Validation of a spectrophotometer-based method for estimating daily sperm production and deferent duct transit¹

D. P. Froman* and D. D. Rhoads†

*Department of Animal Sciences, Oregon State University, Corvallis 97331; and †Department of Biological Sciences, University of Arkansas, Fayetteville 72701

ABSTRACT The objectives of the present work were 3-fold. First, a new method for estimating daily sperm production was validated. This method, in turn, was used to evaluate testis output as well as deferent duct throughput. Next, this analytical approach was evaluated in 2 experiments. The first experiment compared left and right reproductive tracts within roosters. The second experiment compared reproductive tract throughput in roosters from low and high sperm mobility lines. Standard curves were constructed from which unknown concentrations of sperm cells and sperm nuclei could be predicted from observed absorbance. In each case, the independent variable was based upon hemacytometer counts, and absorbance was a linear function of concentration. Reproductive tracts were excised, semen recovered from each duct, and the extragonadal sperm reserve determined by multiplying volume by sperm cell concentration. Testicular sperm nuclei were procured by homogenization of a whole testis, overlaying a 20-mL volume of homogenate upon 15% (wt/vol) Accudenz (Accurate Chemical and Scientific Corporation, Westbury, NY), and then washing nuclei by centrifugation through the Accudenz layer. Daily sperm production was determined by dividing the predicted number of sperm nuclei within the homogenate by 4.5 d (i.e., the time sperm with elongated nuclei spend within the testis). Sperm transit through the deferent duct was estimated by dividing the extragonadal reserve by daily sperm production. Neither the efficiency of sperm production (sperm per gram of testicular parenchyma per day) nor deferent duct transit differed between left and right reproductive tracts ($P > 0.05$). Whereas efficiency of sperm production did not differ ($P > 0.05$) between low and high sperm mobility lines, deferent duct transit differed between lines ($P < 0.001$). On average, this process required 2.2 and 1.0 d for low and high lines, respectively. In summary, we developed and then tested a method for quantifying male reproductive tract throughput. This method makes the study of semen production amenable to systems biology.

Key words: daily sperm production, deferent duct, spectrophotometer, sperm, testis

INTRODUCTION

To date, the term semen quality connotes assays rather than a property subject to control. For example, Wishart (2009) lists 5 types of tests used to assess semen quality. Such tests measure sperm cell shape, integrity, motion, metabolism, and cell surface properties that enable fertilization. In contrast, the quantitative trait sperm mobility (Froman and Feltmann, 1998) affords a means for understanding semen quality in terms of a physiological process subject to genetic selection. Moreover, this perspective makes semen production amenable to analysis by systems biology. In this regard, the male reproductive tract yields a sperm sus-

¹USDA-NIFA-AFRI Award No. 2011-67015-20035 titled “A gene-based, quantitative definition of semen quality.”

2Corresponding author: david.froman@oregonstate.edu

©2012 Poultry Science Association Inc.

Received March 8, 2012.
Accepted June 4, 2012.

2012 Poultry Science 91:2621–2627
http://dx.doi.org/10.3382/ps.2012-02289

© 2012 Poultry Science Association Inc.

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bated by protracted transit through the deferent duct, an organ that holds the bulk of a rooster’s extragonadal sperm reserve (ESR; Lake, 1984). Sperm transit through the deferent duct is estimated by dividing ESR by daily sperm production (DSP), where

\[ \text{DSP} = \text{testis weight} \times \left( \text{sperm g}^{-1} \cdot \text{testis d}^{-1} \right). \]

Daily sperm production is readily estimated by homogenizing a testis in the presence of Triton X-100 and then using a hemacytometer to determine the concentration of homogenization-resistant nuclei in the homogenate (Amann, 1981). This method requires one to make serial dilutions of a testis homogenate, mount a coverslip upon a hemacytometer, and then fill the space between coverslip and hemacytometer with diluted homogenate so that volume is uniform among samples, and then allow the loaded hemacytometer to sit in a humid environment before counting so that nuclei can sink to the chamber bottom without loss of sample volume by evaporation. In practice, this can limit replicate size within experiments. We desired a method applicable to the study of populations. Therefore, the present work was performed to determine whether a reliable alternative technique could be developed for estimating DSP and thereby deferent duct transit.

**MATERIALS AND METHODS**

**Experimental Birds**

Roosters were procured from experimental lines of New Hampshire chickens maintained by the Oregon Agricultural Experiment Station. Birds within low and high sperm mobility lines (n = 600 birds per line) were reared and caged with the approval of the Oregon State University Institutional Animal Use and Care Committee in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edition (FASS, 2010). Test subjects were killed by cervical dislocation before reproductive tract removal.

**Reproductive Tract Excision**

Each rooster was placed on its back once postmortem reflexes ended. Skin was removed from the breast, lower back, thighs, and from around the vent. Each knee joint was cut and the lower legs discarded. The tail was removed with poultry shears. Next, the abdominal cavity was opened and viscera exposed. The colon was tied with string at 2 sites several centimeters apart and then severed in between. Viscera were deflected from the body cavity, and the back was severed anterior to the synsacrum. The lower back containing the left and right reproductive tracts and with thighs attached was rinsed with cold tap water to remove blood and feathers from inner and outer surfaces, respectively. Testes were removed by separating the epididymis from the inner body wall, clamping the deferent ducts at the caudal pole of the testis with hemostats, and then cutting the deferent duct above the clamp. Deferent ducts were dissected free from the inner body wall and the outer surface of the cloaca with the aid of a 2× magnifier lamp (Ted Pella Inc., Redding, CA). Deferent ducts were excised by detaching the receptaculum from the cloaca and parcloacal vascular body (see Baumel, 1979; Lake, 1981), clamping the posterior receptaculum, and then cutting the interface between the posterior receptaculum and cloaca. Finally, adhering adipose tissue and ureters were trimmed from excised deferent ducts.

**Extragonadal Sperm Reserve**

A standard curve was developed for estimating the sperm concentration in semen procured from the deferent duct as follows. A 40-mL volume of pooled, ejaculated semen was mixed and subdivided into 2-mL microcentrifuge tubes. Semen was centrifuged at 20°C for 20 s at 15,000 × g in a Microfuge 22R Centrifuge (Beckman Coulter Inc., Fullerton, CA). Seminal plasma supernatants were pooled and filtered through a 0.2-µm Acrodisc filter (VWR International, San Francisco, CA). Volumes of concentrated semen were pooled, and sperm cell concentration was determined with a hemacytometer. Next, sperm-free seminal plasma and concentrated semen were mixed to yield 500-µL volumes that contained 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% (vol/vol) concentrated semen. A 2-mL volume of 3% (wt/vol) sodium chloride was added to each of 50 polystyrene cuvettes from a Repipet II dispenser (VWR International). Thereafter, a 10-µL volume of semen was added to a cuvette (n = 5 replicates per standard) with a Rainin M-25 positive displacement pipette (Mettler-Toledo Inc., Columbus, OH). Each cuvette was covered with a small square of Parafilm (VWR International), contents were mixed by inversion, and absorbance at 550 nm measured with a Barnstead/Turner SP-830 spectrophotometer (VWR International). Data points approximated a linear function. Therefore, the parameters of \( y(x) = \alpha + \beta(x) \) were estimated by linear regression (Sokal and Rohlf, 1969c).

The ESR was estimated as follows. First, the clamp was removed from the receptaculum. The deferent duct was suspended vertically over a 15-mL plastic centrifuge tube by holding the attached hemostat horizontally. Then the receptaculum was nicked with a pair of iris scissors and then lowered to make contact with the inner surface of the tube. Gentle but firm pressure was applied to the anterior end of the deferent by holding the duct between thumb and forefinger. Pressure was maintained as the hemostat was rotated clockwise. As deferent duct length decreased, the hemostat was lowered to maintain stationary contact between the cut end of the deferent duct and the collecting tube. The volume of exuded semen was estimated by setting a Rainin M-1000 positive displacement pipette (Mettler-Toledo Inc.) to 0 µL, placing the capillary tip within the semen, slowly rotating the drive wheel backward until...
the semen sample was transferred from tube to capillary, and then reading the volume aspirated. Sperm concentration was then determined as outlined above. The extragonadal sperm reserve was estimated by multiplying the volume of exuded semen by the sperm concentration therein.

**Testis Homogenization**

Upon removal, each testis was weighed on a TR-602 toploading balance (Denver Instrument Co., Arvada, CO), wrapped in aluminum foil, and frozen on a block of dry ice. Once frozen, testes were stored in liquid nitrogen vapor before homogenization, which was performed as follows. Each frozen testis was placed within a 250-mL graduated cylinder. Next, 0.05% (vol/vol) Triton X-100 and 0.02% sodium azide in 150 mM sodium chloride (homogenization medium) was added to procure a 10% (wt/vol) suspension upon homogenization. The frozen testis and homogenization medium were poured into a 1-L stainless-steel container, the container was covered with a lid, and contents homogenized with four 30-s pulses in a Waring blender (VWR International). The homogenate was poured through 4 layers of cheesecloth into a 250-mL Erlenmeyer flask.

**Daily Sperm Production**

A standard curve was developed for estimating the concentration of homogenization-resistant sperm nuclei as follows. First, a testis homogenate was prepared as described above. Second, nuclei were washed and concentrated by centrifugation through 15% (wt/vol) Accudenz (Accurate Chemical and Scientific Corporation, Westbury, NY) as follows. A 30% (wt/vol) stock solution of Accudenz stock was prepared with 3 mM KCl containing 5 mM N-tris-[hydroxymethyl]methyl-2-amino-ethanesulfonic acid (TES), pH 7.4, as the solvent. This stock solution was used to prepare a 15% (wt/vol) Accudenz solution by dilution with homogenization medium. A 20-mL volume of this solution was placed within a 50-mL conical centrifuge tube and underlaid with 1 mL of 30% (wt/vol) Accudenz. A 20-mL volume of homogenate was overlaid upon the 15% (wt/vol) Accudenz solution, and the tube was centrifuged at 10°C for 60 min at 1,259 × g. Supernatants were discarded. A stock suspension was prepared by diluting washed nuclei to 10 mL, (5) determining the concentration of nuclei within this suspension by measuring the absorbance of a 1:4 dilution, (6) correcting for dilution, volume, (i.e., the number of nuclei in the homogenate based upon the number in the 20-mL subsample), and recovery, and (7) dividing the predicted number of homogenization-resistant nuclei by 4.5 d (de Reviers and Williams, 1984).

**Fluorescence Microscopy**

Washed homogenization-resistant nuclei were evaluated by fluorescence microscopy as follows. A 10 mg/mL solution of Hoechst 33342 was purchased from Invitrogen (Carlsbad, CA). A 10-µL volume of dye was mixed with a 90-µL volume of sperm nuclei in microcentrifuge tube. The diluted dye was allowed to react with sperm nuclei for 10 min at room temperature. Thereafter, the tube was centrifuged at 10,000 × g for 10 s at 20°C. The supernatant was discarded and pelleted nuclei were suspended in a 400-µL volume of homogenization medium. Stained nuclei were visualized under oil.

**Experiment 1**

Deferent duct transit was compared between left and right reproductive tracts from sexually rested roosters (n = 12). Test subjects were taken from the Oregon State University low sperm mobility line. Once reproductive tracts were excised, testes were weighed and frozen. The ESR of each tract was estimated as outlined above. Frozen testes were homogenized, DSP estimated, and deferent duct transit predicted as outlined above. A randomized complete block design (Sokal and Rohlf, 1969c) was used to compare testis size, efficiency of sperm production (sperm g⁻¹-tests d⁻¹), and deferent duct transit between left and right reproductive tracts.

**Experiment 2**

Deferent duct transit was compared between the Oregon State University low and high sperm mobility
lines of chickens (n = 11 roosters per line) as follows. Sperm mobility was measured according to Froman et al. (1999). Left reproductive tracts were excised from sexually rested roosters as described above. Each testis was weighed and frozen. Then the ESR was estimated as outlined above. A randomized complete block design (Sokal and Rohlf, 1969c) was used to compare sperm mobility, efficiency of sperm production, deferent duct sperm concentration, and deferent duct transit between lines.

RESULTS AND DISCUSSION

Our first task was the construction of a standard curve from which sperm concentration could be predicted for ESR estimation. According to Lake (1984),

Figure 1. Absorbance as a function of sperm cell concentration. Whole semen was centrifuged at 15,000 \( \times g \) for 20 s at 20°C to concentrate sperm. A hemacytometer was used to determine sperm concentration within this stock suspension. Next, the stock was diluted with seminal plasma to provide a 10-fold range in concentration. Absorbance was measured after mixing a 10-\( \mu \)L volume of semen with a 2-mL volume of 3% (wt/vol) saline. Open circles represent observed absorbance (n = 5 observations per concentration). The solid line represents the straight line relationship \( y(x) = 0.0558 + (0.1212)(x) \). The slope and y-intercept were estimated by the method of least squares.

Figure 2. Homogenization-resistant sperm nuclei recovered from a whole testis homogenate. The testis was stored at \(-80^\circ\)C before homogenization. A Waring blender (VWR International, San Francisco, CA) was used to homogenize the testis in isotonic saline containing 0.05% (vol/vol) Triton X-100. Nuclei were washed by centrifugation through 15% (wt/vol) Accudenz (Accurate Chemical and Scientific Corporation, Westbury, NY) prepared with homogenization medium. Washed nuclei were stained with Hoechst 33342 (Invitrogen, Carlsbad, CA).

Figure 3. Absorbance as a function of sperm nuclei concentration. Homogenization-resistant nuclei were procured by centrifugation through 15% (wt/vol) Accudenz (Accurate Chemical and Scientific Corporation, Westbury, NY) prepared with homogenization medium. A hemacytometer was used to determine the concentration of homogenization-resistant nuclei within a stock suspension. The stock suspension was diluted with homogenization medium to provide a 12-fold range in concentration. Open circles represent observed absorbance (n = 5 observations per concentration). The solid line represents the straight line relationship \( y(x) = 0.0546 + 0.0329(x) \). The slope and y-intercept were estimated by the method of least squares.

Figure 4. Correlation between testis weight and daily sperm production (DSP). Each open circle denotes a data pair (n = 46 testes). The solid line denotes the regression equation: DSP = 0.155 + (0.071) (testis weight). The product-moment correlation coefficient was 0.956.
sperm concentration ranges between 5 and $7 \times 10^9$ mL$^{-1}$ before ejaculation. Consequently, we needed a sperm concentration range between 5 and $7 \times 10^9$ mL$^{-1}$ before ejaculation. Our choice of instrumentation took into account the opinion of de Reviers and Williams (1984), who deemed the spectrophotometer to be a superior means for estimating sperm cell concentration. As shown in Figure 1, a linear relationship ($r = 0.999$) was observed between sperm cell concentration and absorbance. Absorbance data were precise as evidenced by dispersions of replicate observations (Figure 1). Moreover, the range afforded estimation of sperm concentration within undiluted semen from the deferent duct. Thus, use of a positive-displacement pipette to measure deferent duct semen volume in combination with a standard curve (Figure 1) enabled estimation of the ESR.

As explained within the Introduction, estimation of deferent duct transit requires 2 variables in addition to ESR: testis weight and daily sperm production. Whereas testis weight was measured with a top loading balance, quantification of daily sperm production required a 3-step process: (1) homogenization of a whole testis, (2) isolation of homogenization-resistant sperm nuclei from the homogenate, and (3) estimation of nucleus concentration within the isolate. The 20-mL volume of homogenate from which nuclei were procured represented 12% of the homogenate on average. Mean recovery was 77% (n = 12; CV = 5.9%). Fluorescence microscopy confirmed the efficacy of the isolation step (Figure 2). Based on these statistics, we deemed the isolation step subject to modest correction and to be repeatable. As shown in Figure 3, a linear relationship ($r = 0.999$) was observed between the concentration of homogenization-resistant sperm nuclei and absorbance. As such, this relationship afforded a standard curve for estimating the concentration of sperm cell nuclei within an isolate following testis homogenization.

Standard curves afforded a parsimonious approach for estimating ESR and daily sperm production with a spectrophotometer. The first application of this approach is summarized in Table 1. Thus, the first experiment was performed to determine whether transit differed between the left and right deferent ducts. No difference was observed between left and right tracts with respect to testis weight, efficiency of sperm production, ESR, or deferent duct transit time ($P > 0.05$). In contrast, each of these variables varied among males ($P < 0.05$). Our experimental method detected differences in reproductive tract output among males within a population. Mean values were consistent with data summarized by Lake (1984) as well as de Reviers and Williams (1984), i.e., an ESR equivalent to several days of sperm production and 80 to 120 $\times 10^6$ sperm per gram of testes. We limited our analysis to one tract per male in the second experiment in view of the similarity observed between the left and right reproductive tracts.

The second experiment was performed to compare reproductive tract output between experimental lines of chickens selected for sperm mobility. Data are summarized in Table 2. Sperm mobility data were included to document the difference between lines. Testis weight was measured but omitted from Table 2 because we knew beforehand that this variable differed between lines (D. P. Froman, unpublished data). We have attributed this difference to a founder effect because testis weight was not correlated with sperm mobility in the random bred population from which the low and high sperm mobility lines were developed ($r = 0.01$; Froman and Feltmann, 1998). Deferent duct transit differed between lines ($P < 0.001$). In high line males, the ESR was equivalent to testis output, which meant that transit required 1 d (Table 2). In contrast, deferent duct transit was twice as long in low line males. In this regard, estimates were consistent between experiments.

Whereas efficiency of sperm production was comparable between lines ($P > 0.05$), the deferent ducts of low line males contained almost 1.4-fold more sperm per unit volume in comparison to high line males ($P < 0.001$). Moreover, sperm from low line males spent

<table>
<thead>
<tr>
<th>Tract</th>
<th>Testis weight (g)</th>
<th>Efficiency of sperm production ($\times 10^6$ sperm/g of testis)</th>
<th>Extragonadal sperm reserve ($\times 10^9$ sperm)</th>
<th>Deferent duct transit (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>18.1 ± 3.16</td>
<td>79.9 ± 5.11</td>
<td>3.1 ± 0.84</td>
<td>2.2 ± 0.49</td>
</tr>
<tr>
<td>Right</td>
<td>17.2 ± 4.24</td>
<td>80.3 ± 5.13</td>
<td>2.7 ± 0.84</td>
<td>2.0 ± 0.48</td>
</tr>
</tbody>
</table>

Table 1. Analysis of male reproductive tract throughput

Table 2. Comparison of reproductive tract output between low and high sperm mobility lines of New Hampshire chickens

<table>
<thead>
<tr>
<th>Line</th>
<th>Sperm mobility (absorbance units)</th>
<th>Efficiency of sperm production ($\times 10^6$ sperm/g of testis)</th>
<th>Deferent duct sperm concentration ($\times 10^9$ sperm/mL)</th>
<th>Deferent duct transit (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.046 ± 0.0205$^A$</td>
<td>78.2 ± 4.76</td>
<td>7.4 ± 0.88$^A$</td>
<td>2.2 ± 0.80$^A$</td>
</tr>
<tr>
<td>High</td>
<td>0.533 ± 0.0878$^B$</td>
<td>80.8 ± 6.23</td>
<td>5.4 ± 0.53$^B$</td>
<td>1.0 ± 0.34$^B$</td>
</tr>
</tbody>
</table>

$^A,B$Means within a column differed at $P < 0.001$.

$^1$Each value is a mean ± SD (n = 11 sexually rested roosters per line).
approximately twice as much time within the deferent duct before ejaculation ($P < 0.001$). These observations support our hypothesis that sperm mobility phenotype depends upon a genetic predisposition that puts sperm cells at risk as they pass through the deferent ducts. The glycolytic pathway is compromised in low line sperm as evidenced by sperm cell proteome analysis (Froman et al., 2011). However, this genetic predisposition might affect deferent duct ontogeny, musculature, or innervation in addition to sperm cell glycolysis. At present, a limited glycolytic ability coupled with prolonged deferent duct transit appears to increase the probability of oxidative stress and thereby premature mitochondrial failure. However, the relationship between deferent duct transit and sperm mobility phenotype must be evaluated apart from any potential founder effect, and this was not the goal of the present research. Rather, our goal was to test the suitability of an alternative method for estimating daily sperm production and deferent duct transit.

We accomplished this goal in view of our standard curves and data obtained from the curves. In addition, a posteriori data analysis (Figure 4) corroborated the utility of our method. The product-moment correlation coefficient (Sokal and Rohlf, 1969a) for testis weight versus daily sperm production was 0.956. Data from experiments 1 and 2 were combined ($n = 46$ testes) for this analysis. In comparison, de Reviers and Williams (1984) estimated daily sperm production with a hemacytometer following testis homogenization. The dispersion of data points along our predicted regression line reflects the reliability of the spectrophotometric method.

In conclusion, a new definition of semen quality can be inferred from our experimental outcomes. To date, semen quality has been described in terms of components (Wishart, 2009). However as shown in Table 3, semen quality now can be defined in terms of reproductive tract throughput. In this case, the throughput of mobile sperm from high line males was 16.8-fold greater than low line males even though daily sperm production was 1.3-fold greater in low line males due to testis size. This new definition affords an opportunity for improving reproductive efficiency because semen quality becomes subject to genetic selection.

### Table 3. Semen quality as affected by the combined effects of reproductive tract throughput and the proportion of mobile sperm ejaculated

<table>
<thead>
<tr>
<th>Line</th>
<th>Observed testis output $^2$ ($\times 10^6$ sperm/d)</th>
<th>Observed deferent duct transit $^3$ (d)</th>
<th>Predicted throughput $^4$ ($\times 10^9$ sperm/d)</th>
<th>Predicted proportion of mobile sperm ejaculated $^5$ (p)</th>
<th>Predicted output of mobile sperm $^6$ ($\times 10^6$ sperm/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>1.74</td>
<td>2.2</td>
<td>7.9</td>
<td>0.06</td>
<td>47.4</td>
</tr>
<tr>
<td>High</td>
<td>1.31</td>
<td>1.0</td>
<td>13.1</td>
<td>0.61</td>
<td>799.1</td>
</tr>
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</table>

$^1$Observed values are averages from the left reproductive tracts of 11 sexually rested roosters per line.

$^2$Output = testis weight $\times$ (sperm/g of testis per d).

$^3$Transit = extraglandal sperm reserve $\div$ [testis weight $\times$ (sperm/g of testis per d)].

$^4$Throughput = testis output $\div$ deferent duct transit.

$^5$Estimated by first measuring sperm mobility in absorbance units and then solving this equation: absorbance units = $-0.0065 + (0.9137)(p)$.

$^6$Output of mobile sperm = throughput $\times$ p.

### REFERENCES


