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Research Article

The Effect of Proctodeal Gland Foam, and Depth and Frequency of Artificial Insemination on Fertility and Hatchability of Japanese Quail (*Coturnix japonica*)*

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Abstract: The effect of the depth of semen deposition in the female reproductive tract, the addition of proctodeal gland foam to the net semen, and insemination frequency on fertility and hatchability of Japanese quails was investigated. In 2 experiments semen was collected from 30-32 males using the method of male stimulation by female. A group of 30-32 females was used in the fertility trials.

In the first experiment semen obtained from group 1 was collected in empty glass tubes, while from group 2 it was collected in tubes containing Lake's extender kept at 20 °C. Semen obtained from each group was deposited intravaginally or intrauterinely (4 groups of females were created, each comprising 8 specimens). In the second experiment, freshly collected semen was mixed with proctodeal gland foam and used for intravaginal insemination; semen from group 1 was inseminated 2×/week, while semen from group 2 was inseminated 3×/week.

Keeping net (undiluted) semen or semen diluted with Lake's extender for 15 min at room temperature reduced the number of live normal spermatozoa in relation to freshly collected semen. Egg fertility was higher after intravaginal insemination in comparison to intrauterine insemination (49.3% vs. 23.0% when undiluted semen was used, and 49.8% vs. 32.4 for semen diluted with Lake's extender).

Intravaginal insemination 3×/week with 20 µl of semen mixed 1:1 with proctodeal gland foam resulted in significantly higher ($P < 0.01$) egg fertility compared to insemination 2×/week (82.7% vs. 44.1%); the former was similar to that obtained in the naturally mated group (86.1%).

Key Words: Japanese quails, semen quality, artificial insemination, fertility, hatchability

Introduction

The first experiments on artificial insemination in Japanese quails were undertaken almost 50 years ago (1,2). Success in this method of reproduction depends on many factors, of which the semen quality, insemination dose (with particular attention to the number of live, morphologically normal spermatozoa), time interval between semen collection and artificial insemination, depth of semen deposition in the female reproductive track, and insemination frequency are the most important.

In insemination practice the use of freshly collected, diluted, or preserved semen is an important factor. In the case of diluted semen, the composition of diluent, dilution rate, and length of storage play crucial roles. Moreover, skilful and gentle treatment of birds during semen collection and insemination, as well as the efficiency of the procedure, are important when fertility output is considered.

The proctodeal foam gland, present in the dorsal wall of the male proctodeum (3), produces, in the course of ejaculation a frothy, usually milky-white fluid, which plays an important role in quail reproduction. The gland is sexually dimorphic (large in mature and sexually active males, rudimentary in females) and its development, as well as intensity of foam production, depends on testosterone stimulation (4,5). Males with a well-developed gland are characterized by high fertilizing potency and produce dense, white semen (6). It was

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indicated by the above-mentioned authors that during mating, semen is mixed with the foam prior to deposition in the female reproductive tract. Ikeda and Taji (7) suggested that the foam serves as a medium for spermatozoa transportation within the oviduct. The positive effect of the foam on spermatozoa fertilizing ability was described by Cheng et al. (4), who reported that in naturally mated quails 98.0% fertility was accomplished by males with a well-developed proctodeal gland, while fertilizing ability of males with a cauterized proctodeal gland was 26.0%. The authors suggested that the presence of naturally or artificially introduced foam extends the duration of fertility. Results published by Fujihara and Koga (8) demonstrated that foam addition to neat quail semen increased egg fertility from 42.9% to 55.7%. The effect of adding proctodeal foam to diluted quail semen on spermatozoa and on egg fertility has not been investigated.

Ogasawara and Huang (2) were the first authors to use artificial insemination to hybridize the Japanese quail with red jungle fowl. They obtained 13.3% fertility and 6.2% hatchability with intravaginal insemination, while with intrauterine insemination they achieved 36.6% and 2.7%, respectively. Wentworth and Mellen (1), when using intravaginal (9), intraperitoneal (10), or intrauterine insemination methods (1), obtained 23.1%, 45.0%, and 18.8% fertility rates, respectively, whereas in quails mated naturally the fertility rate was 54.3%. McFarquhar and Lake (11) reported 90% fertility for eggs collected from the second to fifth day after intrauterine insemination, but only 38.5% for eggs collected from first to tenth day after intrauterine insemination. Marks and Lepore (12), using a modified method described by Burrows and Quinn (9), obtained fertility ranging between 45% and 71%, depending on the insemination frequency. In other experiments of the above-mentioned authors (12), after intravaginal insemination with 10 µl neat quail semen or 20 µl semen diluted with Ringer's I, Ringer's II, and Locke's and Tyrode's solution, fertility was 67.5%, 62.1%, 68.0%, and 72.3%, respectively. Different procedures for artificial insemination of quail described in the literature also resulted in fertile eggs and healthy chicks; however, data thusly obtained are far from fertility rates recorded in naturally mated quail flocks.

In the present study the effect of the addition of proctodeal gland foam, and depth and frequency of artificial insemination on spermatozoa, fertility, and hatchability of Japanese quails was investigated.

Materials and Methods

General Data on Experiments 1 and 2

Sexually mature Japanese quails (*Coturnix japonica*) were used. Males used as semen donors were kept in individual cages (0.32 × 0.44 × 0.24 m), females used for artificial insemination and those assigned to naturally mated groups (male to female ratio was 1:4) were kept in 5-tier battery cages (1.0 × 0.55 × 1.75 m), and females used for male stimulation (usually 5 sexually matured females that displayed tolerance reflex on presence of a male) were kept in colony cages (0.60 × 0.45 × 0.48 m). All cages were placed in 22 °C rooms. During the reproductive period birds were treated with a 14 h light/10 h dark cycle. Water and feed typical for reproductive quails (21%-22% crude protein; 11.2 MJ energy) were available ad libitum and supplemented with minerals and vitamins.

Semen was collected with the male stimulation by female method (13), 2-3×/week, at 2-4-day intervals. Of the 60 males selected on the basis of proctodeal foam gland size and their response to stimulation, 32 were used. Ejaculate volume, blood or fecal contamination, concentration, and morphology of spermatozoa from freshly collected semen were evaluated. The concentration of spermatozoa was calculated in a hemocytometer after staining with eosin 3% NaCl solution; motility was evaluated under a light microscope.

The integrity and morphology of spermatozoa (300 cells per slide) were examined in nigrosin-eosin smears and evaluated at 1250× under a light microscope (Jenaval, Carl Zeiss, Jena, Germany). Spermatozoa were attributed to 7 classes (14). The results of morphological evaluations were expressed as the percentage of a particular class of spermatozoa (300 cells = 100%).

The quail insemination procedure followed the methodology of Burrows and Quinn (9), and Marks and Lepore (12). The female was taken from the cage and placed in the operator's left hand with the bird's breast located in the palm, whereas the wings and legs were lifted. The tail was delicately raised with the thumb in order to expose the cloaca vent; then slight pressure was applied to the abdomen with the thumb and forefinger of the right hand until eversion of the latter part of the oviduct occurred. After vaginal eversion, a thin plastic pipette (inner diameter 0.5 mm) with semen was inserted intravaginally (Figure) to a depth of about 1 cm, near the

utero-vaginal junction (UVJ), or intrauterinely, i.e. 0.5 cm behind the utero-vaginal junction. In both methods, during semen deposition the hand pressure was released resulting in cloaca withdrawal while the pipette remained in place. Semen was blown through the pipette while connected to a rubber-hose. The fertility trials included 32 females.

Insemination was performed 2-3×/week, 4-5 h after switching the light on and immediately after semen collection or dilution (with extender or foam). Insemination dose for fresh, undiluted semen was 10 µl and 20 µl for diluted semen (diluted with Lake's extender or foam).

The fecundity rate (number of eggs, fertility rate, and hatchability rate of set and fertile eggs) was determined for clean, properly formed eggs. Eggs were collected 2-3×/day on the second day after the first insemination until the fourth day after the last insemination, and were stored at 12 ºC and set weekly in a Masalles incubator type 60HS. All fertile eggs were transferred to the hatchery on the 14th day and incubated until hatching (16-17 days) in order to determine hatchability results.

Differences in the number of particular classes of spermatozoa resulting from semen dilution or foam addition, as well as the fertility and hatchability rates obtained after intravaginal or intrauterine insemination were analyzed by ANOVA and Duncan's multiple range tests (SAS system, general linear models procedure).

Detailed methodology

Experiment 1: The effect of depth of semen deposition in the female reproductive track on fertility and hatchability results.

In order to limit semen collection time and thus to maintain a high quality of semen, males were randomly divided into 2 groups (1 and 2, 15 males each). The semen of group 1 was left undiluted and group 2 semen was diluted 1:1 (v/v) with 0.2 ml of Lake's extender (15) stored at 20 °C. When the volume of collected ejaculates exceeded 0.2 ml, the sample content was supplemented with diluent in order to obtain the final dilution ratio of 1:1. Morphology of the spermatozoa was evaluated in each group before and immediately after insemination. The females were divided into 4 groups (A, B, C, and D, 8 females in each group) and were inseminated 3×/week. The females in groups A and C were inseminated intrauterinely with the semen of groups 1 (undiluted) and 2 (diluted), and the females in groups B and D were inseminated intravaginally with the semen of groups 1 and 2, respectively.

The control group (E) consisted of 2 males and 8 females mated naturally. At the beginning of the experiment the birds were 6 months old.

Semen was collected from every male 24 times; 13 times before insemination and 11 for insemination

Figure. Insemination of a Japanese quail.

purposes. Prior to semen collection foam was removed from the proctodeal gland by delicately squeezing the lateral wall of the cloaca, which allowed its collection and use for insemination purposes; semen was collected without foam.

The number of live, morphologically normal spermatozoa was calculated in each insemination semen sample, and fertility and hatchability rates were determined.

Experiment 2: The effect of insemination frequency on fertility and hatchability results.

The males were randomly divided into 2 groups: group 1 (12 males, semen collected 2×/week), and group 2 (20 males, semen collected 3×/week). Females were placed in group A (n = 20, inseminated with semen from group 1, 2×/week) and group B (n = 20, inseminated with semen from group 2, 3×/week). The semen of both groups was mixed with foam immediately after collection and used for intravaginal insemination of the 2 groups of females. Each female was inseminated 10 times. The control group (C) consisted of 3 males and 12 females mated naturally. At the beginning of the experiment all birds were 8 months old. Semen quality was estimated before and after mixing with foam, and fertility and hatchability rates were determined.

Results

Experiment 1: The effect of the depth of semen deposition in the female reproductive track on fertility and hatchability results.

The average volume of pooled ejaculates collected from group 1 was 280 µl (range: 250-300 µl) and 300 µl (range: 250-400 µl) for group 2; the difference between groups was not significant.

Sperm concentration in group 1 semen averaged 1.22 $\times 10^9$ ml^{-1} (range: 0.68-1.66 $\times 10^9$ ml^{-1}) and 1.63 $\times 10^9$ ml^{-1} (range: 0.82-2.7 $\times 10^9$ ml^{-1}) for group 2; the difference between groups was significant ($P < 0.05$).

Morphology of spermatozoa in semen collected into Lake's extender was slightly less favorable in comparison to fresh, undiluted semen (group 1). The percentage of live normal cells in the diluted semen was significantly lower (range: 62.3%-81.3%) ($P < 0.05$) than in the undiluted semen (range: 70.0%-88.7%). Moreover, the number of defective cells (mainly macrocephalic and bent-neck) was higher in diluted semen than in undiluted semen; however, the difference was not significant.

Although the time interval between semen collection and insemination of the last female was short (about 15 min), a significant decrease ($P < 0.01$) in semen quality, both in fresh and diluted samples, was observed (Table 1). Compared to semen evaluated immediately after collection, the number of live normal spermatozoa decreased by 10.5% in undiluted semen and 7.0% in diluted semen.

Fertility and hatchability of set eggs collected from artificially inseminated groups were significantly lower ($P < 0.01$) than those of the control group (Table 2). Fertility in the control group ranged from 60% to 100%, while in the artificially inseminated groups, regardless of the type of semen and insemination method, fertility ranged from 0% to 100%.

The average number of live morphologically normal spermatozoa in the insemination doses of fresh, undiluted semen (groups A and B) was 9.7 million, vs. 11.8 million in diluted semen (groups C and D).

A significant effect ($P < 0.01$) of insemination method on fertilizing ability of quail spermatozoa was observed. A comparison of the results of the intravaginal insemination groups (B and D) and intrauterine insemination groups (A and C) pointed to the evident advantage of intravaginal insemination, regardless of the type of semen used. In the intravaginal insemination groups the average fertility rate was 21.7% higher (49.53% vs. 27.82%) and the hatchability rate of set eggs was 19.2% higher (42.42% vs. 23.22%) than in the intrauterinely inseminated groups.

The type of semen used for insemination did not affect the fertilizing ability of quail spermatozoa. The differences in average fertility (36.47% vs. 40.62%), hatchability of set eggs (30.05% vs. 53.39%), and hatchability of fertile eggs (82.89% vs. 87.13%) obtained after insemination with fresh and diluted semen, respectively, were not significant.

Experiment 2: The effect of insemination frequency on fertility and hatchability results.

The average volume of single ejaculates collected from both groups of males was 20 µl (range: 15-26 µl) and sperm concentration was 2.27 $\times 10^9$ ml^{-1} (range: 1.94-2.72 $\times 10^9$ ml^{-1}) in group 1, and 2.45 $\times 10^9$ ml^{-1} (range: 2.12-2.80 $\times 10^9$ ml^{-1}) in group 2; the differences

Table 1. Comparison of spermatozoa morphology in fresh undiluted semen and semen diluted with Lake's extender, evaluated prior to and after insemination (n = 11; mean ± SD).

Classes of spermatozoa (%)	Group 1 Undiluted semen		Group 2 Semen diluted with Lake's extender	
	After collection	After insemination	After collection	After insemination
Live in total	88.67^{A}* ± 3.39	80.18^{B} ± 6.49	84.36 ± 4.93	80.48 ± 5.02
Live normal	79.03^{Aa} ± 5.26	68.57^{B} ± 8.23	73.25^{Ab} ± 5.41	66.25^{B} ± 6.01
Macrocephalic	1.81 ± 0.62	1.72 ± 0.56	2.41 ± 1.15	2.22 ± 1.23
Bent-neck	2.19 ± 0.98	3.18 ± 0.15	2.95 ± 1.53	3.36 ± 1.80
Other defects	5.59 ± 2.74	6.72 ± 3.28	5.73^{a} ± 3.14	8.65^{b} ± 4.61

*Values in lines followed by different superscripts differ significantly (A,B P < 0.01; a,b P < 0.05).

Table 2. Fertility and hatchability results of Japanese quails inseminated intrauterinely or intravaginally with fresh or diluted semen.

Female groups	Male group	Set eggs (n)	Fertility (%)	Hatchability of eggs (%)	
				Set	Fertile
A (N, IU)*)	I	213	23.00^{Aa} ± 21.2	19.25^{A} ± 18.9	83.67 ± 45.9
B (N, IV)	I	223	49.33^{B} ± 21.2	40.36^{B} ± 23.3	81.82 ± 29.9
C (L, IU)	II	222	32.43^{Ab} ± 23.3	27.03^{A} ± 23.9	83.33 ± 41.1
D (L, IV)	II	199	49.75^{B} ± 24.2	44.72^{B} ± 24.4	89.90 ± 30.7
E (Control)		250	96.80^{D} ± 22.5	88.40^{C} ± 21.9	91.32 ± 17.3

* N: net (undiluted semen); L: diluted semen; IU: intrauterine insemination; IV: intravaginal insemination.
Values in columns followed by different superscripts differ significantly (A,B: P < 0.01; a,b: P < 0.05).

between the groups were not significant. Furthermore, there was no difference in the number of live morphologically normal spermatozoa in the semen of the groups (Table 3).

Foam addition to freshly collected semen caused a significant decrease (P < 0.01) in the number of live normal cells. This decrease was 23.9% in group 1 and 27.4% in group 2 (Table 3).

Fertility in the naturally mated control group in Experiment 2 (86.1%; Table 3) was not as high as in Experiment 1 (Table 2) and did not differ significantly when compared to the results of females inseminated 3×/week. Quail insemination performed 2×/week was not sufficiently effective. Fertility and hatchability of set eggs were significantly lower (P < 0.01) than in the group inseminated 3×/week and the group that mated naturally (Table 3).

Discussion

Quail semen, unlike rooster, turkey, or drake semen, is devoid of seminal plasma (16); therefore, its collection and in vitro storage for artificial insemination is very difficult.

The dilution of freshly collected poultry semen usually positively affects the quality of spermatozoa stored in vitro at temperatures above 0 °C, extending their viability and fertilizing potency. In the present experiment semen collection into tubes containing Lake's extender did not reduce the unfavorable effect of storage. The number of live normal spermatozoa in diluted semen decreased, on average, by 7% after 15-min storage at room temperature. The significantly detrimental effect of in vitro storage on undiluted semen and the beneficial effect of the addition of extender on morphology and fertilizing ability of spermatozoa were observed by Chelmonska et

Table 3. Percent of live morphologically normal spermatozoa, and fertility and hatchability results, depending on quail insemination frequency (n = 10; means ± SD).

Items		Group I /A Insemination 2×/week	Group II /B Insemination 3×/week	Group C Natural mating
Semen samples	Freshly collected	48.6 ± 9.4	45.8 ± 12.4	-
	With foam addition	24.7 ± 11.5	18.4 ± 12.4	
	After insemination	21.0 ± 7.3	17.9 ± 6.1	
Fertility (%)	Fertility (%)	44.1A ± 30.7	82.7B ± 20.3	86.1B ± 13.9
	Set eggs (n)	216	205	207
	Hatchability of:			
	set eggs (%)	34.7A ± 27.0	69.1B ± 23.9	71.3B ± 21.8
	fertile eggs (%)	78.8 ± 30.7	83.5 ± 19.7	82.8 ± 21.5

Values in lines followed by different superscripts differ significantly (A,B: $P \leq 0.01$).

al. (17) for Muscovy drakes. Similar results were observed by Lukaszewicz (18) for rooster semen diluted in different media.

It should be mentioned that the quality of semen produced by 8-9-month-old males (Experiment 2) was not as good compared to semen collected from 4-month-old quails (13). The percentage of normal spermatozoa ranged from 53.5% to 62.8% in ejaculates of younger quails and from 45.8% to 48.6% in the semen of 8-9-month-old males, while ejaculate volume and spermatozoa concentration were similar. Additionally, the fertility rate obtained after insemination with semen of 4-month-old quails mixed with foam (91.4% on average) was higher compared to the later period of the reproductive cycle (82.7%). The observation that semen quality decreases during the reproductive season was confirmed for other poultry species (19,20).

Opinions related to the role and importance of foam secreted by the proctodeal gland of male quails on reproduction efficiency differ. Amano and Watanabe (21), Kobayashi et al. (22), and Ogawa et al. (23) reported that quail spermatozoa tend to form clusters in diluents commonly used for poultry semen. Conversely, Fujihara et al. (16) suggested that clustered spermatozoa are released by contact with foam, which may have a favorable effect on fertility. Foam's effect on spermatozoa viability, independent of storage temperature, was also observed. Cheng et al. (24) reported that spermatozoa stored with foam at ambient temperature maintained satisfactory motility for 90 min, while without foam it was lost within 10 min. Fujihara and Koga (8), and Fujihara et al. (16) described the unfavorable effect of foam at low temperatures (0-3 °C), which was also confirmed by the results of our experiments (unpublished data).

In the present work, the addition of foam to freshly collected ejaculates resulted in a decrease, by as much as 6.0%, in the number of live morphologically normal cells; but contrary to undiluted semen or semen diluted with Lake's extender, length of time between semen collection and insemination did not affect spermatozoa morphology.

Changes in quail spermatozoa motility and morphology observed at different stages of the artificial insemination process caused similar changes in the fertilizing ability of treated cells. Average fertility results obtained in the present work after artificial insemination varied from 23.0% to 82.7%, depending on the character of inseminated semen, depth of semen deposition in the oviduct, and insemination frequency. The fertility of eggs produced by females inseminated with undiluted semen was significantly lower (23.0%; $P < 0.01$) compared to the eggs of females inseminated with semen diluted with Lake's extender (32.4%).

It has to be emphasized that placement of semen had a very important impact on fertility results. Irrespective of semen character (net, undiluted, or diluted), the results obtained after intravaginal insemination were significantly higher ($P < 0.01$) in comparison to intrauterine insemination. Chick hatchability from fertile eggs was not affected by the method of insemination. In contrast to this, the comparative studies on intravaginal and intramagnal insemination described by Ogasawara and Huang (2), Wentworth and Mellen (1), and McFarquhar and Lake (11) indicated that deep insemination was more effective. Ogasawara and Huang (2) obtained 13.3% and 36.6% fertility rates in eggs collected from the 2nd to 10th day after intravaginal and intramagnal insemination, respectively; however, a negative effect of intramagnal insemination on laying intensity and egg number was observed. Wenworth and Mellen (1), when injecting semen into the anterior region of the magnum, obtained a 77.5% egg fertile rate and only 23.1% after intravaginal insemination; but, infections of the oviduct, decreased number of eggs, and hen mortality were noted. In the present experiment, no unfavorable effects of deep intrauterine insemination on infection occurrence or egg numbers were observed; however, egg fertility was lower compared to the above-cited authors' results. It can be assumed that deep penetration of the posterior region of the uterus creates unfavorable conditions for spermatozoa (reducing their transport along the oviduct) and, consequently, fertility efficacy. Similarly, Surai and Wishart (25) reported that rooster semen deposited at a depth of 5-6 cm (not 2-3 cm, as is the usual practice) decreases fertility results. Marks and Lepore (12), with the same method, obtained a 56.1% egg fertility rate (range: 45.3%-72.0%) when inseminating with 20 µl of undiluted semen 3 times over 4 days.

Insemination frequency is another important factor affecting success in artificial insemination. In chicken and turkey reproduction, artificial insemination is usually performed every 7-10 days. Our experiment indicated that in quails, even twice a week insemination is insufficient; resulting in a 44.1% egg fertility rate, compared to the 82.7% obtained with insemination performed 3×/week. In further experiments on insemination frequency, Lepore and Marks (26) obtained 52.0%, 36.0%, and 22.0% fertility in eggs collected up to the seventh day after the first intravaginal insemination performed 3×, 2×, or 1×/week. The authors mentioned above increased the insemination dose of undiluted quail semen from 2.5 µl to 15 µl and obtained a proportional increase in egg fertility (from 55.6% to 69.3%), but the difference in fertility rates was not significant. In our experiments, 2×/week intravaginal insemination with 10 µl of fresh, undiluted semen or 20 µl of semen diluted with Lake's extender resulted in 49.3% and 49.5% fertility.

Although the addition of proctodeal gland foam to freshly collected semen decreased the number of live morphologically normal spermatozoa, its beneficial effect on fertilizing ability was evident. In the present experiment, intravaginal insemination carried out 3×/week with 20 µl of semen mixed 1:1 with foam resulted in 82.7% egg fertility, which is almost equal to the results of natural mating (86.1% on average). However, in our other experiment (13), when semen of 4-month-old quail mixed with foam was inseminated 3?/week, average egg fertility was 91.4%. Similarly, Fujihara and Koga (8) concluded that quail semen mixed with foam resulted in higher fertility (55.9%) and longer duration of fertility (5.8 days) compared to females inseminated with undiluted semen (42.9% and 5.2 days). Moreover, quail females mated naturally with normal males or cauterized males whose semen was artificially supplemented with foam after copulation produced fertile eggs for 7-8 days, while females mated with cauterized males without foam supplementation produced fertile eggs for up to 5 days (4).

The conclusion that may be drawn from these results is that fertility levels comparable to those of natural mating can be obtained when quail females are inseminated regularly (3×/week) with good quality semen supplemented with proctodeal gland foam.

An average quail fertility rate of 86.1% as the result of artificial insemination encourages the authors to continue studying the effect of proctodeal gland foam on the viability and fertilizing ability of quail spermatozoa suspended in different diluents and cryoprotective agents, which constitutes an initial and basal step of cryopreservation.

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