EXTENSIVE studies have been conducted on stallion seminal characteristics by Nishikawa (1959), who concluded that seminal volume and gelatinous material were the only two of seven parameters significantly affected by season. Pronounced seasonal variation in concentration of ergothioneine, citric acid and semen volume was observed by Mann, Leone and Polge (1956). Skinner and Bowen (1968) reported that as daylight decreased reaction time lengthened, concentration of spermatozoa and motility increased and ejaculatory volume and citric acid content declined. Season has also been reported to affect stallion fertility (Wagenaar and Grootenhuis, 1953).

Comparisons of physical and chemical characteristics have been made between first and second ejaculates of bovine semen (Pickett and Komarek, 1967; Seidel and Foote, 1969), but relatively few comparisons are available on stallion semen. Hendrikse (1966) reported that second ejaculates from stallions of normal fertility contained a lower sperm concentration, fewer total sperm and a smaller volume than first ejaculates.

Second ejaculates are generally helpful in studying seminal physiology and sexual behavior. The purpose of this study was to determine the influence of month and stallion on selected characteristics of first and second ejaculates of stallion semen.

**Experimental Procedure**

Two ejaculates were collected within 4 hr. at approximately weekly intervals from two 2-year-old and one 3-year-old Quarter Horse, one 4-year-old and one 5-year-old Thoroughbred stallion. The ejaculates were obtained at approximately equal intervals from December 1967, through November 1968.

The artificial vagina used for collecting semen and the method of separating the gelatinous material (gel) have been described (Pickett, 1968; Komarek et al., 1965). Immediately after collection, the gel-free semen (hereafter referred to as semen) was transferred to a graduated cylinder maintained at 37°C and the volume was recorded. The semen was mixed and subsampled for the various measurements. The gel was not studied in this investigation. Only one stallion consistently produced gel, even during the breeding season.

Percent progressive motility of spermatozoa in raw semen and semen extended 1:20 in 5% glucose was estimated at 200 magnifications with a phase contrast microscope. In conjunction with the motility estimates, the degree of clumping of spermatozoa was estimated in the raw semen. Numerical designations of 0, 1, 2 and 3 were used to indicate none, slight, moderate and severe clumping, respectively.

Sperm concentration per milliliter was estimated photometrically (Pickett, 1968), and pH was determined with a combination glass/reference electrode, digital pH meter. Freezing-point depression was determined on 2.0 ml of seminal plasma obtained by centrifuging 3.0 ml of semen at 12,000 x g for 3 minutes.

The influence of season on sexual behavior was studied by measuring reaction time and copulation time in seconds and number of mounts required per ejaculate. Reaction time was the total time the stallion was in visual contact with the mare until the beginning of copulation. Copulation time was from the moment of entry into the artificial vagina until the stallion started to dismount after
ejaculation. If either of the paired ejaculates had missing values, both ejaculates were eliminated from the analysis for that particular characteristic. The first three collections in each month, for each stallion in which paired ejaculate observations were available, were utilized in the analysis. From the possible 180 paired ejaculates, a range of 165 to 173 observations per criterion was obtained.

Due to unequal subclass numbers, these data were analyzed by 2- and 3-way analyses of variance, using the method of fitting constants (Snedecor and Cochran, 1967). Each characteristic was analyzed separately for each ejaculate to determine the effect of month and stallion. Then a 3-way analysis for each characteristic was performed using ejaculates, months and stallions as main effects. Statistical significance between months (figures 1-11) was tested by Tukey's Range Test (Shortest Significant Range) (Snedecor and Cochran, 1967). Variation due to month and stallion was removed from the standard deviations presented in table 1, while the standard deviations in figures 1 through 11 represent total variation by month, including variation among stallions.

Results and Discussion

Means of 11 characteristics, across months and stallions for first and second ejaculates,
Figure 2. Monthly variation in number of spermatozoa per ml. in millions (N=170).
Figure 3. Monthly variation in total sperm per ejaculate (N=170).
Figure 4. Monthly variation in percent progressively motile sperm (N=165).
Figure 5. Monthly variation in percent progressively motile sperm diluted 1:20 in 5% glucose (N=167).
Figure 6. Monthly variation in degree of clumping of stallion spermatozoa (N=171).
Figure 7. Monthly variation in pH (N=172).
Figure 8. Monthly variation in freezing-point depression (N=172).
Figure 9. Monthly variation in mounts per ejaculation (N=171).
Figure 10. Monthly variation in reaction time per ejaculate (N=170).
Figure 11. Monthly variation in copulation time ($N=172$).
are presented in table 1. The mean volume of semen was 36.6 and 33.1 ml for first and second ejaculates, respectively. The results by ejaculate by month are presented in figure 1. The range was 25 ml in December to 50 ml in March. The mean volume of 36.6 ml was lower than other studies (Day, 1940; MacLeod and McGee, 1950), the means of which ranged from 50 to 73 milliliters. Although not always stated in these reports, most collections were made during the breeding season and included the volume contributed by the gel fraction. Therefore, the results of this study are similar to those studies when gel volume and season are considered.

There were significant (P<.01) month and stallion effects on semen volume for both first and second ejaculates, but no difference (P>.05) between first and second ejaculates. These results are contrary to those of Henrikse (1966), who found that initial ejaculates were larger than those collected later the same day.

The number of spermatozoa per milliliter was different (P<.05) among months for first but not second ejaculates, while the variation due to stallion was extremely large (P<.001). There was also a highly significant difference between first and second ejaculates. The range among stallions across months was 142.4 to 507.5 and 66.1 to 336.7 x 10^6/ml for first and second ejaculates, respectively. The range for first and second ejaculates among months across stallions was 278.8 to 450.5 and 164.1 to 291.4 x 10^6/ml (figure 2), respectively.

Total sperm per ejaculate was affected (P<.01) by month, stallion and ejaculate. First ejaculates collected in May contained 12.7 billion spermatozoa compared to 6.6 billion in November (figure 3). This difference of 6.1 billion represented a 52% reduction. The largest number of spermatozoa in second ejaculates was in June and the lowest was in December, 6.7 vs. 3.1 x 10^9, respectively, a decrease of 46.2%. Although definite proof is lacking, this reduction in sperm output per ejaculate probably represents a decrease in spermatogenesis. This conjecture does not agree with Skinner and Bowen (1968); they stated "that androgen secretion in the stallion is strongly influenced by daylight length, whereas spermatogenesis is not influenced to any noticeable extent." Nishikawa (1959) found no significant seasonal differences in total sperm output from five stallions collected "throughout" the year. In contrast,
Mann et al. (1956) reported a dramatic increase in sperm per ejaculate during May and June.

Second ejaculates across months and stallions (table 1) contained only 49.7% of the number of spermatozoa found in first ejaculates. This approximate 50% reduction in the number of sperm in second ejaculates was quite consistent. For example, the range by month across stallions was 41.4 to 62.2% (figure 3), and by stallion across months was 41.0 to 60.2%. Thus, second ejaculates from stallions that have been sexually rested for at least a week will contain approximately one-half the number of spermatozoa found in the first ejaculate. When the deviation is greater than 10%, the following is suspected: (1) incomplete ejaculation, (2) low sperm reserves, (3) depletion of sperm reserves or, (4) sperm have accumulated in the reproductive tract. Second ejaculates from bulls properly prepared for collection generally contain more than 50% of the number of cells in the first ejaculate (Pickett and Komarek, 1967). It is possible that this ratio in stallions could change as more information is accumulated on methods of preparation and their influence on sexual behavior.

The mean of $12.7 \times 10^9$ spermatozoa per first ejaculate collected in May was in good agreement with the 11.8 and $14.3 \times 10^9$ reported by MacLeod and McGee (1950) and the $13.6 \times 10^9$ observed by Komarek et al. (1965), while the $6.3 \times 10^9$ calculated from Day's data (Day, 1940) is in better agreement with the $6.6 \times 10^9$ obtained in November (figure 3).

Motility estimations made on spermatozoa in raw semen and semen diluted 1:20 in 5% glucose are presented in figures 4 and 5, respectively. The variation due to month was much more pronounced ($P<.01$) than the effect of stallion. Stallion effect was not significant for first ejaculates and only at the 5% level of probability for second ejaculates. The difference between first and second ejaculates was not significant ($P>.05$). The mean motility values and general trends were similar, regardless of method of estimation. In general, motility was highest in winter and spring and lowest in summer and fall; whereas Krause and Grove (1967) observed a marked decrease in motility during early winter.

Motility appeared to be inversely related to the degree of clumping (figure 6), and clumping appeared to be directly related to those months when the animals would be undergoing the transition from the nonbreeding to the breeding season. However, coefficients of correlation between clumping and motility were low. Adjustment of these correlations (Van Duijn, 1968) to clarify these relationships may be necessary.

It is suspected that if clumping had been prevented by using an appropriate extender, the highly significant monthly effect might have been reduced or eliminated. Similar questions have been posed by other investigators (Skinner and Bowen, 1968). If this is the case, other procedures should be used for estimating motility.

The significant ($P<.01$) month and stallion effects on degree of clumping are presented in table 1. There was more ($P<.01$) clumping in first than in second ejaculates. More frequent clumping in first ejaculates might be due to the fact that gel is produced more frequently in first ejaculates, and/or first ejaculates contain a greater number of spermatozoa. There was less clumping in both first and second ejaculates in January (figure 6), while the greatest degree was observed in October and July, respectively.

The pH of first ejaculates (figure 7) was not significantly influenced by month, but there were differences ($P<.01$) among months for second ejaculates. The opposite was true for stallion effect, i.e., differences ($P<.01$) due to stallion were observed in first but not second ejaculates. The first ejaculate pH of 7.43 was lower ($P<.01$) than the 7.60 of second ejaculates. This difference may be due to a greater contribution by certain accessory glands and/or a smaller contribution by the epididymal contents, which follows the observation of Hendrikse (1966) that higher pH's were associated with large ejaculates. Further evidence for this was obtained in this study by a significant ($P<.01$) correlation of $-0.46$ between sperm concentration ($10^6$/ml) and pH, and a correlation ($P<.01$) of $-0.64$ between semen volume and sperm concentration per milliliter in first ejaculates.

Nishikawa (1959) also found no seasonal effect on pH, but the effect on second ejaculates was not reported. The value of 7.4 for first ejaculates was higher than the 6.6 reported by Nishikawa (1959) but was in agreement with others (Hendrikse, 1966; MacLeod and McGee, 1950; Rajamannan, Zemjanis and Ellery, 1968).

Variation in freezing-point depression due to month (figure 8) just exceeded significance
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(P<.05) for both first and second ejaculates; while variation due to stallion was greater (P<.01) in second than first ejaculates (P<.05). There was no difference (P>.05) between the means of 0.620 and 0.622 for first and second ejaculates, respectively. The mean freezing-point depression was slightly higher than those previously reported (Pecnikov, 1956; Rajamannan et al., 1968).

There was a highly significant difference among months and stallions with respect to mounts per ejaculation for both ejaculates. In addition, it required more (P<.01) mounts to collect first than second ejaculates, 1.8 vs. 1.5, respectively. Stallions required an average of 1.4 mounts per ejaculate in natural service and 2.2 with the artificial vagina (Wierzbowksi, 1958); while Asbury and Hughes (1964) collected 130 ejaculates from 52 stallions which took 1.38 mounts per ejaculate. These observations are all within the range shown in figure 9. The range for first ejaculates was 1.2 to 2.4 in April and October, respectively, but for second ejaculates September was lowest (1.0) and January highest (2.1). These data tend to agree with the observations of Wierzbowksi and Hafez (1961) that the number of mounts per ejaculate was characteristic of an individual. However, within individuals there were some extreme variations (table 1), particularly for first ejaculates.

Reaction times were significantly different among months and among stallions for first ejaculates. In second ejaculates, only stallions were different (P<.01). It required more time (P<.01) to collect first than it did second ejaculates, 415 vs. 212 sec., respectively. This observation was contrary to those of Wierzbowksi (1966), who reported that rams, bulls and stallions were slower on the second ejaculate. In their study, the mean reaction time was 206 sec. in August compared to 819 in January (figure 10). A faster reaction time for second ejaculates might mean that the most effective stimuli were not provided for collection of the first ejaculate (Pickett, 1968).

Copulation time (figure 11) was least affected by month or stallion of all the characteristics measured. However, it appeared to be highly individualistic, which was borne out by the rather large standard deviations. There was no difference in copulation time between first and second ejaculates.

The unadjusted standard deviations presented in figures 1 to 11 are consistent with the observations of other investigators (Day, 1940; Pecnikov, 1956; Wagenaar and Grootenhuis, 1953; Wierzbowksi and Hafez, 1961) that characteristics of stallion semen are extremely variable. Although season accounts for considerable variation, additional research is needed to further identify other factors contributing to this extreme variation.

Summary

Eight seminal and three behavioral characteristics were measured during and immediately following collection of first and second ejaculates of semen from each of five stallions over a 12-month period. The number of paired ejaculates per criterion ranged from 165 to 173. The parameters studied were: volume of gel-free semen, sperm concentration per milliliter, total sperm per ejaculate, motility in raw and diluted semen, degree of clumping, pH, freezing-point depression, number of mounts per ejaculate, reaction time and copulation time. A highly significant difference (P<.01) among months was found for all measurements on first ejaculates except sperm concentration per ml, pH, freezing-point depression of seminal plasma and copulation time. Sperm concentration per ml and freezing-point depression were significant at the 5% level of probability. Variation due to stallion was significant (P<.01) for all characteristics of first ejaculates except motility, freezing-point depression and copulation time, and freezing-point depression was different at the 5% level of probability.

The trends were similar for second ejaculates, with respect to the influence of month and stallion (table 1). Differences (P<.01) between the means of first and second ejaculates were observed for all criteria except semen volume, motility, freezing-point depression and copulation time.

Literature Cited


Day, F. T. 1940. The stallion and fertility—The


