailing" blob of albumen is generally removed in this manner.

6. Carefully spread the piece of PL on a microscope slide, trying to avoid wrinkles. Whether or not the PL breaks, attempt to get as much of the PL as flat as possible.

7. Add 5µL of 10µL DAPl/mL onto the PL and cover with a cover-slip, avoiding trapped air. [An alternative method is to pre-stain the piece of PL in stain solution for 20 to 30 min before spreading on the slide. The mounted piece of PL is then dried onto the microscope slide with a hot-air dryer (taking care not to over dry - a glossy sheen should be maintained by the PL.

8. The slide is ready to view after 15 min. If likely to be left for 2 to 3 h, place the slides in the refrigerator. If longer, wait until the samples have dried for 15 to 30 min and then seal the edges of the cover-slip with nail varnish. The samples can then be kept for 24 h in the refrigerator.

9. For microscopy, locate the PL under low power with light optics and then focus with the 40x objective before switching to fluorescence mode. The sperm nuclei have a characteristic "comma" shape and are readily distinguished from other fluorescing material (Figure 1).

Figure 1. Observed is a turkey sperm after staining with *bisbenzimide*. The nucleus is blue, and the acrosome (conical shaped organelle at the tip of the sperm head) and the cobblestone-like mitochondria of the mid-piece (below the nucleus) remained unstained.

10. Count the sperm nuclei found in 50 randomly selected fields. When there are no sperm in a field, ensure that you are still focused in the plane of the PL by looking for other features such as nuclei from other cells, artifacts, or reflection by the folds of the PL. Always focus up and down to cover the full thickness of the PL.

11. View a micrometer or hemocytometer or other cell-counting grid at the same magnification used for counting sperm so that the diameter of the field of view can be ascertained.

CALCULATIONS

The area of the PL viewed is 50 πr^2, where r is the radius of the field of vision under the 40x objective. For example, is 50 x 3.14 x 0.412 or 26 mm^2. For the total number of sperm nuclei per PL, use the total area of the yolk, calculated as 4 πr^2.

INTERPRETATION

For chickens, when less than 0.2 sperm/mm^2 are found in the outer PL, the egg is likely to be unfertilized. Furthermore, the daily reduction in sperm numbers/outer PL is around 30% per day. So, if 10 sperm/mm^2 are found on day 1, we can expect fertilized eggs for around 10 d. (Broken down by each successive egg/day the same hen would expect to have 7.0, 4.9, 3.4, 2.4, 1.7, 1.1, 0.8, 0.6, 0.4, 0.3, and 0.2 sperm/mm^2.)

For turkeys, unfertilized eggs are likely when they have less than 0.2 sperm/mm^2. The daily reduction rate is much slower at around 10% so that for a given number of outer PL sperm, the turkey hen will lay fertilized eggs for a longer period.

The number of SST sperm can be calculated from the total numbers of outer PL sperm by equations that have been derived for both chickens and turkeys (see references below).

REFERENCES

Brillard, J.P. 1993. Sperm storage and transport following natural mating and artificial insemination. Poultry Sci. 72:923-928.

Brillard, J.P., and M. R. Bakst, 1990. Quantification of spermatozoa in the sperm storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer. BioI. Reprod. 43:271-275.

Wishart, G. J., 1987. Regulation of the length of the fertile period in the domestic fowl by the numbers of oviductal sperm. J. Reprod. Fertil. 80:493-498.

Wishart, G. J. 1995. New approaches to evaluating male and female fertility. Pages 207-223 in: Proceedings First International Symposium on the Artificial Insemination of Poultry. M. R. Bakst and G. J. Wishart, ed. The Poultry Science Association, Savoy, IL.

Chapter VII. Predicting Fertility

Section 4. Determination of Holes Made by Sperm in the Perivitelline Layer of Laid Eggs: The Sperm Penetration Assay

R. Keith Bramwell and Ann M. Donoghue

PURPOSE

At the time of fertilization, the ovulated ovum is enveloped by a thin, fibrous investment called the inner perivitelline layer (PL). Sperm digest a path through the PL in order to reach the germinal disc (GD) and fertilize the ovum. The sperm penetration assay provides a means to quantitatively determine the number of sperm penetration sites (sperm-holes) in the PL overlying the GD. When performed with freshly laid eggs it can be used as a predictive measure of fertility. The original procedure, which was developed for chicken eggs (Bramwell et al., 1995), has been adapted to turkey eggs (Donoghue, 1996).

MATERIALS AND METHODS

Materials Needed

Small dissecting scissors

Small forceps

Bowls

Disposable pipettes

Microscope slides & cover slips

Paper towels

Needles or probes (for straightening PL)

Yolk separator (optional)

Disposable bags for discarding albumen and yolk contents

Disposable laboratory gloves

Equipment

Light microscope (4x and 10x objectives)

Chemicals

Sodium chloride, NaCI, M.W. 58.44

Schiff's reagent (wear gloves when handling)

Formalin (3 - 5% solution in PBS, use with caution

Reagents

1% NaCl:

Dissolve 10 g NaCI into 1 L deionized water

1x PBS buffer:

8.00 g of NaCl
0.20 g of KCl
1.44 g of Na_2HPO4
0.24 g of KH_2PO4
Dissolve in 800 ml of distilled H_2O
Adjust the pH to 7.4 with HCl or NaOH
Add distilled H2O to 1 liter.

PROCEDURE

NOTE: Differences in the following two techniques reflect more the preferences of the individual investigators, rather than necessary adaptations due to differences in germinal disc or egg size and structure.

Procedure for Chicken Eggs

1. Separate ovum (yolk) from the egg albumen either with a yolk separator or by decantation with eggshell halves (cool eggs work best). Egg yolk separators reduce broken yolks.

   NOTE: Eggs can be stored in a refrigerator or an egg storage room for 1-2 weeks. However, the longer the eggs are stored the weaker the PL becomes, and the likelihood of 'breaking' the yolk and losing the PL increases.

2. Gently place the yolk on a *smooth* paper towel and gently roll the yolk or dab at the GD until the GD area is free from visibly adhering albumen. Failure to clean the GD area well will cause problems in identifying PL-holes after staining.

3. Transfer the yolk into a bowl with enough 1% NaCl to completely submerge the yolk and GD for at least 5 minutes. Time is not critical at this point.

4. After incubation in the NaCl solution, orient the GD upwards and puncture the underside of the yolk to relieve internal pressure of the yolk contents. This step aids in preventing the yolk membrane from curling up around the incision site.

5. With the fine dissecting scissors, carefully cut the PL overlying the GD in a square with each side *at least* 1 cm in length.

6. With the fine forceps, grasp a cut edge of the PL and carefully slide it off the yolk and then vi*gorously* shake the PL section in the NaCl solution to free it from adhering yolk material (adhering yolk or thick albumen obstructs the visualization of the sperm holes after staining). When using fresh eggs, the PL section is strong and can withstand the cleaning of the adhering debris.

7. Transfer the PL section to a microscope slide and straighten it with needles, or small

probes or fingers. (Adding 1 - 2 drops of water often makes straightening somewhat easier). It is important to keep the GD area as smooth as possible without wrinkles, tears and overlapping.

8. Add a few drops of the prepared formalin solution (3 - 5% formalin) to the PL sections, let sit for 10 to 20 seconds. It is not necessary to remove the formalin after fixation.

9. Add a few drops of Schiff's reagent (undiluted) and allow ample time for the PL section to take up the stain. The time required to stain properly is dependant upon the age and strength of the reagent used. It can take as little as a few seconds to a minute or more. (If the stain is too dark it can make viewing holes and locating the GD somewhat difficult).

10. Gently rinse the excess solution from the slide by tipping the slide over an empty container and allow the excess solution to "flow off" the surface of the slide. Carefully add several drops of a clear solution (either the NaCl solution or water) to the tilted slide and again, allow the solution to "flow off" the slide. You may need to repeat this several times until the fluid draining from the slide is mostly clear. Be careful when the rinsing solution is added so the PL section does not slip off the microscope slide.

11. Gently place a cover-slip over the PL section and carefully press on the cover-slip to remove air bubbles. (Note: do not move cover-slip after placed on the slide, it may cause wrinkling of the PL section). In some cases a cover-slip is not needed, especially if the slides are counted shortly after preparation.

12. Place slide on the microscope and locate the GD on low power (4x) and count holes on higher power if needed (10x). Count only those holes in the circular GD region (See Figure 1).

Procedure for Turkey Eggs

1. Make a small hole in the shell in mid-section the egg. Carefully insert tip of scissors into hole and cut the entire circumference of the eggshell using only the tip of the scissors to avoid cutting the ovum (yolk).

2. After removing the top half of the shell, decant (pour off) as much albumen as possible and then locate the GD (it should appear as a dense whitish disc about 4 mm in diameter on the surface of the yolk). If the GD is not observed, carefully rotate the yolk until the GD is centered on the yolk.

   (NOTE: It is better to first "wet" a *spoon or* a *small spatula with albumen before rotating the yolk.)*

3. Gently pour the yolk into your hand to further separate the yolk from albumen, making sure to "wet" your hand as much as possible with albumen as it falls out. If not visible, the GD should be centered on the yolk.

4. Cut away the chalazae using small dissecting scissors.

5. Remove as much of the chalaziferous layer (the thick albumen immediately surrounding the yolk) as possible. This can be done by allowing a paper tissue to fall on the yolk's surface and then gently peeling it off the yolk. This removes a portion of the chalaziferous layer and gives the yolk a dry appearance. If not done carefully, the PL will tear rendering releasing the yolk contents.

6. Place the yolk with the GD centered and up in a deep weigh boat or Petri dish.

7. Add enough 1% NaCI to totally submerge the yolk and quickly proceed to the next steps.

8. Center the GD and with fine scissors cut a piece of PL about 15 x 25 mm.

9. Using fine forceps and starting at an outer corner, tease the PL away from the yolk being careful not to tear the PL.

10. While holding the PL with the fine forceps, swirl it gently in a beaker of PBS until it opens up and the PL appears clean of yolk.

11. Drop several drops of PBS onto a microscope slide and mount the PL onto the slide. Straighten out all wrinkles and folds.

12. Once the PL is mounted, pipette a few drops of 3 -5 % formalin onto the section (enough to cover the section). Let the PL sit for 10 to 20 sec, then tip the slide onto its side to allow the excess formalin to drain onto a paper towel. If the PL is too wet, it will slide off the microscope slide.

13. Pipette a few drops of Schiff's reagent onto the PL (enough to cover the section).

14. Let it stain until a purple coloration begins to appear (several seconds), then tip the slide onto its side to allow excess stain to drain off onto a paper towel.

15. Wipe away any excess fluid around the PL with a paper tissue.

16. OPTIONAL: Place a cover-slip over the PL.

17. Under bright field microscopy at 40x, PL holes caused by sperm digestion can easily be detected. Locate the germinal disc and center in field of view. Count all the holes in this one field of view (Figure 1).

    (NOTE: We find that once the PL completely dries, the cover slip does not stay on the slide during extended storage. Slides can be stored without cover slips if care is taken.)

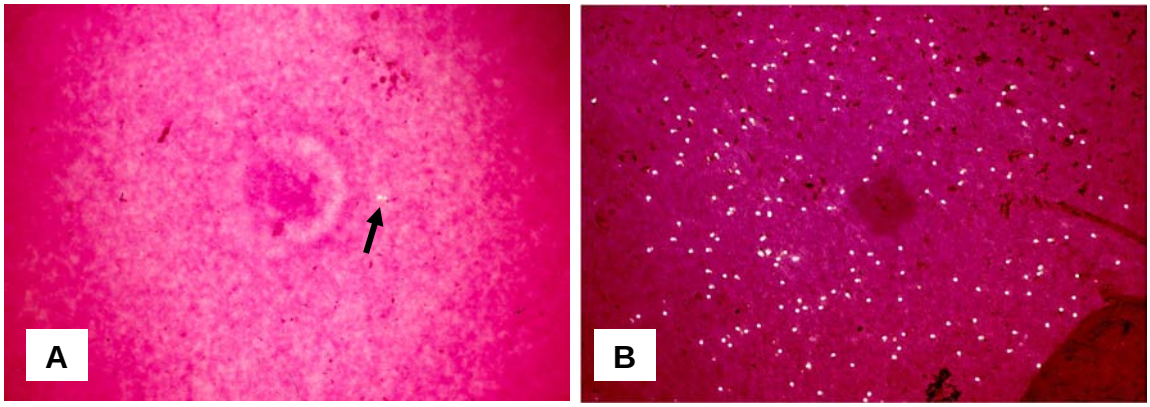


Figure 1. Representative chicken PL preparations. In Figure 1a, the arrow indicates two sperm holes. In contrast, numerous sperm holes are observed in Figure 1b.

REFERENCES

Bramwell, R. K., H. L. Marks, and B. Howarth, Jr., 1995. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. Poult. Sci. 74:1875-1883.

Donoghue, A. M., 1996. The effect of 24 hour in vitro storage on sperm hydrolysis through the perivitelline membrane of ovipositioned turkey eggs. Poult. Sci. 75: 1035-1038.

Chapter VIII. Sperm Function Assessment

Section 1. *In Vitro* Sperm-Egg Interaction Assay Utilizing Inner Perivitelline Layer from Laid Chicken Eggs

Laura Robertson and Graham J. Wishart

PURPOSE

This is an *in vitro* sperm quality assay in which sperm are co-incubated with inner perivitelline layer (IPL) from laid eggs. The number of sperm holes per unit area of the IPL are then viewed under dark-field optics and quantified as a measure of sperm activity. The IPL samples for the assay are separated from the outer perivitelline layer (OPL) by acid hydrolysis. This assay can be used for ranking individual males on the basis of their semen quality and is especially recommended for monitoring the quality of stored semen.

MATERIALS AND METHODS

Materials

Fresh infertile eggs (not more than 36 h old)
Semen sample
Beakers, 100-mL capacity large beaker for waste
Small bowls for separated yolk (e.g., 5-cm high, 3.5-cm diameter base and 8-cm diameter lip)
Petri dishes (e.g, 9-cm diameter)
Blunt forceps
Sharp forceps
Polycarbonate vials, 20-mL capacity
Microscope slides and cover-slips
Micropipettor and tips for dispensing 10 to 1,000 µL

Chemicals

Sodium chloride (NaCI)
([N-tris] hydroxymethyl) methyl-2-aminomethane sulfonic acid (TES)
Hydrochloric acid (HCI)
Distilled water
Dulbecco/s Modified Eagles Medium (modified with 25 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesolfonic acid (HEPES)

Equipment

40 ºC shaking water bath

37 ºC incubator

Microscope with dark-field optics and l0x objective

Video camera, monitor, and video printer attached to microscope (optional)

pH meter

Reagents

1% (1 g/100 mL) NaCI

150 mM NaCI with 20 mM TES, pH 7.4 (NaCI-TES)

0.01 M HCI

PROCEDURE

Separation of IPL from Laid Eggs

1. Crack egg open over the waste beaker retaining the yolk in one half of the shell.

2. Decant the yolk from one half of the shell to the other several times to remove the albumen, taking care not to break the yolk.

3. Place the isolated yolk in a small dish and wash several times with 1 % NaCl to remove any excess albumen. Remove stubborn pieces of albumen using blunt-ended forceps.

4. Place the yolk in a 100-mL beaker containing 75 mL of 0.01 M HCI and place the beaker in a 37 ºC incubator for 1 h.

5. After 1 h, remove the beaker from the incubator and decant the HCI into the waste beaker, taking care not to burst the yolk. Transfer the yolk to a small bowl and burst the perivitelline layer (PL) using sharp forceps.

6. Place the isolated PL in a Petri dish containing 1 % NaCI. Wash the PL in several changes of 1% NaCI, until all yolk particles are removed, and spread the PL out in a Petri dish containing fresh 1% NaCI. The IPL can now be distinguished from the OPL by its thinner, more transparent, appearance. This is more easily achieved if the Petri dish is placed on a dark surface. The two layers can then be separated using sharp forceps. The isolated IPL can be stored in Petri dishes containing NaCI-TES for up to 24 h at 5 ºC.

Sperm-Egg Assay

1. Dilute 1 part fresh semen in 3 parts NaCI-TES and store at 40 ºC in a shaking water bath for 10 to 60 min before assay. Stored semen should also be subjected to a similar 10-min aerobic incubation before assay.

2. Cut fragment of the isolated IPL to approximately a 0.5 cm x 0.5 cm square and add to a polycarbonate vial containing approximately 1.25×10^7 sperm in 1 mL MEM (generally 10 µL of diluted semen in 1 mL MEM).

3. Incubate at 40 ºC for 5 min in a shaking water bath.

4. Remove the IPL from the vial and wash in 1% NaCI to remove any unbound sperm.

5. Carefully spread the IPL onto a microscope slide using forceps and avoiding wrinkles. Cover with a cover-slip.

6. View immediately using dark-field optics under 10x objective. The holes appear as dark circles of around 50 µm diameter.

7. Either count the number of holes per microscope field manually or take images of fields of view and then count the holes. Count at least three areas chosen at random. Calculate the average for the three counts.

8. Use a hemocytometer (or other microscopic grid) to define the dimensions of the microscope field described by the 10x objective or of the image.

CALCULATIONS

Calculate the area of the microscope field or the image and express the results as number of holes per square millimeter of IPL. See Figure 1 for representative IPL sperm hole images.

FURTHER COMMENTS

For semen from individual male chickens, we have correlated the results of this test with other sperm quality parameters, which are, in turn, correlated with fertility (Robertston et al., 1996). We have shown that this test is a much more accurate monitor of the likely fertilizing ability of chicken and turkey semen stored at 5 ºC and for cryopreserved chicken sperm (Wishart et al., 1995).

It is important to compare the activity of two or more semen samples on IPL from the same egg, as there are between-egg differences. It is also necessary to monitor the variation between duplicates to ensure statistical significance.

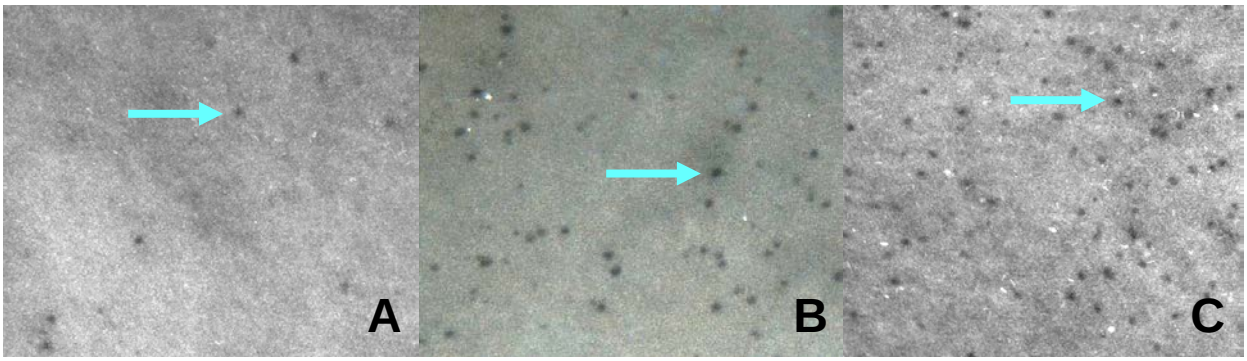


Figure 1. Representative inner perivitelline layer (IPL) with varying numbers of hydrolysis points (sperm holes). Arrows denote representative sperm holes. Panel A: < 10 sperm holes/mm^2. Panel B: ~ 50 sperm holes/ mm^2. Panel C: ~ 90 sperm holes/ mm^2. (*Photographs provided by Julie A. Long)*

REFERENCES

Kido, S., and Y. Doi. 1988. Separation and properties of the inner and outer layers of the vitelline membrane of hen's eggs. Poult. Sci. 22:71-77.

Robertson L., H. L. Brown, H. J. Staines and G. J. Wishart. 1997. Characterization and application of an avian in vitro spermatozoa-egg interaction assay using the inner perivitelline layer from laid chicken eggs. J. Reprod Fertil. 1997 110:205-211

Wishart, G. J., L. R. Robertson, and L. Wood, 1995. Sperm:egg interaction in turkeys: mechanisms and practical applications. Turkeys 43:19-22.

Chapter VIII. Sperm Function Assessment

Section 2. ATP Assay

Julie A. Long

PURPOSE

This is a specialized protocol for determining the adenosine triphosphate (ATP) content of poultry sperm, which can be used an indirect assessment of sperm motility. The mitochondria generate the sperm cell's supply of ATP, which is used as a source of chemical energy to power sperm motility.

MATERIALS AND METHODS

Materials

Plate reader capable of luminescence detection
White micro titer plate
Eppendorf tubes
Pipettor, 1-10 µL delivery, and corresponding pipette tips
Pipettor, 100- µL delivery, and corresponding pipette tips
Multi-channel pipettor, tips
Pipettor, 1 mL delivery, and corresponding pipette tips
Sigma ATP standard (cat. # FL-AAS; St. Louis, MO)
Sigma ATP assay mix (cat#. FL-AAM)
Sigma ATP assay mix dilution buffer (cat. # FL-AAB)
Sigma Phosphatase Inhibitor Cocktail 2 (cat. # P 5726)

Solutions

Sigma ATP assay mix stock:

1. Add 5 mL sterile Milli-Q water to 1 vial of lyophilized assay mix and gently invert to mix (**DO NOT VORTEX**).

2. Place on ice and let stand 60 minutes.

3. Put 1 mL aliquots in dark brown Eppendorf tubes and place at -20 °C. Aliquots can withstand several freeze/thaw cycles.

Sigma ATP Standard stock:

1. Add 10 mL sterile Milli-Q water to 1 vial of lyophilized Sigma ATP standard and swirl to mix. Generally supplied as 0.9 mg per vial, however check quantity and adjust volumes as needed (see step 7 in Assay Procedure).

2. Place 500 µL aliquots into Eppendorf tubes and place in -20 °C freezer. Aliquots can withstand several freeze/thaw cycles.

Sigma Assay Mix Dilution Buffer:

1. Remove 1 vial of lyophilized dilution buffer from the freezer.

2. Add 50 mL sterile Milli-Q water to the vial and mix.

3. Reconstituted buffer is stable for at least a year.

4. Store at -20 °C.

Sigma Phosphatase Inhibitor Cocktail 2:

1. Supplied as a 100x stock solution.

2. Store at 4 °C.

PROCEDURES

Sample Preparation

1. Remove the dilution buffer from the freezer to thaw.

2. Dilute the sperm to 1x 10^9 with dilution buffer. Final volume will depend on amount of starting sample.

3. Add Sigma Phosphatase inhibitor cocktail to a final concentration of 1x (e.g., 10 µL to a 1 mL sample) and allow sample to incubate at room temperature for 30 minutes (samples can be frozen @ -70 °C at this point for future analysis).

4. Place samples in a boiling water bath for 10 minutes.

5. Centrifuge samples for 5 minutes at full speed in the microfuge and collect supernatant. The supernatant is the final sample. Samples can either be run fresh or placed @-70 °C for future analysis.

Assay Procedure

1. Remove the dilution buffer from the freezer (if not done for sample prep) and thaw. Thawing can be hastened by placing vial in beaker of room temperature water.

2. When the dilution buffer has thawed, remove 1 aliquot of assay mix stock and 1 aliquot of standard and allow them to thaw.

3. Label Eppendorf tubes for samples (if not prepared in advance).

4. Remove a 96 well white micro titer plate and determine number of wells to be used, e.g., # samples + # standards + # blanks *3 (each is run in triplicate).

5. Calculate the total volume of working assay mix needed. The assay mix stock is diluted 1:10 with assay mix dilution buffer before use. Take the total number of wells to be used and multiply by 0.1. For example, if all 96 wells will be used, 96 * 0.1 is 9.6, so 9.6 mL of working assay mix will be needed. Always make a slightly larger volume than actually needed, so for this example, make 10 mL. For a 1:10 dilution, 9 mL assay mix dilution buffer plus 1 mL assay mix stock will give 10 mL working assay mix.

6. Add 50µL sterile distilled water to each well needed.

7. Label Eppendorf tubes for the standard curve as follows: 1.25, 2.5, 5, and 10, 20, 40, 80 and 160. Prepare the standards using the following serial dilution: add 964 µL Milli-Q water to the 160 tube and 500 µL Milli-Q water to the remaining tubes. To the 160 tube, add 36 µL ATP standard stock @0.9 mg/mL. This gives 5400 pmol/mL (160 pmol in 25 µL). Take 500 µL of this solution and add to the 80 tube. Continue the same serial dilution scheme for the rest of the standards. For blanks, use 25 µL assay mix dilution buffer.

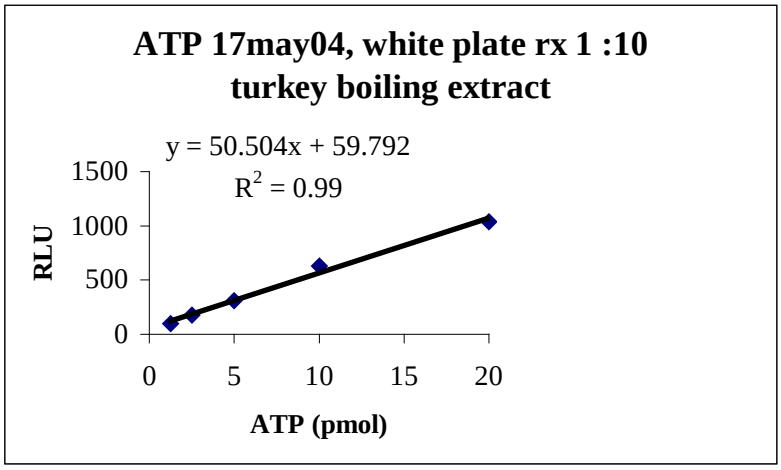
8. If the samples were frozen after treatment with inhibitor but not boiled, then take out of freezer and immediately place in boiling water for 12 minutes. Centrifuge for 5 minutes at full speed and collect the supernatant. If the samples were processed before freezing, then remove from the freezer and thaw by immediately placing them in boiling water for 5 minutes.

9. Add 25 µL of either standard, blank or sample to the appropriate wells.

10. Add 100 µL working assay mix to all wells using the multi-channel pipettor. Swirl plate to mix, and place in plate reader. Read plate on a plate reader within 5 minutes after pipetting in the working assay mix.

Standard Curve Calculations

1. Using Excel set up a spreadsheet and place the pmol values from the standard curve in one column and the raw data from the printout from the plate reader in another. You can place all the raw data in and let the program calculate the means, or calculate by hand and just type in the means. Once the means are in, then select chart, x-y scatter and highlight the data. You can also select trend line and get the formula and R^2 value included in graph. Example below:



2. For the unknowns, use the straight-line equation from the standard curve to calculate pmol per 25 µL sample. Then multiply that value by 40 to get pmol/10^9 sperm. Report values as nmol/10^9 sperm if, after multiplying, values are over 1000.

REFERENCES

Long J. A. and H. D. Guthrie. 2006. Validation of a rapid, large-scale assay to quantify ATP concentration in spermatozoa. Theriogenology. 65:1620-1630.

Appendix 1. Sperm Mobility – The Commercial Version

David. P. Froman

INTRODUCTION

Sperm mobility is a quantitative trait (Froman and Feltmann, 1998) with h^2 = 0.30 in the chicken (Froman et al., 2002). An exclusively maternal additive genetic effect was observed when heritability was estimated. Initially, this effect was attributed to the mitochondrial genome (Froman and Kirby, 2005). However, genome-wide SNPlotyping has identified a locus of interest on the Z chromosome (Rhoads and Froman, unpublished data). At the time of writing, research is underway that will relate genotype to sperm mobility phenotype. Our goal is to explain the sperm cell proteome, which in terms of gene expression, differs between low and high sperm mobility roosters.

Sperm mobility denotes the net movement of a sperm cell population from one place to another at body temperature. It is noteworthy that such movement is against resistance. In other words, whereas mobile sperm must be motile, not all motile sperm are mobile. This is a key distinction that imparts biological significance to sperm mobility measurements. One can infer that sperm move against something akin to muco-ciliary transport on the vaginal surface (Solomon, 1983; Bakst et al., 1994). Even though the resistance imposed in vitro is wholly artificial, such resistance is telling. Sperm mobility is quantified as outlined in Figure 1.

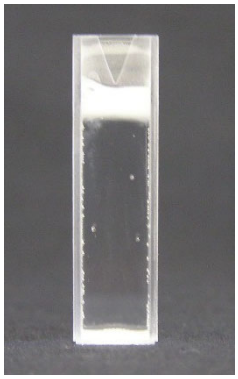


Figure 1. Mobile sperm enter 6% (wt/vol) Accudenz® from an overlaid sperm suspension (Froman and Mclean, 1996). Accudenz® is a non-ionic, biologically inert cell separation medium. The medium's density affords an artificial resistance to sperm cell movement between media. For example, immotile or poorly motile sperm do not penetrate the Accudenz® solution. In contrast, sperm penetrate rapidly at body temperature if their velocity is $\geq$ 30 µm s^{-1}. Such sperm interact with light when a beam is passed through the Accudenz® solution after a 5-min incubation at 41° C. The magnitude of this signal is proportional to the number of mobile sperm within the overlaid sperm suspension. The total number of sperm within the cuvette is controlled by applying a constant volume containing a defined number of sperm.

In essence, a sperm mobility measurement reflects an effective insemination dose. This can be explained as follows. If the overlay is a 300 µl-volume containing 500 million sperm ml^{-1}, then each assay estimates the size of the mobile subpopulation within a population of 150 million sperm. Mobile subpopulations of chicken sperm range in size from 0 to 90%.

Such variation in sperm mobility among males prompts the question: what is the relationship between in vitro sperm mobility and fertility? As shown in Figure 2, male fertility is a *function* of sperm mobility phenotype when hens are inseminated artificially.

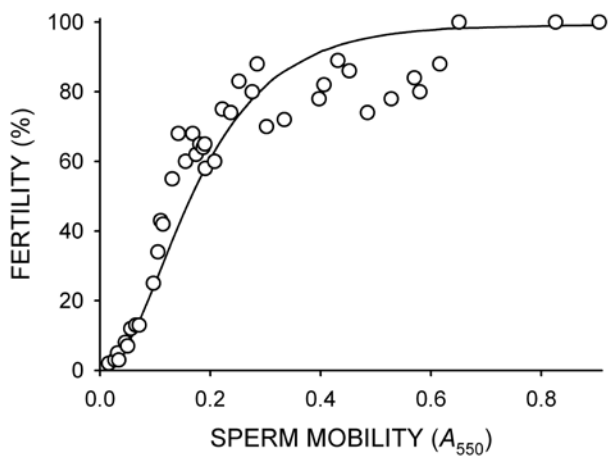


Figure 2. Relationship between sperm mobility phenotype and male fertility. Open circles denote data pairs (n = 48 broiler breeder males). Sperm mobility was measured by passing monochromatic light (550 nm) through the Accudenz® layer to measure change in absorbance (*A*). Residual semen was used to inseminate 8 to 12 hens with 80 million

viable sperm per hen. Eggs were collected for 7 d, incubated for 4 d, and then broken and examined. Plotted data approximated Richard's function, a skewed logistic function. The solid line denotes the fitted curve (from Froman et al., 1999).

On one hand, male fertility is determined by multiple interacting variables. These include testis size and function, libido, health, social status, and frequency of successful copulations in addition to sperm mobility phenotype. However, sperm mobility has been proven to be a critical determinant of male fertility in chickens and turkeys in both non-competitive and competitive fertility trials (Froman et al., 1997; Donoghue et al., 1998; Birkhead et al., 1999; Donoghue et al., 1999; Froman et al., 1999; King et al., 2000; Donoghue et al., 2003). It is also noteworthy, that sperm mobility phenotype, unlike most other variables affecting fitness, can be quantified readily with a living bird.

In essence, sperm are self-propelled DNA delivery vehicles. Surprisingly, the quality of self propulsion varies among males. In review, such variation is readily measured by the sperm mobility assay. Whereas sperm mobility phenotype can be predicted by an interaction of variables measured by computer-assisted sperm motion analysis (CASA; see Figures 3 and 4), neither technology as sophisticated as CASA nor as simple as the sperm quality index can predict sperm mobility based upon instrument readout (Froman et al., 2003). Finally, it is noteworthy that sperm mobility phenotype has been shown to be independent of age (Froman and Feltmann, 1998; Holsberger et al., 1998; Bowling et al., 2003; Froman, 2006) but subject to genetic selection (Froman et al., 2002).

The demonstration that male fertility can be determined by sperm mobility phenotype (Fig. 2) represents a landmark discovery. Predicting male fertility from sperm motility has been a long sought goal (Wishart, 2009). However, one must realize that the meaning of sperm motility has changed with time. Prior to CASA, motile sperm typically meant a percentage of sperm within an ejaculate *progressively advancing at some velocity based* upon subjective evaluation. Such measurements were often made at room temperature. This practice resulted in artifact because sperm velocity is exquisitely dependent upon temperature (Froman, 2003). Computer-assisted sperm motion analysis broadened the meaning of sperm motility because the term now encompasses the number of motile sperm within an ejaculate as well as the attributes of individual motile cells. These attributes include velocity and trajectory. This realization prompts a critical question: why has CASA, an objective and powerful technique, failed to predict the fertility of individual males?

The excerpt below is from the *Poultry Science* article in which the sperm mobility assay was validated and first described (Froman and McLean, 1996).

"In summary, our overall experimental goal was to determine whether we could develop an objective sperm motility assay that would: 1) approximate physiological conditions, 2) require simple, portable equipment, 3) be applicable to individual males, and 4) yield repeatable, biologically significant results. Each of these stipulations was met. . . Even so, measurement of poultry sperm motility by sperm penetration of Accudenz® does not afford information about individual sperm. Indeed, it is an assessment of the mobility of a sperm population. Nonetheless, it is sperm mobility rather than sperm motility per se that enables sperm sequestration within the hen's sperm storage tubules."

Subsequent work sought underlying mechanisms that explained sperm behavior following ejaculation (Froman and Feltmann, 2000; Froman 2003; Froman et al., 2003; Froman and Feltmann, 2005; Froman and Kirby, 2005; Froman et al., 2006). This search entailed use of CASA, and the application of this technology enabled the author to understand how two independent variables could be used to construct a "snap shot" of a motile sperm cell population (Fig. 3).

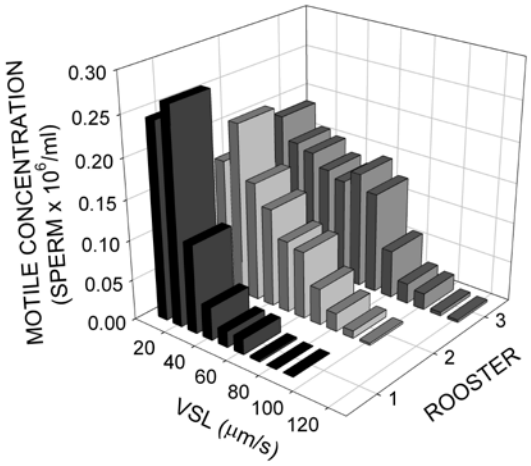


Figure 3. Straight line velocity (VSL) distributions constructed for roosters with different sperm mobility phenotypes. Roosters 1, 2 and 3 were characterized *a priori* by low, average, and high sperm mobility, respectively. Motile concentration and VSL were measured at body temperature with a Hobson SpermTracker. VSL estimates for individual sperm were assigned to frequencies using increments of 10 μm s^{-1}. (Froman et al., 2003).

The possibility that sperm mobility phenotype was determined by the *shape* of a VSL distribution was tested using broiler breeder males representing the observed phenotypic range. VSL distributions were generated for test subjects, upper tail area estimated (VSL > 30 μm/s), and sperm mobility phenotype plotted as a function of upper tail area. The outcome is show in Figure 4 below.

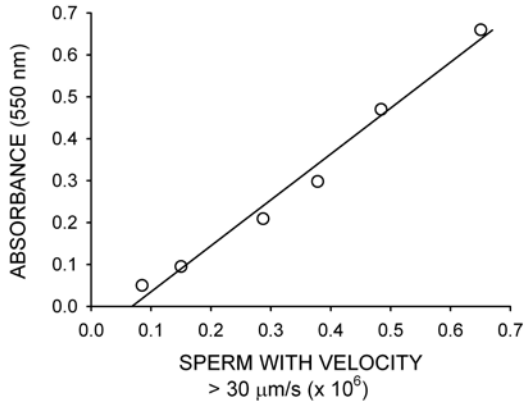


Figure 4. Linear relationship (r = 0.997) between the area within the upper tail area of a VSL distribution and in vitro sperm mobility. The size of the mobile subpopulation was proportional to absorbance at 550 nm. Each open circle denotes a data pair. The solid line represents the function $y(x) = \alpha + \beta(x)$.

In summary, the sperm mobility assay predicts male fertility because it affords a context in which the consequences of variation in sperm velocity become evident in time.

MEASURING SPERM MOBILITY

The commercial sperm mobility assay was validated by the author using pedigree line meat-type chickens (Froman, 2006). The assay, which uses cuvettes containing 6% (wt/vol) Accudenz, is sold by Animal Reproduction Systems (www.arssales.com). Note that 6% (wt/vol) Accudenz was used to field test pedigree line turkey toms (Donoghue et al., 2003). The assay components are shown in Figure 5.

Figure 5. Model 596a Chicken Mobility Analyzer with peripheral equipment and supplies. Items include a saline dispenser, polystyrene cuvettes, pipets, a thermocouple to insure reagent temperature, a timer, sperm mobility buffer, 12 x 75 mm polypropylene tubes in which sperm suspensions are prepared, prepackaged 6% (wt/vol) Accudenz® in sealed cuvettes, warming blocks, a stable 41° C heat source, and a power strip. The Model 596a has a port for connection to a lap top computer. The mobility buffer is TES-buffered saline containing 2 mM Ca^{2+}.

The preparation for the sperm mobility assay entailed the following steps. Mobility buffer was prepared by dissolving the contents of a small sealed package into a bottled sodium hydroxide solution. This step provides an isotonic buffered saline solution containing calcium ions. Note that the motility of chicken sperm, and presumably turkey sperm, depends upon extracellular Ca^{2+} cycling through sperm cell mitochondria (Froman and Feltmann, 2005). The Accudenz® cuvettes were prewarmed prior to arrival in-house. This step requires approximately 30 min. Warming blocks containing prewarmed Accudenz® cuvettes (white seals) and 2 cuvettes filled with mobility buffer (blue snap-on lids) were bundled in bubble-wrap immediately before departure. Accudenz® cuvettes are arranged in arrays of 100 within styrofoam containers packaged within cardboard boxes. Bundled loaded blocks were placed within an empty cuvette box to further minimize heat loss during transport. The equipment and supplies were transported in a covered plastic tub that was sanitized between work sites. In-house set-up entailed arranging a work station upon a portable folding table. Bundled warming blocks were unwrapped and returned to the surface of the heat source, multiple 12 x 75 polypropylene tubes were placed upon the remainder of the exposed heated surface, the Model 596a Mobility Analyzer was blanked with 3% saline, and reagent temperature was double checked with a thermocouple connected to a digital thermometer in order to assure that reagents were at 41° C. In-house set up required approximately 10 to 15 min.

Assays were performed batch-wise as follows. Five 3.4-ml volumes of 3% saline were dispensed into polystyrene cuvettes aligned within a styrofoam cuvette container. A 100-µl volume of neat semen was placed within a 12 x 75 mm polypropylene tube identified by semen donor cage number. Such tubes were held temporarily at room temperature within a polypropylene test tube rack. A positive-displacement pipet was used to remove an11.4-µl volume of semen for determination of sperm concentration. This volume was extruded into the saline, the pipet tip was wiped clean with a Kimwipe, and the cuvette's contents were mixed. The Model 596a Chicken Mobility Analyzer estimates sperm concentration, in billions, and estimates the buffer volume required for preparation of the sperm suspension. Once buffer volumes were determined for one batch of samples, seals were removed from 5 cuvettes within an active row (see Fig. 5). Next, the buffer volume required for the first sample was pipetted into a pre-warmed 12 x 75 mm polypropylene tube, a 50-µl semen sample was added immediately, contents mixed by finger vortexing, a 300-µl volume of the sperm suspension overlaid upon the first cuvette within in the series, and the timer started. This procedure was performed sequentially so that cuvettes were loaded on the minute over the course of a 4-min interval. Thereafter, the cuvette that had served as a reservoir for pre-warmed mobility buffer (lower left-hand corner of heating block pair in Fig. 5) was refilled and sealed. The cap from its counterpart was removed in preparation for the next series of samples. This practice assured that pre-warmed buffer was always on hand for each iteration. After 5 minutes of incubation, each loaded Accudenz® cuvette was removed from the warming block, placed within the well of the Model 596a Chicken Mobility Analyzer, and sperm mobility quantified. While the measurement was being made, a pre-warmed Accudenz® cuvette was moved into the space left within the active row. Likewise, a cuvette at ambient temperature was moved into the heating block. Such cuvettes were positioned with caps turned at a 90° angle to distinguish them from prewarmed cuvettes within the heating blocks (see Fig. 5). In view of warming rate and time required to cycle through all cuvettes within the warming blocks, this practice assured a pre-warmed cuvette for each assay. As sperm mobility measurements neared completion for one cohort, a series of new cuvettes were filled with saline for sperm concentration determination in the next cohort. In this fashion, two people can perform approximately 100 assays per day.

INTERPRETATION OF RESULTS

Observations typically vary among days when repeated measures are made on the same test subject. This variation may be attributed to the nature of an ejaculate, one's semen collection technique, and assay error, which involves estimation of sperm cell concentration, sample dilution, and incubation interval. Such variation is expected with any assay. However, the extent to which observations vary can mean the difference between a useful and useless assay. The

percentage of total variance associated with test subjects can be increased by making duplicate measurements per ejaculate. This is not always possible in commercial breeding programs. Nonetheless, the power of the sperm mobility assay is best appreciated when replicate observations are made per ejaculate. This principle is illustrated in Tables 1 and 2 below. Semen donors (n = 8) were from low and high sperm mobility lines of chickens maintained at Oregon State University. Males were ejaculated once per week for three consecutive weeks, the sperm mobility assay was performed as outlined above, and were data analyzed by nested analysis of variance. A summary of the ANOVA is shown in Table 1. Raw data are shown in Table 2. Note that the Model 596a Chicken Mobility Analyzer's readout ranges from 0 to 90 sperm mobility units as opposed to 0.000 to 1.000 absorbance units. However, the principle of operation is the same: a beam of light is passed through the Accudenz solution after a 5-min incubation at body temperature. Sperm mobility is proportional to the extent to which this beam is disrupted by sperm within the light path. The relationship between sperm mobility units and absorbance units is shown in Figure 6.

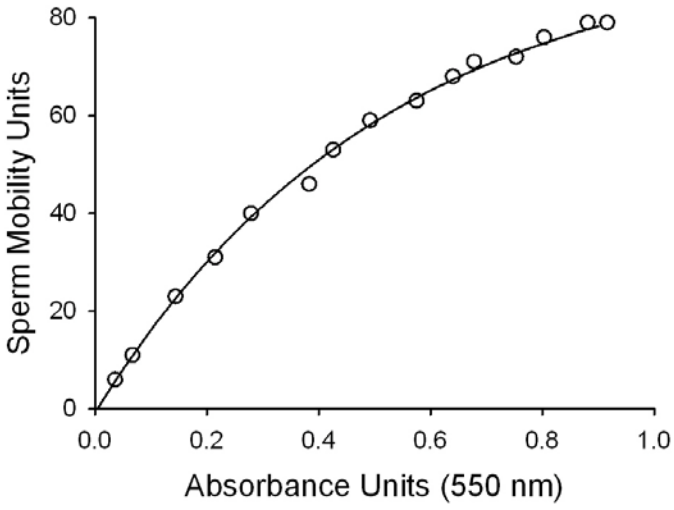


Figure 6. Sperm mobility units (measured with an Animal Reproduction Systems Model 596a Chicken Mobility Analyzer) as a function of absorbance units (measured with a spectrophotometer). Each open circle is a data pair generated with sperm suspended in 6% (wt/vol) Accudenz. The line denotes the predicted curve:

$$y(x) = 94.83 - [(95.67)(e^{-1.9468(x)})].$$

Whereas each instrument detects a phenotypic range, the Mobility Analyzer expresses this range in terms of whole numbers, which tend to be more readily understood by people at large.

Table 1. Summary of nested ANOVA evaluating variation associated with test subject, time, and assay effects.

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-value	Percentage of total variance
Rooster	7	21342	3048.9	35.15****	90.2
Day	16	1388	86.7	4.14***	6.0
Observational error	24	502	20.9		3.8

$^{****}P < 0.0001$.
$^{***}P < 0.001$.

In this illustration, the highly significant difference among roosters should come as no surprise in view of their genetic background. In contrast, the significant day effect could be viewed as

problematic; for if the day upon which the assay is performed affects male phenotype, then the utility of the assay could be in question on any given day. Therefore, the significant time effect warrants explanation.

The author has observed a significant time effect in studies ranging in duration from a few days to many months. For example, when phenotype was proven to be independent of age using test subjects characterized with average or high sperm mobility (Froman, 1998), individual observations varied within kind during the experiment. In other words, distinct ranges were observed between groups of test subjects. However, such variation does not limit the value of phenotypic distinctions among test subjects at one point in time. Consider males 6 and 7 in Table 2. Observations within the second pair for male 6 are roughly three times larger than those within the first pair. Nonetheless, when this male is viewed over the three week interval, this phenotype was consistent with that assigned *a priori* many weeks in advance. Likewise, the first observations for Male 7 are only 75% of those observed two weeks later. Nonetheless, the range in his scores falls well within that observed for high line males as a group. Thus, his initial categorization was accurate relative to subsequent observations that varied a bit with time. In summary, one finds with practice that repeated measurements fall within *ranges* and that these ranges denote genuine differences among males within a flock. This principal is illustrated in the Table 3.

Table 2. Sperm mobility of roosters selected from experimental lines of New Hampshire chickens.[1]

		Sperm Mobility Scores by Ejaculate					
Male	Line	1		2		3	
1	High	57	57	60	60	67	46
2	High	51	46	70	67	54	42
3	High	34	36	42	33	45	50
4	Low	18	12	24	18	22	17
5	Low	3	3	8	6	5	6
6	Low	5	3	14	11	4	5
7	High	44	45	67	57	61	58
8	Low	13	10	26	21	18	14

[1]Each rooster was ejaculated weekly, and sperm mobility was measured in duplicate per ejaculate with the Animal Reproduction Systems commercial assay.

Table 3. Phenotypic distinctions based upon repeated measures.[1]

Rooster[1]	1st Score	2nd Score	3rd Score	4th Score	Average Score	Phenotype Relative to Population
1	6	4	4	4	4	low
2	16	17	18	21	18	average
3	40	20	59	53	43	high
4	9	31	21	7	17	average

[1]Data from Froman (2006). Sperm mobility was measured once per week over the course of 4 consecutive weeks. The population mean was 20 units (n = 65 roosters).

Based upon average scores, each test subject could be assigned to one of three phenotypes: low, average, or high. However, two test subjects had consistent scores through time whereas two did not. In general, poor sperm mobility can be reliably detected from a single measurement. In contrast, repeated measures are best for assigning either an average or high phenotype. In

the author's experience, and as shown in Table 3, some average males have very consistent readings. However, other males are average thanks to arithmetic, i.e. [9 + 31 + 21 + 7] / [4] = 17. To date, the basis for such variation remains unexplained.

The variation in test results explained above is largely due to the nature of semen samples because variables such as reagent pH, osmolarity, and density, along with incubation temperature and time can be controlled within very narrow limits. As mentioned, estimation of sperm cell concentration and sample dilution are sources of experimental error. However, the precision of one's technique can be assessed by applying a simple test. Once a set of measurements are made, the contents of each cuvette can be mixed and a second reading taken. This practice serves to estimate the total number of sperm loaded into a cuvette. In the case of the data shown in Table 2, the mean $\pm$ standard error was 75 $\pm$ 0.6 units (n = 48 observations). Thus, the number of sperm overlaid upon the Accudenz® solution was uniform among the cuvettes used in this experiment. This uniformity reflects reliable estimation of sperm cell concentration among ejaculates. When nested ANOVA was performed with these second readings as data, neither a male nor day effect was observed ($P > 0.05$), an outcome that confirmed experimental precision.

CONCLUDING REMARKS

The sperm mobility assay was first described by Froman and Mclean (1996). The assay arose from the desire of a major professor to help his graduate student answer a significant biological question in an objective manner within a laboratory of modest means. Specifically, we wanted to ascertain whether sperm motility contributed to subfertility associated homozygosity for the rose comb allele. At the time, the efficacy of Accudenz as a washing medium had been demonstrated (Froman and Thursam, 1994). In doing so, a serendipitous observation was made: when the room was warm, sperm entered 12% (wt/vol) Accudenz prior to centrifugation even though the sperm suspension overlay had not mixed with the underlying Accudenz solution. Consequent-ly, Froman and Mclean (1996) arrived at a concentration of 6% (wt/vol) Accudenz by trial-and-error. The ability of the sperm mobility assay to distinguish between hetero- and homozygotes for the rose comb allele, i.e. fertile and subfertile roosters, prompted a question: could the assay be used to detect differences among normal, fertile roosters? The answer to this question proved to be affirmative.

As discussed by King et al. (2000), Accudenz concentrations ranging from 2 to 6% (wt/vol) have been used in turkey research. However, the most stringent assay was proven to be based upon a 6% (wt/vol) solution of Accudenz. Stringency is also enhanced by using a glucose-free buffer, although reagents containing glucose can be used effectively. The author was awarded U.S. Patent Number 5,866,354 in 1999 for his development and validation of the sperm mobility assay. The patent was licensed by Animal Reproduction Systems in Chino, CA, and development of a commercial assay followed. Froman and Mclean (1996) ended their manuscript with the following assessment.

"Our experiments have demonstrated the potential for selecting semen donors based upon sperm motility and the establishment of simple, objective criteria for assessing the quality of semen . . . ."

These goals have been met broadly over the course of the last 14 years and within commercial contexts. Based upon experimental outcomes, semen quality can be defined as the number of mobile sperm produced per male per day. Moreover, semen quality is subject to genetic selection when so defined. The author's long-term goal is the identification of gene networks that affect expression of this complex trait.

REFERENCES

Bakst M. R., G. Wishart, and J.-P. Brillard, 1994. Oviducal sperm selection, transport, and storage in poultry. Poult. Sci. Rev. 5:117-143.

Birkhead, T. R., J. G. Martinez, T. Burke, and D. P. Froman, 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc. Roy. Soc. London B 266:1759-1764.

Bowling E. R., D. P. Froman, A. J. Davis, and J. L. Wilson, 2003. Attributes of broiler breeder males characterized by low and high sperm mobility. Poult. Sci. 82:1796-1801.

Donoghue A. M., D. R. Holsberger, D. P. Evenson, and D. P. Froman, 1998. Semen donor selection by in vitro sperm mobility increases fertility and sperm storage in the turkey hen. J. Androl. 19:295-301.

Donoghue A. M., T. S. Sonstegard, L. M. King, E. J. Smith, and D. W. Burt, 1999. Turkey sperm mobility influences paternity in the context of competitive fertilization. Biol. Reprod. 61:422-427.

Donoghue A. M., D. J. Kirby, D. P. Froman, S. P. Lerner, A. N. Crouch, L. A. King, D. J. Donoghue, and T. S. Sonstegard, 2003. Field testing the influence of sperm competition based on sperm mobility in breeder turkey toms. Brit. Poult. Sci. 44:498-504.

Froman, D. P., 2003. Deduction of a model for sperm storage in the oviduct of the domestic fowl (*Gallus domesticus*). Biol. Reprod. 69:248-253.

Froman, D. P., 2006. Application of the sperm mobility assay to primary broiler breeder stock. J. Appl. Poult. Res. 15:280-286.

Froman, D. P. and A. J. Feltmann, 1998. Sperm mobility: A quantitative trait of the domestic fowl (*Gallus domesticus*). Biol. Reprod. 58:379-384.

Froman, D. P. and A. J. Feltmann, 2000. Sperm mobility: Phenotype in roosters (*Gallus domesticus*) determined by concentration of motile sperm and straight line velocity. Biol. Reprod. 62:303-309.

Froman, D. P. and A. J. Feltmann, 2005. Fowl (*Gallus domesticus*) sperm motility depends upon mitochondrial calcium cycling driven by extracellular sodium. Biol. Reprod. 72:97-101.

Froman, D. P., A. J. Feltmann, M. L. Rhoads, and J. D. Kirby, 1999. Sperm mobility: A primary determinant of fertility in the domestic fowl (*Gallus domesticus*). Biol. Reprod. 61:400-405.

Froman, D. P and D. J. McLean DJ, 1996. Objective measurement of sperm motility based upon sperm penetration of Accudenz®. Poult. Sci. 75:776-784.

Froman, D. P. and K. A. Thursam, 1994. Desialylation of the rooster sperm's glycocalyx decreases sperm sequestration following intravaginal insemination of the hen. Biol. Reprod. 50:1094-1099.

Froman, D. P., E. R. Bowling, J. L. Wilson, 2003. Sperm mobility phenotype not determined by sperm quality index. Poult. Sci. 82:496-502.

Froman, D. P., A. J. Feltmann, and D. J. McLean, 1997. Increased fecundity resulting from semen donor selection based upon *in vitro* sperm motility. Poult. Sci. 76:73-77.

Froman, D. P., J. C. Wardell, and A. J. Feltmann, 2006. Sperm mobility: Deduction of a model explaining phenotypic variation in roosters (*Gallus domesticus*). Biol. Reprod. 74:487-491.

Froman, D. P., A. J. Feltmann, M. L. Rhoads, and J. D. Kirby, 1999. Sperm mobility: A primary determinant of fertility in the domestic fowl (*Gallus domesticus*). Biol. Reprod. 61:400-405.

Froman, D. P., T. Pizzari, A. J. Feltmann, H. Castillo-Juarez, and T. R. Birkhead, 2002. Sperm mobility: Mechanisms of fertilizing efficiency, genetic variation, and phenotypic relationship with male status in the domestic fowl. Proc. Roy. Soc. London B 269:607-612.

Holsberger, D. R., A. M. Donoghue, D. P. Froman, and M. A. Ottinger, 1998. Assessment of ejaculate quality and sperm characteristics in turkeys: Sperm mobility phenotype is independent of time. Poult. Sci. 77:1711-1717.

King, L. M., J. D. Kirby, D. P. Froman, T. S. Sonstegard, D. E. Harry, J. R. Darden, and A. M. Donoghue, 2000. Efficacy of sperm mobility assessment in commercial flocks and the relationship of sperm mobility and insemination dose on fertility in turkeys. Poult. Sci. 79:1797-1802.

Solomon, S. E., 1983. Oviduct. in: Physiology and Biochemistry of the Domestic Fowl, Volume 4, BM Freeman (ed.). Academic Press, London, UK. Pages 379-419.

Wishart, G. J., 2009. Semen quality and semen storage. In: Biology of Breeding Poultry, Poultry Science Symposium Series Volume 29, P Hocking (ed.). Carfax Publishing Company, Abingdon, UK. Pages 151-178.

Appendix 2. Composition of Lake's Pre-freeze diluent

Lake's Pre-freeze Diluent

Magnesium acetate	0.7 g 3.2 mM
Sodium glutamate	19.2 g or 114 mM
Potassium acetate	5.0 g or 51 mM
Fructose	8.0 g or 44 mM
B.E.S.	1.0 g or 4.7 mM
P.V.P.	3.0 g or 0.3%
Gluthathione	0.5 g or 1.3 mM

Add above to 900 mL distilled water and mix. When dissolved, pH to 7.1. Q.S. to 1liter and check osmolarity. Should be ~340 mmol/kg. Filter and aliquot into glass bottles. Store aliquots @ 4°C.

Appendix 3. Composition of Lake's Glycerol Cryodiluent

Lake's Cryodiluent with 33% Glycerol (vol/vol)

Magnesium acetate	0.7 g or 3.2 mM
Sodium glutamate	19.2 g or 114 mM
Potassium acetate	5.0 g or 51 mM
Fructose	8.0 g or 44 mM
B.E.S.	1.0 g or 4.7 mM
P.V.P.	3.0 g or 0.3%
Gluthathione	0.5 g or 1.3 mM
Glycerol	330.0 mL or 33%

Add above (except glycerol) to 600 mL distilled water and mix. When dissolved, pH to 7.1. Then add the 330 mL glycerol and mix. Q.S. to 1 liter with Milli-Q water. Filter and aliquot into glass bottles. Store aliquots @ 4ºC.

Chapter VI. Semen Cryopreservation

Section 2 Addendum. Cryopreservation of Turkey Sperm Using DMA

Julie A. Long

PURPOSE

This protocol for cryopreservation of turkey sperm using dimethylacetamide (DMA) as the cryoprotectant was recently developed in collaboration with Dr. Henri Woelders (Institute for Animal Science and Health, The Netherlands) and Dr. Phillip Purdy (ARS National Germplasm Program). The primary difference between this protocol and the one described for freezing rooster semen (Chapter VI, Section 2) is the use of straw packaging rather than forming pellets that are subsequently stored in cryovials.

MATERIALS AND METHODS

Equipment and Supplies

Pipettor, 1 or 2 mL delivery, and corresponding pipette tips

Pipettor, 200 µL delivery, and corresponding pipette tips

Cryopreservation straws, 0.5 mL size

Sealing beads to fit 0.5 mL straws or straw sealing powder

Conical tubes, 50 mL size

Rack to hold conical tubes

Thermometer

Container suitable to hold water at 5 °C and a conical tube rack

Latex gloves

Long-handled forceps, 2 pair

Cryogloves

Goggles

Rack to hold cryopreservation straws (horizontally)

Rubber tray suitable to hold 5-6” depth of liquid nitrogen

Canes or goblets for straw storage in nitrogen tank

Styrofoam box with lid

Wire rack (4 cm tall) to fit in Styrofoam box and hold horizontal straw racks

Ruler

Cryomarker or sharpie

Chemicals

Lake's pre-freeze diluent (see Appendix 2 for components), pre-cooled to 5°C

dimethylacetamide (DMA), pre-cooled to 5°C

Liquid nitrogen

PROCEDURES

Semen Preparation, Cooling and Freezing

1. Collect semen from a maximum of 5 toms, visually inspect the samples and pool good quality samples in a conical tube pre-cooled to 5 °C. Do not use semen containing blood, fecal material or other contaminants.

2. Measure the volume of the pooled semen, immediately dilute 1:1 with 5 °C Lake's pre-freeze diluent, gently swirl to mix and return to the 5 °C water bath.

3. Repeat steps 1-2 until all the toms have been collected. Keep track of the volume as more semen is added and then diluted.

4. Place conical tube with diluted semen in 5 ºC walk-in cold room or refrigerator and cool for 30 min. While waiting, skip down to steps 10-12 and prepare the freezing box.

5. Put on a pair of latex gloves for the remaining steps of the freezing protocol. DMA is easily absorbed through the skin.

6. Add a volume of cold (5 ºC) DMA to yield a final concentration of 8% DMA and gently mix with the pipettor tip. For example, for a raw semen volume of 5.0 mL, you would have added an equal volume of Lake's pre-freeze diluent (5.0 mL) for the initial 1:1 dilution, giving a total volume of 10 mL in the conical tube. Multiply the total volume by 0.08 to determine the volume of DMA to add. In this example, the volume of DMA would be 0.8 mL.

   Important: DMA is slightly toxic to sperm cells; therefore do not add the DMA until the freezing apparatus is set up.

7. Allow the semen/cryodiluent mixture to equilibrate at 5 ºC for no longer than 1 min and begin filling pre-cooled straws. Diluted semen should not be allowed to re-warm to room temperature. If a refrigerator is used, work inside as far as possible.

8. Fill straws with 0.5 mL of semen using a 1.0 mL pipettor, ensuring that the semen reaches the cotton plug on the closed end of the straw and that no air bubbles are introduced into the straw. Do not use any partially-filled straws (changes the freezing rate). When processing a large number of straws, filled straws may be laid horizontally on a flat surface until all are filled before beginning the sealing process.

9. Seal each straw with a bead or straw powder (following manufacturer's instructions) and place on the horizontal straw rack, working until the rack is filled.

10. Calibrate a Styrofoam box by marking walls at 4 cm from the bottom of the box (should be same height as the wire racks), place the wire rack inside, and fill to the marked line with liquid nitrogen, wearing protective cryogloves and goggles.

11. Place the Styrofoam cover on, and allow 10 min to come to thermal equilibrium (temperature gradient ranges from ambient temperature at cover to -196 ºC at the liquid nitrogen surface).

12. After thermal equilibration, remove the cover from Styrofoam box and check that the level of liquid is still 10 cm from the bottom. Add more liquid nitrogen if needed before beginning the straw filling process (step 8).

13. Carefully place the horizontal racks containing the sealed straws on the wire rack, making sure not to allow straws to fall into liquid nitrogen, and replace cover. (Goal is to freeze straws in the vapor at 4.5 cm above the liquid.)

14. Leave straws on rack for 30 min, remove the cover and, using forceps, tip the horizontal rack to allow straws to fall into liquid nitrogen.

15. Keep straws under liquid nitrogen all times while packing into canes or goblets for storage in the nitrogen tank. At most, straws can be out of the liquid for no more than 6 sec without adversely raising the temperature.

16. There will be some semen leftover after step 8. Measure & record the sperm concentration to determine volume needed for insemination after thawing.

Semen Thawing and Insemination

1. Semen thawing should be conducted as close to the hen enclosure as possible.

2. Prepare a 4 to 5 ºC water bath by first measuring the water temperature and then adding ice until the desired temperature is achieved. Monitor and add ice as needed during the thawing process.

3. Using long-handled forceps, remove straws from liquid nitrogen, immerse straws completely under the surface of the water bath and vigorously agitate for 30 sec.

4. Use a dry paper towel to wipe any water on the straw before cutting the straw. Direct contact with water will damage sperm cells.

5. Hold the straw over the collection tube and cut the beaded end. Most of the thawed semen will flow into the tube. Cut the cotton plug end to release any semen remaining within the straw. A 1 mL pipette may be used to force residual semen from the straw, if needed.

6. Semen should be inseminated within 2 min of thawing. With typical sperm concentration of 9 billion sperm/mL in raw semen, the dilution steps in this protocol will yield approximately 2.5 billion sperm in each straw. An insemination volume of 0.133 mL would equal 300 million sperm per insemination dose, or 3-4 hens per straw. Best practice is to thaw ~ 4 straws at a time, inseminate the maximum number of hens (e.g. 12-16), and return to thaw 4 more straws for a second insemination, repeating until all hens are inseminated. This keeps the total time from thawing to insemination under 2 minutes.

Chapter VII. Predicting Fertility

Section 4 Addendum. Determination of Holes Made by Sperm in the Perivitelline Layer of Laid Eggs: The Sperm Penetration Assay

Murray R. Bakst

PURPOSE

Since the 2010 publication of the 2nd edition of the workbook, two additional methods for isolating the perivitelline layer (PL) have been described for the sperm penetration assay. The first method below was presented by Dr. Radivoje Spasojevic and colleagues at the 2010 MPF Convention; while the second method below will be found in the following journal article - M. Bakst, J. Eastridge, and I. A. Malecki, **The inner perivitelline layer sperm-hole assay: use of filter paper rings for the isolation of the perivitelline layer overlying the germinal disc and new observations on its morphology** - to be published in *Journal of Applied Poultry Research* in 2014.

Method #1 (Spasojevic, et al., 2010)

Figure 1: Ovum is placed in petri dish with blastoderm clearly visible. Cut the PL around the blastoderm such that the diameter of the cut PL containing the blastoderm is slightly larger than the width of the slide;

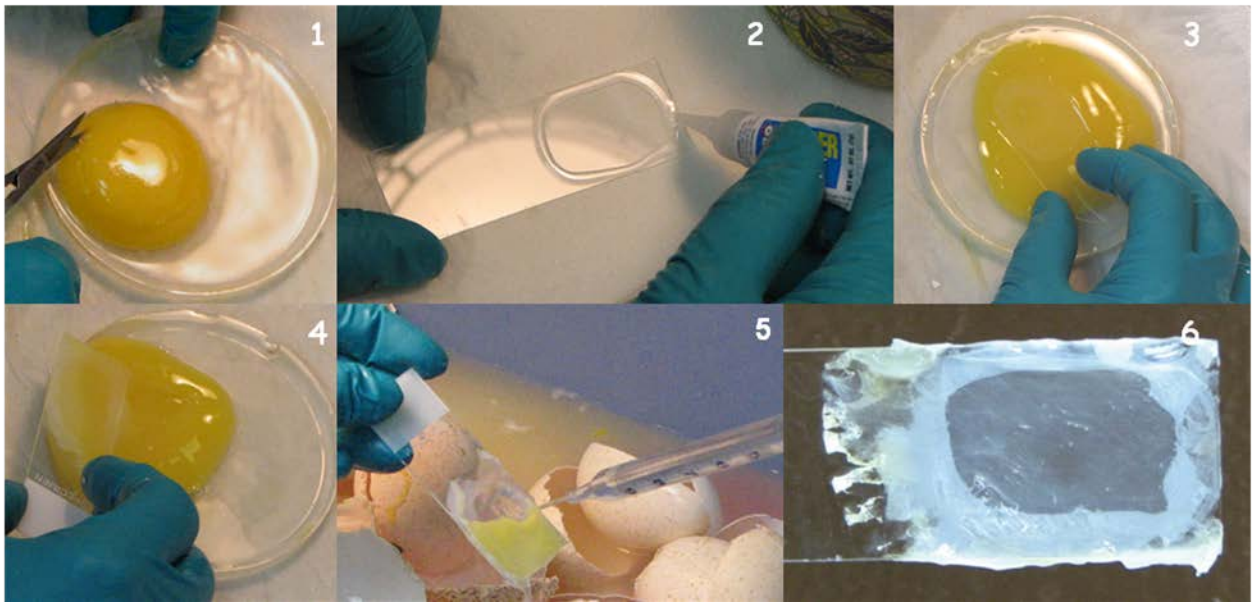


Figure 2: Super-glue is placed on a clean slide;

Figure 3: Place slide with super-glue firmly on the ovum with the blastoderm centered in the circle of super-glue. Hold for a few seconds until glue sets;

Figure 4: Lift the PL with the blastoderm off the ovum;

Figure 5: Using a pipette, gentle wash off all adhering yellow yolk and the white yolk of the blastoderm;

Figure 6: The slide with the adhering PL is ready to be fixed and stained.

The benefits of this method are that it is both fast and the PL is clean and wrinkle free.

Method #2 (Bakst, et al., 2014)

1. Prepare filter-rings before starting the PL isolations. Filter-rings are made from Whatman #1 or #5 filter paper (GE Healthcare Life Sciences, Piscataway, NJ) and cut with an outside diameter of 15 mm and an inside diameter of 6 mm, the latter made with a paper hole puncher;
2. With the blastoderm exposed, grasp a filter-ring with forceps and place on the ovum with the blastoderm centered in the ring;
3. Cut around the outside diameter of the filter-ring;
4. Using forceps, grasp the outer edge of the filter-ring and lift so that the filter-ring, PL, and adherent yolk are perpendicular to the surface of the yolk, and then quickly totally immerse in a petri-dish containing saline;
5. The petri-dish with the filter-ring supporting the PL is placed under a stereomicroscope, and while viewing at low magnification (0.75X to 1.5X), the yellow and white yolk were removed by flushing a gentle stream of saline over the entire filter-ring.
6. The filter-ring with the cleaned PL is then placed on a glass-slide for fixation and staining.

This technique is fast and the PL is clean and wrinkle free. In addition, it can be used with quail, duck, goose, chicken, and turkey eggs.