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Methods for the Prediction of Breeding Success in Male Cynomolgus Monkeys (*Macaca fascicularis*) Used for Reproductive Toxicology Studies

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The cynomolgus monkey (*Macaca fascicularis*) may be the species of choice for nonclinical reproductive toxicology studies because of the comparability of its reproductive processes to man, similarity of pharmacologic response to various test substances, and decreased probability of immunogenic response to biological therapeutic products. For success in conducting these studies, the male and female monkeys used need to be characterized thoroughly. This study focused on the evaluation of 42 male cynomolgus monkeys as potential breeders for reproductive toxicology studies. Parameters evaluated included age, body weight, testicular volume, serum testosterone levels, ejaculate volume, and sperm parameters (count, motility, and morphology). The results showed that physical parameters (age, body weight, testicular volume) were a good starting point for selection of potential breeder males. However, additional endpoints (testosterone; ejaculate volume; sperm count, motility and morphology; mating behavior) were also helpful as part of an overall 'weight of evidence' approach to optimize selection of breeder males. In light of the data obtained in this study, 29 of 42 of the males evaluated were used with excellent success as breeder males for reproductive toxicology studies, resulting in an overall pregnancy rate of 36% when mated with sexually mature females. The successful breeder males were at least 6 y old, weighed at least 5 kg, had testicular volumes of at least 25 ml and serum testosterone levels of 1 to 10 ng/ml, and produced ejaculates with large numbers of sperm (median: 502×10^6 per ejaculate) of high quality (few morphologic defects and most sperm highly motile).

Testing for potential reproductive toxicology effects is often a required part of safety assessment for pharmaceutical drug candidates, especially those intended for use in women of childbearing potential. Most of this testing is done in rats, mice, and rabbits. However, in some cases nonhuman primates are the most appropriate species for reproductive toxicology studies, because of the comparability of the reproductive processes to those of humans, similarity of pharmacologic response to various test substances (e.g., biologics, recombinant proteins), and decreased probability of immunogenic response to biological therapeutic products. Cynomolgus monkeys are the nonhuman primate species used most commonly for reproductive studies, because of their availability and the fact that they will breed year-round (rhesus macaques are seasonal breeders).

The use of monkeys for reproductive toxicology testing presents several unique challenges. Menstrual cycles and gestation periods are long (approximately 28 d and 5.5 mo, respectively), resulting in studies of long duration. Obtaining adequate numbers of pregnancies is difficult. A typical pregnancy rate in a mating-dependent study is 20% to 40% (compared with 80% to 90% in rodents), and the abortion rate is usually 15% to 20%.[3] Furthermore, monkeys nearly always have single offspring, making the sample size limited for data interpretation. Because of these considerations, laboratories conducting nonhuman primate reproductive toxicology research should make every effort to ensure that sexually mature males and females with optimal fertility are used for these studies. This attention will help address potential logistical issues (that is, time required to reach target number of pregnancies) and ethical issues (that is, use of minimum number of animals).

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It is difficult to ensure that monkeys are sexually mature when purchased or imported from suppliers of animals. Typically, the animals are requested as 'sexually mature' or 'proven breeders' from the suppliers. Documentation of previous breeding success is sometimes provided with the animals. However, age and weight generally are the only qualifications used by the suppliers. Age and weight can provide a reasonable 1st indication of sexual maturity.[10] But to ensure the sexual maturity of male or female monkeys for use as breeders in reproductive toxicity studies, and increase the probability of success in mating trials, additional evaluations are needed. For female monkeys, sexual maturity can adequately be determined by ensuring the presence of regular menstrual cycles of normal duration. For males, however, no single outward sign can guarantee sexual maturity. To optimize selection of male monkeys as breeders, a more comprehensive evaluation of factors related to male fertility has been used by some laboratories.[5,13]

The present study investigated multiple factors that could be used to evaluate male cynomolgus monkeys for potential success as breeders in reproductive toxicology studies. A 'weight of evidence' approach based on evaluation of multiple factors was used to optimize selection of breeder males. These factors included physical parameters (age, body weight, testicular volume) and functional parameters related to male fertility (serum testosterone, ejaculate volume, sperm quality). In light of the data obtained in this study, 13 of 42 males purchased as sexually mature or proven breeders were excluded from consideration as potential breeders. The remaining 29 males selected through the screening procedures were used with excellent success as breeders for reproductive toxicology studies, leading to an overall pregnancy rate of 36% in the females to which they were bred.

Materials and Methods

Animals. We evaluated 42 experimentally naïve male cynomolgus monkeys for possible use as breeder males for reproductive toxicology studies by mating them to sexually mature females (evidence of regular menstrual cycles). The males (3 shipments) and females (multiple shipments) were obtained from Scientific Resources International (Reno, NV) and Covance Research Products (Alice, TX). Sexually mature males and females were requested from the suppliers, who were asked to include records of previous breeding history (if available). Previous breeding history was available for only 8 of the males and for a negligible number (<5%) of the females. Upon delivery, the animals were quarantined at Charles River Laboratories Preclinical Services (Sparks, NV) for at least 31 d as required by the Centers for Disease Control and Prevention, and then were further acclimated to the facility for at least 3 mo prior to experimental use. During quarantine the animals were confirmed by serology or polymerase chain reaction assay or both to be negative for simian retrovirus, simian immunodeficiency virus, and simian T-cell leukemia virus. The males were at least 3.8 y old and weighed at least 3.4 kg at the start of the study. The females to which the males were mated were at least 3.5 y old and weighed at least 3.0 kg.

Mating. Menstrual cycles of the females were determined by daily vaginal swabs during a 3- to 4-mo prestudy period; at least 3 cycles were determined for each female prior to mating. Matings were conducted by introducing a female near the time of ovulation (approximately midcycle) to the male breeder in his cage (double-size), and cohabitating the mating pair for 3 consecutive days. Mating pairs were selected at random, consistent with standard procedures for Good Laboratory Practice reproductive toxicology studies. The 2nd day of mating was considered day 0 of gestation. Pregnancy was confirmed in the females by ultrasonography (Ultramark 440C, Medison, Seoul, Korea) on days 20 and 25 of gestation.

Experimental procedures. Treatment of the animals was in accordance with the laboratory's standard operating procedures, which adhere to the regulations outlined in the Animal Welfare Act and the conditions specified in the *Guide for Care and Use of Laboratory Animals*.[6] The study protocol was approved by the institutional animal care and use committee prior to the initiation of the procedures.

To determine testicular volume, male monkeys were sedated with ketamine HCl (to effect), and calipers were used to measure the length and width of each testicle. The volume (ml) of each testicle was calculated according to the formula for an ellipsoid object.[2,11,12] At the same time, 1 or 2 blood samples (2 ml each) were collected from each male for evaluation of serum testosterone levels; collection time was standardized to between 0900 and 1500. The serum testosterone levels were evaluated by David Hess (Oregon National Primate Research Center, Beaverton, OR) using a chemiluminescence assay. The normal range for testosterone was considered to be 1 to 10 ng/ml, with peak values of 20 ng/ml during normal episodic secretion.[4]

Semen was collected using penile electroejaculation,[9] with minor modification. This method was preferred to avoid the loss of sperm into the bladder that can occur when using rectal probe stimulation.[8,9] Prior to conducting the procedure, all males were acclimated on at least 2 occasions to the nonhuman primate restraint chairs and other aspects associated with the procedure, to minimize possible stress. When possible (most males), 2 semen samples were collected approximately 1 wk apart. A maximum of 4 attempts to collect semen were made at each session, by using the smallest stimulation possible, according to standard operating procedure. If no semen sample was obtained, the males were given several days rest prior to the next attempt for sample collection. Ejaculates were collected into preweighed vials to determine ejaculate volume by weight.

Approximately 20 µl of the liquid portion of the ejaculate was collected and placed in a plastic culture dish containing 1 ml 1% bovine serum albumin in phosphate-buffered saline that was prewarmed to a temperature of approximately 38 °C. Approximately 20 µl of the diluted sperm sample was placed on a prewarmed microscope slide, cover-slipped, and evaluated by light microscopy for the presence of sperm and motility and morphology determinations. The motility rating scale used was: 1, sperm present, no motility; 2, sperm have little or weak movement; 3, sperm show a mixture of weak movement and some rapid movement; 4, most sperm have strong, rapid movement; 5, all sperm have strong, rapid movement. Morphologic assessment of the sperm included evaluation of the head, midpiece, and tail. The remaining ejaculate was trypsinized to dissolve the coagulum, and a diluted sample of the semen was evaluated for sperm count by using a hemocytometer. Sperm count was calculated as total number of sperm per ejaculate, which provides a better indicator of testicular output than does sperm concentration per milliliter.[1]

In light of evaluation of the previously described parameters, we selected 34 of the original pool of 42 animals for use in mating in reproductive toxicology studies that required pregnant females (that is, developmental toxicology and peri- and postnatal toxicology studies). The breeding results presented here reflect the use of these males over approximately 2 y (November 2003 through September 2005) on 4 reproductive toxicology studies.

Statistics. Graphical and statistical analysis of the data was conducted using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Linear regression and correlation (Pearson) analyses were performed for age, body weight, and testicular volume data. Additional evaluation of these data and of testosterone levels and sperm parameters were performed by Student *t* test. All tests were 2-sided and conducted at a significance level of $P \leq 0.05$.

Results

The relationship of age and body weight for all of the males (n = 42) that were considered for use as potential breeders is shown in Figure 1A. The males ranged in age from 3.8 to 9.5 y and in body weight from 3.4 to 10.3 kg. There were 8 males at the low end of the age and body weight distributions, compared with the other males on study. All 8 of these males were younger than 6 y and weighed less than 5 kg; values of approximately 6 y old and 5.0 kg were cutoffs for inclusion in the main cluster of the remaining 34 males. For the 8 males just mentioned, age and body weight (mean ± standard error of the mean) were 5.1 ± 0.3 y and 3.8 ± 0.1 kg, respectively. For the remaining 34 males, these values were 7.4 ± 0.2 y and 6.9 ± 0.2 kg, respectively.

Testicular volume data in relation to body weight are shown in Figure 1B. Testicular volume ranged from 5.3 to 70.3 ml. The 8 males at the low ends of the age and body weight distributions also were at the low end of the testicular volume distribution. Testicular volume for these 8 males was 12.4 ± 2.2 ml (range, 5.3 to 22.6 ml). The testicular volumes for 7 of these males were less than 20 ml, which has been cited as a minimum value consistent with sexual maturity in male cynomolgus monkeys.[3,11,12] For our data, a value of approximately 25 ml was the cutoff for inclusion in the main cluster of males. The 34 males in the main cluster had a testicular volume of 48.7 ± 2.0 ml (range, 25.9 to 70.3 ml).

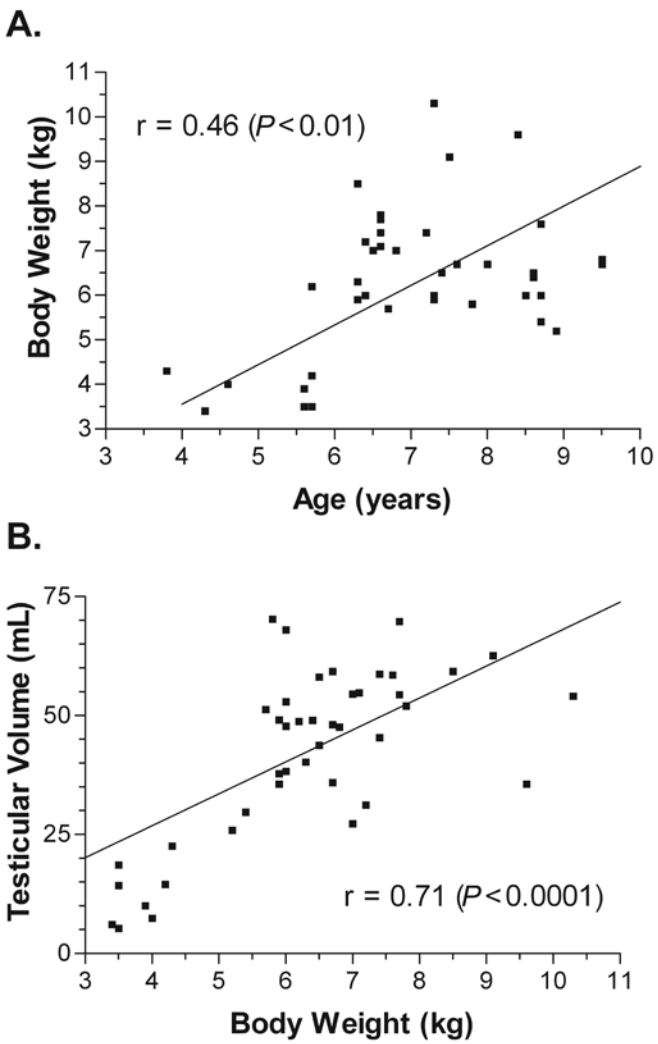

Figure 1. Physical parameters of male cynomolgus monkeys (n = 42) considered for use as breeder males. (A) Distribution and correlation of age to body weight. (B) Distribution and correlation of body weight to testicular volume. Statistical analysis by linear regression and correlation analysis was done; the correlation coefficient (*r*) and its statistical significance are shown.

Statistical analysis confirmed that age, body weight, and testicular volume were significantly lower in the cluster of 8 males identified in the distribution plots, compared with the remaining 34 males (Figure 2A through C). In light of the overall assessment of the physical parameter data, a preliminary classification was made of the 8 males as nonbreeders and the remaining 34 males as potential breeders.

Further refinement in the evaluation of the potential breeding candidacy of all 42 males was obtained by measurement of serum testosterone, ejaculate volume, and sperm parameters (count, motility and morphology). These parameters were consistently lower in the cohort of 8 nonbreeders compared with the 34 breeders (Figure 3A through D); differences between the 2 groups reached statistical significance for sperm count and motility. Group-average serum testosterone in the nonbreeders (4.1 ± 1.1 ng/ml) was approximately 1/2 that in the breeder group, and 2 of the nonbreeders had very low serum testosterone levels (<1.0 ng/ml). Average ejaculate weights (338 ± 153 mg) were approximately 1/2 of those in the breeder candidates, and 1 nonbreeder did not produce an ejaculate at all despite multiple trials. Sperm counts in the nonbreeders were low (130 ± 70 million sperm per ejaculate), as were sperm motility scores (2.2 ± 0.5 on a scale of 1 to 5). Five of the nonbreeders had morphologic defects of sperm, including bent tails; bent midpieces; protoplasmic droplets; and coiled, curled, or looped

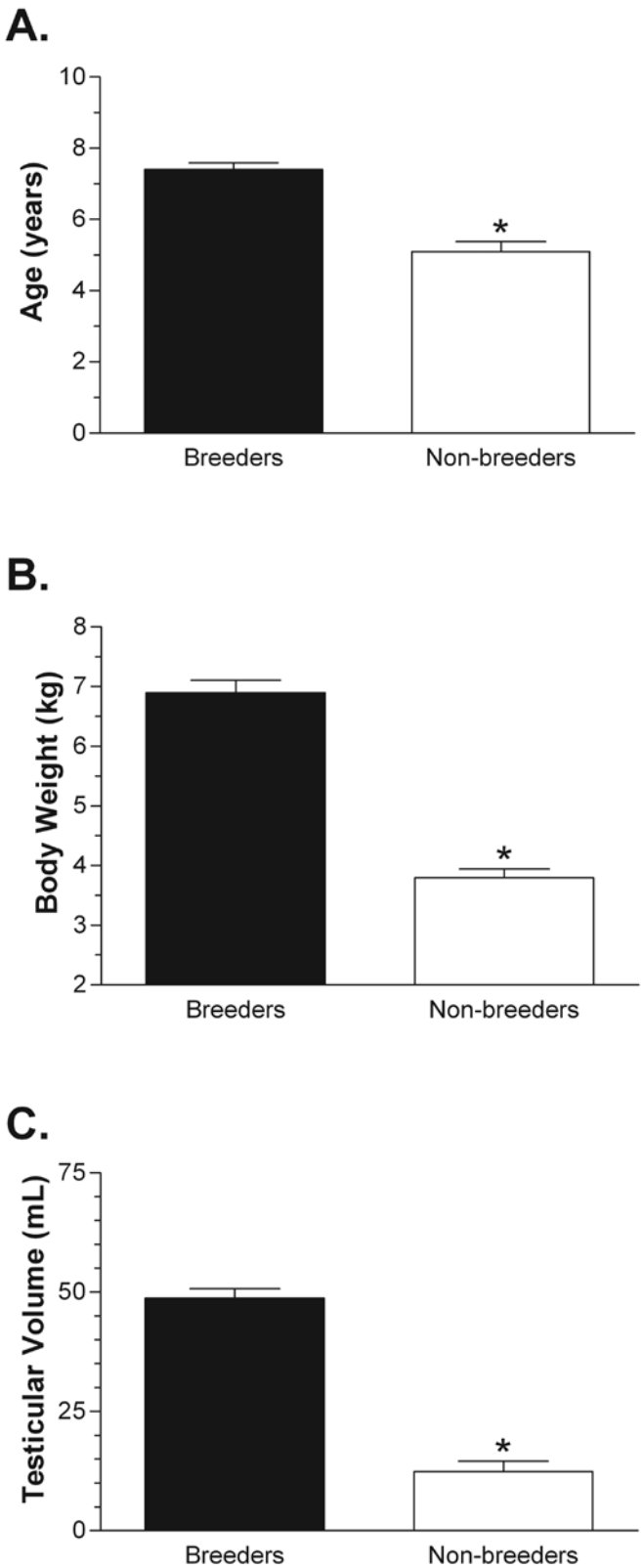

Figure 2. Comparison of (A) age, (B) body weight, and (C) testicular volume in males preliminarily classified as breeders (n = 34; solid bars) or nonbreeders (n = 8; open bars). Data are presented as mean ± standard error of the mean. Asterisks indicate statistical significance (*t* test, $P \leq 0.05$).

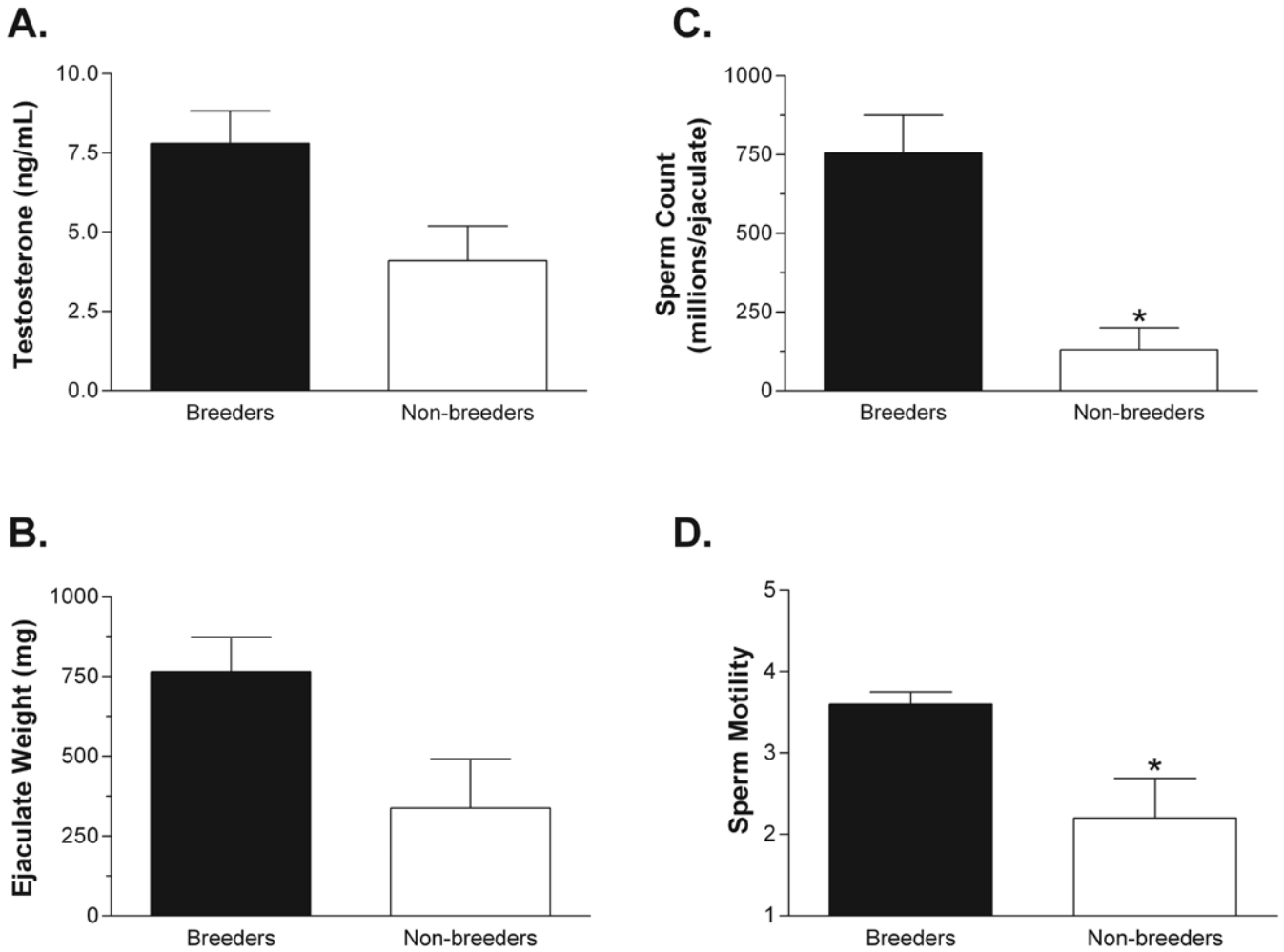

Figure 3. Comparison of (A) serum testosterone, (B) ejaculate volume, and sperm (C) count and (D) motility in males preliminarily classified as breeders (n = 34; solid bars) or nonbreeders (n = 8; open bars). Data are presented as mean ± standard error of the mean. Asterisks indicate statistical significance (*t* test, $P \leq 0.05$).

tails—an estimated 30% to 90% of the sperm had defects. In contrast, all 34 of the males considered as breeder candidates had acceptable serum testosterone levels (7.8 ± 1.0 ng/ml) and produced large ejaculates (764 ± 108 mg; median: 510 mg) with large numbers of sperm (755 ± 120 million sperm per ejaculate; median: 502 million per ejaculate), with sperm of good motility (3.6 ± 0.1 on a scale of 1 to 5; median score: 4) and few morphologic defects.

The 34 males considered to be good candidates for breeding were mated with females in 4 reproductive toxicology studies conducted at our laboratory over approximately 2 y (November 2003 through September 2005). Among these males, 29 of 34 proved to be successful breeders and induced an overall pregnancy rate of 36% in the females with which they were bred (Table 1). The remaining 5 males were unsuccessful as breeders. One of these males showed unacceptably aggressive behavior toward females during its first 2 matings and was removed from the breeder population. The other 4 males produced no pregnancies after as many as 6 matings each and therefore also were removed from the breeder pool. These 4 males appeared comparable to the 29 successful breeders for all parameters evaluated (Table 2), with the exception of low serum testosterone (1.08 ng/ml) in 1 of the unsuccessful breeders.

The overall screening process, based 1st on evaluation of physical parameters (age, body weight, testicular volume) followed by assessment of functional parameters (testosterone and sperm quality), resulted in the selection and effective use of 29 of the original 42 males obtained for breeding purposes (Figure 4).

Discussion

This study demonstrated the effectiveness of a comprehensive 'weight of evidence' approach to evaluate male cynomolgus monkeys for sexual maturity and potential success as breeders for reproductive toxicology studies. This approach included multiple endpoints, including age, body weight, testicular volume, serum testosterone levels, semen production, and sperm parameters (sperm count, motility, and morphology). Of 42 males screened with these procedures, 8 did not pass the initial screening tests to qualify as breeder males. Another male displayed unacceptably aggressive behavior during mating, and 4 males used for breeding were unsuccessful in producing pregnancies after as many as 6 mating trials each. The other 29 males that passed the overall selection process have provided an effective pool of breeders. The overall fertility of the selected males has been excellent for laboratory conditions of mating, resulting in an overall pregnancy rate of 36% when mated one-on-one with sexually mature, cycling females.

The value of using a multifactor, 'weight of evidence' approach for evaluating potential breeders was particularly evident in light of the cohort of 8 males that first appeared suspect when

Table 1. Breeding performance of cynomolgus male monkeys selected through a multiparameter screening process

Group	Pregnancy rate; range[a] (no. of pregnancies/no. of matings)[b]
Successful breeders (n = 29)	36%; 20%–55% (197/553)
Unsuccessful breeders (n = 5)	No pregnancies (0/19)

[a]Based on males with a minimum of 10 mating trials.
[b]Based on total number of matings and pregnancies achieved by all males in group.

Table 2. Comparison of breeder male monkeys with successful and unsuccessful mating performance

Endpoint evaluated	Successful breeders (n = 29)	Unsuccessful breeders (n = 4)[a]
Age (years)	7.6 ± 0.2	7.2 ± 0.7
Body weight (kg)	6.9 ± 0.2	7.5 ± 0.4
Testicular volume (mL)	50.3 ± 2.0	47.3 ± 6.3
Serum testosterone (ng/mL)	8.2 ± 1.1	4.8 ± 2.0[b]
Ejaculate weight (mg)	753.2 ± 121.8	812.5 ± 250.5
Sperm count (×10^6 per ejaculate)	734.1 ± 136.2	847.0 ± 224.1
Sperm motility (scale of 1 to 5)	3.6 ± 0.2	3.8 ± 0.4

Data are presented as mean ± SEM. Statistical analysis by *t* test ($P \leq 0.05$) failed to reveal any significant differences between groups.
[a]Excludes one male with unacceptably aggressive behavior, because it was only mated twice. The other males in this cohort were used for up to 6 matings each.
[b]Group mean influenced by 1 male with value of 1.08 ng/ml.

they were noted as clustering on the low end of distribution ranges for age, body weight, and testicular volume. Five of these males were older than 5.6 y and could have been considered good candidates for breeding. Smedley and colleagues[10] found that approximately 90% of cynomolgus males older than 5.4 y were sexually mature, as assessed by testicular histopathology. Other data have shown that testicular volumes of 10 to 20 ml correlate with sexual maturity in male monkeys.[2,5,11,12] According to this criterion, 1 other male in this cohort could have been considered sexually mature; this animal was only 3.8 y old but had a testicular volume of 22.6 ml. Therefore, on the basis of single endpoints of age or testicular volume, 6 of the 8 males could have been considered for breeding. Instead, in light of our more comprehensive evaluation, we decided not to use any of these males as breeders. The physical parameters of age, body weight, and testicular volume originally detected these 8 males as outliers (Figure 1), and data on serum testosterone and sperm quality (Figure 3) provided further confirmation of their poor candidacy as breeder males. These 8 males never actually were bred to confirm the expectation of low fertility. By our judgment, breeding success would have been unlikely and attempts to breed not consistent with purposeful (ethical) use of either the males or the females to which they would have been mated.

There were 4 males selected as breeders from the screening process that did not produce a pregnancy after as many as 6 matings each. These males did mate (confirmed by observation), and given the overall 30% to 40% pregnancy rate achieved by the other breeders, at least 1 pregnancy was expected after this number of matings. These 4 males therefore were removed from the breeding pool, in favor of other males that were performing better. Interestingly, these 4 nonbreeder males could not be distinguished from the 29 successful breeders in light of any of the physical or functional parameters evaluated (Table 2). It is possible that more subtle influences such as the males' social upbringing (group housing versus individual cages) could have influenced their reproductive performance (for example, mounting behavior, penile penetration, or ejaculation). Mounting behavior was observed for these males, but practicalities of carrying out the mating procedures precluded evaluation of penetration and ejaculation. This situation reinforces the concept that prospective screening for sexual maturity optimizes the likelihood of breeding success but does not guarantee it. Such screening can still assist in decisions regarding optimal use of the animals, however. For example, mature males that do not mate well are excellent candidates for general toxicology or male fertility (nonmating) studies that require sexually mature males.

All of the factors evaluated in this study proved to be useful in the overall evaluation of the males as potential breeders. Age and body weight are traditional starting points, and in our study, the successful breeder males were at least 6 y old and weighed at least 5 kg. Testicular volume has been reported by some investigators as being neither reproducible nor reliable as an indicator of sexual maturity,[10] but we and others[2,5,11,12] found it useful for selection of breeder males. In our study, a testicular volume of approximately 25 ml was the cutoff for inclusion in the successful breeder cohort; others[2,5,11,12] have used values of 10 to 20 ml. Serum testosterone levels are inherently variable due to episodic secretion; nonetheless, males with values <1 ng/ml were obvious outliers. Males successful as breeders had testosterone levels of at least 1 to 10 ng/ml, with peak values of approximately 20 ng/ml. This pattern is consistent with data indicating a correlation between plasma testosterone and sexual maturity in macaques.[7] Semen collection and analysis of sperm quality (count, motility, and morphology) was also a useful contributing factor in evaluation of the males and helped confirm that the cohort of 8 outlier males were likely not good candidates for breeding. Testicular biopsy data can also be used,[5] but there are potential problems with any invasive procedure, and presumably adequate spermatogenesis is reflected in the more easily obtained semen samples.

Of the 42 males purchased for potential use as breeders, only 8 had documentation by the vendor of previous breeding success in the 2 y prior to shipment to our laboratory; 7 of these 8 proved to be successful breeders. The 8th male did not induce a pregnancy after 3 mating trials. Therefore, the mating data supplied by the vendor, although limited to a small number of males, were considered useful as ancillary data in the described 'weight of evidence' approach. Such data cannot be used to set expectations of pregnancy rates in a laboratory setting, however, because the data from vendors will in most cases be from breeding colonies where the males are group-housed with a harem of females and are engaged in natural (versus timed) matings. In such cases, the reported pregnancy rate is likely to be higher than what can be expected in a laboratory setting with timed mating of paired animals.

The pregnancy rate of 36% reported here is considered to be within the anticipated range for mating cynomolgus monkeys under the conditions used for this study (one-on-one matings in single housing). The pregnancy rate obtained is obviously dependent upon both male and female factors. Coinciding with the evaluation of male nonhuman primates as potential breeders as outlined here, it is also paramount to have comprehensive procedures for evaluating the females that will be used for the mating trials. This process involves conditioning the females

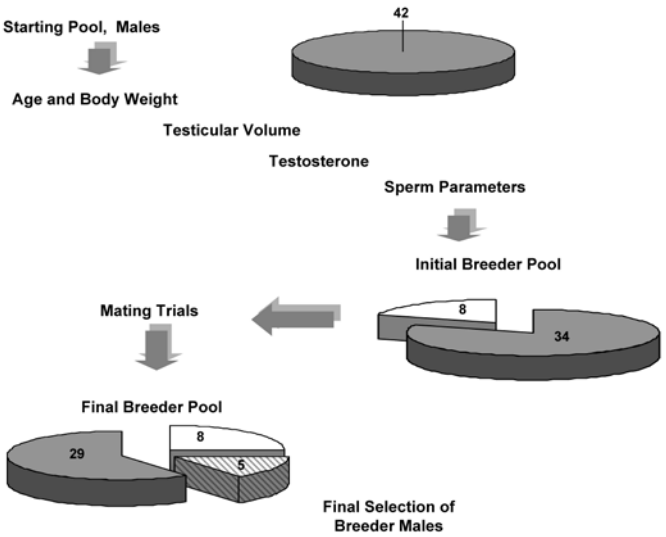

Figure 4. Decision tree to select the final male breeder pool from the overall male population. From the starting pool of 42 male monkeys (solid pattern), 8 (open pattern) were screened out and not used for breeding, whereas 34 animals were retained. Of these 34 males, 5 (striped pattern) were removed from the breeder pool; 1 was unacceptably aggressive in behavior, and 4 did not produce a pregnancy after as many as 6 mating trials each. Therefore, 29 of 42 males eventually made it through the 'weight of evidence' screening process to be used successfully on reproductive toxicology studies.

to the facility and careful characterization of their menstrual cycles during multiple cycles prior to mating. This evaluation determines the typical menstrual cycle length for each female and thereby its optimal cycle time for mating.

In establishing a pool of breeder male monkeys for use in reproductive toxicology studies, the tendency may be to rely on vendor-supplied classifications of the animals, such as 'mature' or 'proven breeders.' However, these classifications generally will be based on only 1 or a few discriminating physical factors such as age, body weight, and testes descent. Vendor-supplied classifications will not encompass a comprehensive evaluation of the multiple factors potentially influencing male fertility, such as testicular volume, testosterone level, ejaculate volume, and sperm quality. Screening of a battery of both physical and functional factors will optimize selection of breeder males, an effort that is well worthwhile because these breeders are a long-term resource that will be used over many years. Use of carefully screened breeder males will decrease the number of matings that need to be conducted to impregnate females for reproductive toxicology studies, thus resulting in more purposeful use of both the males and the females to which they are bred. The successful breeder males from our study were at least 6 y old, weighed at least 5 kg, had testicular volumes of at least 25 ml and serum testosterone levels of 1 to 10 ng/ml, and produced ejaculates with large numbers of high-quality sperm.

Acknowledgments

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