Longer and faster sperm exhibit better fertilization success in Japanese quail

Mei Matsuzaki,[*] Noritaka Hirohashi ,[†] Masaoki Tsudzuki,[‡,§] Mohammad Ibrahim Haqani,[‡] Teruo Maeda,[#,§] Shusei Mizushima,[‖,§] and Tomohiro Sasanami[¶,§,1]

*Program of Food and AgriLife Science, Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima City, Hiroshima 739-8528, Japan; †Oki Marine Biological Station, Education and Research Center for Biological Resources, Faculty of Life and Environmental Science, Shimane University, Oki, Shimane 685-0024, Japan; ‡Laboratory of Animal Breeding and Genetics, Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima City, Hiroshima 739-8528, Japan; §Japanese Avian Bioresource Project Research Center, Hiroshima University, Higashi-Hiroshima City, Hiroshima 739-8528, Japan; #Laboratory of Animal Reproduction, Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima City, Hiroshima 739-8528, Japan; ‖Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan; and ¶Department of Applied Life Sciences, Faculty of Agriculture, Shizuoka University, Shizuoka City, Shizuoka 422-8529, Japan

ABSTRACT In birds, sperm storage tubules (**SST**) located in the utero-vaginal junction are thought to be a site of sperm selection; however, the exact mechanism of sperm selection is poorly understood. Here, we investigated sperm entry into the SST and subsequent fertilization success under a competitive situation created by artificial insemination of a sperm mixture obtained from 2 males. We employed 2 quail strains, a wild-type and a dominant black (**DB**) type, as this allows easy assessment of paternity by feather coloration. We found paternity of embryos was biased toward DB males when a sperm mix with similar sperm numbers from the 2 males strains was artificially inseminated into females. Our novel sperm staining method with 2 different fluorescent dyes showed that the DB-biased fertilization was because of the better ability of DB sperm to enter the SST. Moreover, we found that DB sperm had a longer flagellum and midpiece. These characteristics probably allow sperm to swim faster in a high viscosity medium, which may be a similar environment to the lumen of the female reproductive tract. Our results indicated that sperm competition occurs to win a place in the SST and that filling the SST with their own spermatozoa is a critical step to achieve better fertilization success for the male Japanese quail.

Key words: fertilization, postcopulatory sexual selection, sperm storage tubules, artificial insemination, Japanese quail

2021 Poultry Science 100:100980
https://doi.org/10.1016/j.psj.2021.01.003

INTRODUCTION

Birds and mammals produce considerable amounts of sperm in their lifetimes. As many as one hundred million sperm are ejaculated into the female reproductive tract at each copulation (Kekäläinen and Evans, 2018); however, only a small fraction of sperm that arrive at the site of fertilization are capable of fusing with ovulated oocytes (Florman and Ducibella, 2006; Matsuzaki and Sasanami, 2017). This massive reduction in the number of sperm during the journey to the oocyte can be accounted for, at least in part, by postcopulatory sexual selection operated through sperm competition and cryptic female choice (Firman, 2018). Sperm competition is a process that occurs when sperm from more than 1 male compete to fertilize the same ova (Parker, 1970; Karr and Pitnick, 1999). On the other hand, cryptic female choice arises from a biased utilization of particular sperm in favor of producing the progeny of preferred males (Thornhill, 1983; Birkhead, 1987; Olsson et al., 1999; Firman et al., 2017). It is a great

Received September 22, 2020.
Accepted January 1, 2021.

[1]Corresponding author: atsasan@shizuoka.ac.jp

challenge to experimentally discriminate 2 independent processes, sperm competition and female sperm choice, because they are thought to occur in a coordinated manner and often mutually interact (Kempenaers et al., 2000; Pitnick and Brown, 2000; Lupold et al., 2013).

Most studies investigating the mechanism of postcopulatory sexual selection have focused on the proportion of offspring sired by a particular male when different males have competed with each other for fertilization (Evans and Magurran, 2001; Gasparini et al., 2018). A recent challenge in understanding the postcopulatory process of sperm competition and/or sperm selection has been to develop a technique for visualizing sperm movement, transport, and storage in the female reproductive tract. Using transgenic fruit flies that produce fluorescent sperm (green or red), Manier et al. (2010) observed that paternity of the subsequent clutch after mating with multiple males was biased to the last male the female mated with (last male sperm precedence, **LMSP**) (Manier et al., 2010). Real-time and spatiotemporal observations of live sperm in the females revealed that the resident sperm in the sperm storage organ (i.e., spermathecae and sperm receptacle) were displaced over time by a second male's sperm after remating (Presgraves, 2005). More recently, Laturney et al. (2018) used similar transgenic lines and reported that LMSP in fruit flies was affected by female remating latency (Laturney et al., 2018), indicating that LMSP was modulated not only by the sperms' competitive ability but also by females' active control. These studies demonstrated the great potential of fluorescent sperm have to better understand how postcopulatory sexual selection takes place. However, there have been no reports addressing whether similar techniques can be used in higher vertebrates such as birds and mammals.

One of the most remarkable features of avian reproduction is the ability to store sperm at body temperature (i.e., 41°C) for extended periods of time (e.g., 3 mo in the turkey hen) in sperm storage tubules (**SST**) that are mainly located in the utero-vaginal junction (**UVJ**) (Sasanami et al., 2013). However, the mechanism underlying sperm storage in the SST is largely unknown because these events are completely concealed under the opaque and thick smooth muscle of the female reproductive tract. Before entering SST, ejaculated sperm are selected in the vagina because only 1 to 2% of inseminated sperm enter the SST, most likely because of sperm ejection via female defecation (Bakst, 1994). Thus, in addition to the SST, the vagina is considered to be a major barrier for sperm in terms of successful fertilization. Although we have previously demonstrated the molecular mechanisms underlying sperm motility inactivation in the SST (Matsuzaki et al., 2015) and sperm release from the SST (Ito et al., 2011), the mechanism by which ejaculated sperm enter the SST remains largely unknown. Because the ejaculated sperm have to enter the SST before being transported to the site of fertilization (i.e., the infundibulum part of the oviduct), we hypothesized that sperm competition, as well as sperm selection by females after mating, may occur during the process of sperm entrance into the SST.

In this study, we developed a new method in which the ejaculated sperm obtained from 2 male birds were separately stained with 2 different fluorescent dyes and simultaneously inseminated into the female vagina to investigate the sperm storage in the female reproductive tract under a competitive scenario. This heterospermic insemination appears to be a good strategy for investigating postcopulatory sexual selection because previous studies suggested that this method is effective for assessing the relative fertility of males in chicken and boar (Martin et al., 1974; Martin and Dziuk, 1977; Berger, 1995; Flowers et al., 2016). In addition, we evaluated the relationships between the sperms' SST filling ability and male paternity to clarify whether sperm filling of the SST is a critical event for better male fertilization success. Furthermore, because flagella and midpiece length were reported to influence fertilization success (Bennison et al., 2015; Fisher et al., 2016), we also examined the relationship between these sperm morphological measurements and the previously described parameters.

MATERIALS AND METHODS

Animals and Sperm Collection

All experimental procedures for the care and use of animals were carried out in accordance with approved guidelines of the Animal Care Committees of Shizuoka University (Approval number: 2018A-5) and Shimane University (Approval number: MA-30-13).

Eleven wild-type, 11 dominant black (**DB**) males, and 35 wild-type females were maintained under a photoperiod of 14L:10D (light went on at 5:00) with *ad libitum* access to water and a commercial diet (Hokkaiya Corporation, Toyohashi, Japan) at the Shimane University. The DB males were obtained from a closed population maintained at the Hiroshima University (Higashi-Hiroshima, Japan). Wild-type quails (both males and females) were purchased from Quail Cosmos Inc. (Toyohashi, Japan), and 11 males and 35 females were randomly selected. They were individually maintained (cage size: 20 cm × 20 cm × 30 cm in height, width, and depth, respectively), and we found no obvious differences in behavior between the 2 types of male strains (DB and wild-type). The DB phenotype is an autosomal, incomplete dominant mutation that occurs in the melanocortin receptor gene, and the neonatal plumage of the homozygote shows blackish (or dark brownish) plumage all over the body (Nadeau et al., 2006; Tsudzuki, 2008). In chicks heterozygous for this mutation, the plumage is blackish and can be clearly differentiated from wild-type feather coloration (Figure 1). In addition, leg skin color of heterozygotes is also blackish (Figure 1). Thus, the paternity of the F1 animals is easily assessed by their appearance without subjectivity.

Semen was obtained from the male quails during mating with teaser female before ejaculation according to the procedure of Kuroki and Mori (1997). Briefly, male and

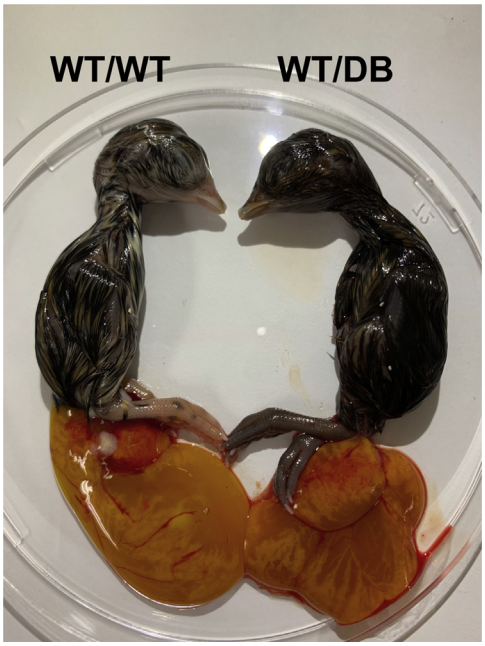

Figure 1. Appearance of wild-type and heterozygote embryos at 15 d incubation. Note that the appearance of wild-type (WT/WT) and heterozygote (WT/DB) embryos are different in terms of the plumage and leg skin color.

female pair was placed in cardboard box, and semen was squeezed out from male cloaca when the mating behavior was expressed. The semen was collected by small spatula and suspended in 1 mL Hanks' balanced salts solution (**HBSS**, GE Healthcare, Chicago, IL) supplemented with 0.8 mmol/L $MgSO_4$, 1.26 mmol/L $CaCl_2$ and 4.2 mmol/L $NaHCO_3$ (pH 7.4, 270–305 mOsm/kg). We removed cloacal gland foam before semen collection because this foam contains prostaglandin $F_{2\alpha}$, and this is known to affect the process of sperm entrance into the SST (Sasanami et al., 2015).

Artificial Insemination and Paternity Assessment

An equal volume of sperm suspensions of each male (20 μL) were mixed. Then, 40 μL of the mixture was placed into a hematocrit tube connected to a mouthpiece and inseminated into the wild-type female's vagina. We collected sperm from wild-type and DB males, and the sperm mixture of each pair was inseminated into 1 to 5 females. Eleven male pairs were made, and totally 35 females were inseminated. After the artificial insemination (**AI**), the sperm number in the residual of each preparation was counted, and each ejaculate was estimated. We counted the sperm number after completing AI because quail sperm rapidly lost their motility during short-term storage under high sperm density. From the day after AI, the oviposited eggs were collected for 7 d and incubated at 37.5°C for 15 d. After incubation, the eggs were opened, and the paternity of the embryo was confirmed by its feather and leg skin color. The DB females were not used because paternity analysis by plumage color in this combination is impossible.

Sperm Staining

The ejaculated sperm obtained from 2 individuals were separately stained with either 10 μmol/L Hoechst 33,342 (Wako pure chemicals, Osaka, Japan) or 10 μmol/L pHrodo-red-AM (Molecular Probe Inc. Eugene, OR) for 2 min, and equal volumes of sperm suspensions of each male (20 μL) were then mixed. In the competitive mode experiments (see below), we stained wild-type and DB sperm with Hoechst 33,342 and pHrodo red-AM, respectively. In noncompetitive mode, sperm isolated from each individual were devided into 2 parts, and stained with 2 different dyes. When the stained sperm were mixed, we found that dye transfer to the adjacent sperm did not occur with both dyes (Figures 2A–2C). In addition, after AI, the resident sperm within the SST could be easily observed and their origin clearly identified (Figure 2D). Thus, this approach allowed us to perform direct observations of sperm in the SST ex vivo, as well as to investigate the sperm storage bias in a competitive condition in birds.

Observation of Resident Sperm in Sperm Storage Tubules After Artificial Insemination

After paternity assessments, 25 female birds were kept for at least 2 wk to deplete the resident sperm in the SST. After the depletion period, the animals were again inseminated for the observation of the resident sperm in the SST.

To evaluate the ability of the sperm of each male to fill the SST in the competitive mode, we collected sperm from 3 pairs of wild-type and DB males, stained, and the sperm mixture of each pair was inseminated into 5 females (15 females in total). After the AI, the sperm number in the residual of each preparation was counted, and each ejaculate was estimated. One hour after AI, the UVJ mucosa around the junction of the uterus and the vagina were dissected and placed in physiological saline. The UVJ mucus membranes containing SST were then isolated with fine forceps and scissors under a stereomicroscope according to the method of Ito et al. (2011). Following steps are described below.

In the noncompetitive mode, the ejaculated sperm were divided into 2 portions and stained with 2 different dyes. They were then mixed together and inseminated into 10 wild-type females. One hour after AI, UVJ isolation was performed as described above. We isolated the sperm from 5 wild-type or five DB males, and the stained sperm mixture was inseminated into single female.

Sperm Motility Analysis

A sperm suspension (10 μL) was placed on glass slides that were prewarmed to 39°C and mounted with coverslips (22 × 22 mm). The glass slides and coverslips were

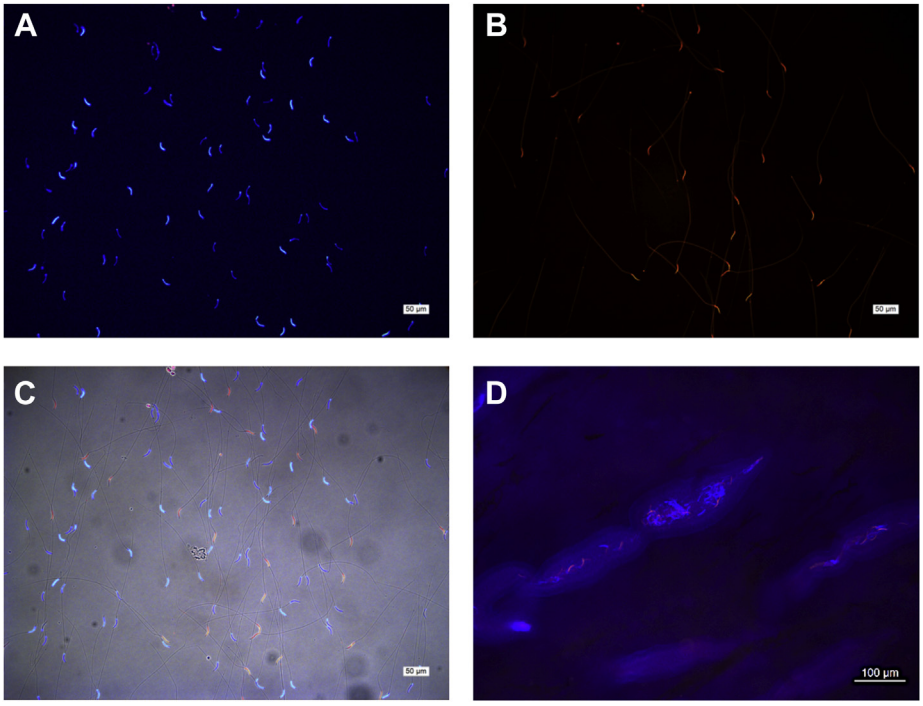

Figure 2. Validation of sperm staining for the competitive sperm storage assay. (A) Hoechst 33,342 detection under the conditions with Ex = 340 nm and Em = 420 nm. (B) pHrodo red-AM detection under the conditions with Ex = 530-550 nm and Em = 575 nm. (C) Merged image of (A), (B), and differential interference contrast image. Representative sperm images of 5 males are shown. Bar = 50 µm. (D) Mixed sperm in the female reproductive tract 1 h after insemination. Note that the sperm labeled with each dye are visible in the lumen of the SST. Representative images of 5 independent experiments are shown. Bar = 100 µm. Abbreviations: SST, sperm storage tubule.

coated with 1% (wt/vol) BSA to avoid sperm attachment to the glass surface. Sperm swimming velocity was measured by **SMAS** (Sperm Motility Analysis System, Detect, Tokyo, Japan), which automatically tracks swimming sperm under a microscope (TE-2000; Nikon, Tokyo, Japan) and calculates their motility parameters such as linear velocity (**VSL**), curvilinear velocity (**VCL**), average velocity (**VAP**), amplitude of lateral head displacement (**ALH**), and beat-cross frequency (**BCF**). The percent of motile sperm was calculated automatically with SMAS. Curvilinear velocity is the average velocity of the sperm head through its real path. These motility parameters were also measured on the sperm stained with Hoechst 33,342 or pHrodo-red-AM to confirm that the 2 fluorochromes did not affect sperm motility differently. As a result, we found no statistically significant difference between the 2 (Table 1). To investigate the effects of medium viscosity on sperm motility, various concentrations of methyl cellulose (0.125, 0.25, 0.5, 1.0, and 2.0% of **MC**, Sigma, St. Louis, MO) were added to HBSS. Then, 10 µL of medium containing various concentrations of MC was placed on prewarmed glass slides, and 2 µl sperm suspension was then added to the medium. After 1 min of incubation for stabilization, sperm motility parameters were measured by SMAS.

Fluorescence Microscopy

One hour after the AI, the UVJ was isolated as described in the section of "Observation of Resident sperm in Sperm Storage Tubules After Artificial Insemination". After washing with PBS, the UVJ mucosa was peeled off from the connective tissue by forceps under a stereomicroscope (M165FC; Leica microsystems, Wetzlar, Germany; 40× magnification), mounted in glycerol, and observed under a fluorescence microscope (BX 51, Olympus optics, Tokyo, Japan) with 20x objective lens (UplanApo20X, NA0.70; BX 51, Olympus). The fluorescence signals of Hoechst 33,342 and pHrodo-red-AM of sperm before

Table 1. Computer-assisted sperm motility analysis of ejaculated sperm stained with Hoechst 33,342 or pHrodo-AM[1].

	Motility (%)	VSL (µm/s)	VCL (µm/s)	VAP (µm/s)	ALH (µm)	BCF (Hz)
Hoechst33342	98.4 ± 1.1	22.9 ± 11	45.3 ± 16	30.1 ± 12	0.9 ± 0.4	9.2 ± 1.5
pHrodo-AM	99.4 ± 1.0	28.8 ± 13	53.4 ± 18	37.5 ± 15	1.1 ± 0.4	9.9 ± 0.5

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; VSL, linear velocity; VCL, curvilinear velocity; VAP, average velocity.

[1]Sperm were isolated from 6 wild-type males. All the data presented here are expressed as means ± SD. There is no statistically significant difference between Hoechst33342- and pHrodo-AM-stained sperm.

and after AI were detected using fluorescence filter cubes U-MWU2 (Olympus optics) and U-MWIG3 (Olympus optics), respectively, and images were recorded on a digital camera (DFC7000 T; Leica microsystems). The total number of SST and the number of SST filled with fluorescent sperm (Hoechst 33,342 or pHrodo-red-AM-positive sperm) were counted, and the filling rate of the SST (% SST filled with sperm) was calculated.

Measurement of Sperm Flagellum Length

To measure sperm flagellum length, sperm were fixed and stained by the procedure of Korn et al. (2000) (Korn et al., 2000). Briefly, the ejaculated sperm were suspended in HBSS and stained with 0.1% (wt/vol) amido black in 5% (vol/vol) acetic acid for 10 min, then mounted on glass slides. According to the classification by Korn et al. (2000), we separately measured the midpiece length, where the central axoneme was enveloped by a mitochondrial sheath and tail length, which corresponded to an area devoid of amido black labeling (Figure 3). As shown in Figure 3, mitochondrial sheath extended for more than 80% of the overall flagellum length. Overall flagellum length was calculated by the sum of the midpiece and tail lengths. The sperm were photographed, and the measurements of midpiece and tail lengths were manually performed by image J software (version 1.50i, https://imagej.nih.gov/ij/). Because the measurement of flagellum is not performed automatically, we measured 127 and 125 sperm obtained from randomly selected 5 wild-type and 5 DB males, respectively.

Data Analysis

All statistical data analyses were performed with the statistical package R version 3.3.3 (The R Foundation for Statistical Computing Platform, https://cran.r-project.org/bin/macosx/). The AI data were analyzed by a binomial test to verify the paternity bias. Sperm motility parameters of SMAS (VCL, VSL, VAP, ALH, and BCF) and sperm length were analyzed by the F-test followed by the t test after confirmation of distribution normality using the Shapiro-Wilk test. Percentage data of sperm motility were arcsine-transformed before analysis. The data of competitive sperm storage assay were tested with Wilcoxon's sign test to confirm the predominance of each male.

Generalized linear mixed models were made to investigate whether male type affected sperm motility parameters in the viscous medium. Data were fitted to a gamma distribution with a log link function using the "glmer" function of "lme4" package. The medium viscosity (MC), male type (type: as a dummy variable of 0 for wild-type and 1 for DB, respectively), and their interaction (MC*type) were regarded as a fixed effect, and the individual differences were considered to be a random effect. The best fit model selection was performed by comparison of Akaike's information criterion values generated from models fit by maximum likelihood. To evaluate the statistical significance of fixed effects and generate P-values, targeted likelihood ratio tests comparing models with and without MC*type were conducted. In all statistical analysis, differences were considered significant at $P < 0.05$.

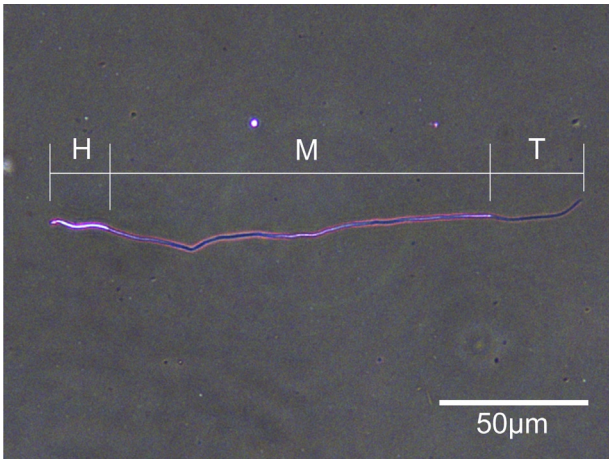

Figure 3. Light micrograph of Japanese quail sperm stained with amido black. Head (H), midpiece (M), and tail (T) are shown. Note that the junction of the head and midpiece or midpiece and tail are clearly differentiated. Bar = 50 μm.

RESULTS

Relationships Between Fertilization Success and Sperm Storage in SST

As mentioned in Materials and Methods, we confirmed that the sperm number of wild-type ejaculate was similar or greater than that of DB. The ratios of the number of sperm (wild-type/DB) in 11 replicates were 2.91, 3.84, 1.75, 1.46, 0.73, 4.13, 0.68, 0.91, 4.93, 0.96, and 5.79, respectively. The paternity was determined by the feather coloration of 15-day-old embryos. The AI assays revealed that paternity was significantly biased toward DB males (Table 2, $P = 4.6 \times 10^{-4}$). In fact, 71 of 107 embryos (66.4%) were found to be fathered by DB males. Although biased fertilization from day 2 to 7 for DB was not statistically significant, more than 60% embryos were fathered by DB. We also found that fertility decreased to 50% after day 7 (Table 2).

From these observations, we hypothesized that the emergence of paternity bias could be related to the pattern of sperm storage in the SST. To test this hypothesis, we first performed AI under noncompetitive mode. When the ejaculates of wild-type males were divided into 2 portions, stained differently with the dyes and then mixed and inseminated into the female's vagina, the sperm filling rate in the SST did not differ significantly after 1 h of AI (Figure 4A). The results were similar when DB sperm were used (Figure 4B). These results indicated that staining with different dyes does not have any adverse effects on the process of sperm entry

Table 2. Paternity analysis after artificial insemination[1].

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Total
No. of eggs	21	33	28	27	29	16	14	168
No. of fertilized eggs	16	24	20	14	17	9	7	107
Fertility (%)	76.2	72.7	71.4	51.9	58.6	56.3	50.0	63.7
No. of wild-type offspring	4	9	8	4	6	3	2	36
No. of DB offspring	12	15	12	10	11	6	5	71
Paternity of wild-type (%)	25.0	37.5	40	28.6	35.3	33.3	28.6	33.6
Paternity of DB (%)	75.0[2]	62.5	60	71.4	64.7	66.7	71.4	66.4[3]

Abbreviations: DB, dominant black.
[1]Totally, 35 females were artificially inseminated with mixed sperm obtained from 11 wild-type and DB males (22 males in total).
[2]Indicates statistically significant difference between wild-type paternity ($P = 0.04$).
[3]Donates statistically significant difference between wild-type paternity ($P = 4.6 \times 10^{-4}$).

into the SST and that sperm under noncompetitive conditions evenly enter into the SST.

Using this method, sperm from wild-type and DB males were examined. The ratios of the number of sperm (wild-type/DB) in the 3 pairs used were 1.38, 1.85, and 0.71, respectively. As shown in Figure 4C, when the sperm filling rates were compared between wild-type males and competitor DB males, we found DB sperm were preferentially stored in the SST ($P = 1.3 \times 10^{-2}$).

Sperm From the Dominant Black Strain Swam Faster in a Viscous Medium and Had Longer Flagella

In the initial assessment, we confirmed that 2 fluorochromes, Hoechst 33,342 and pHrodo-AM, did not affect sperm motility, differently (Table 1). As shown in Table 3, none of the parameters examined showed significant differences between the 2 groups when assayed in a

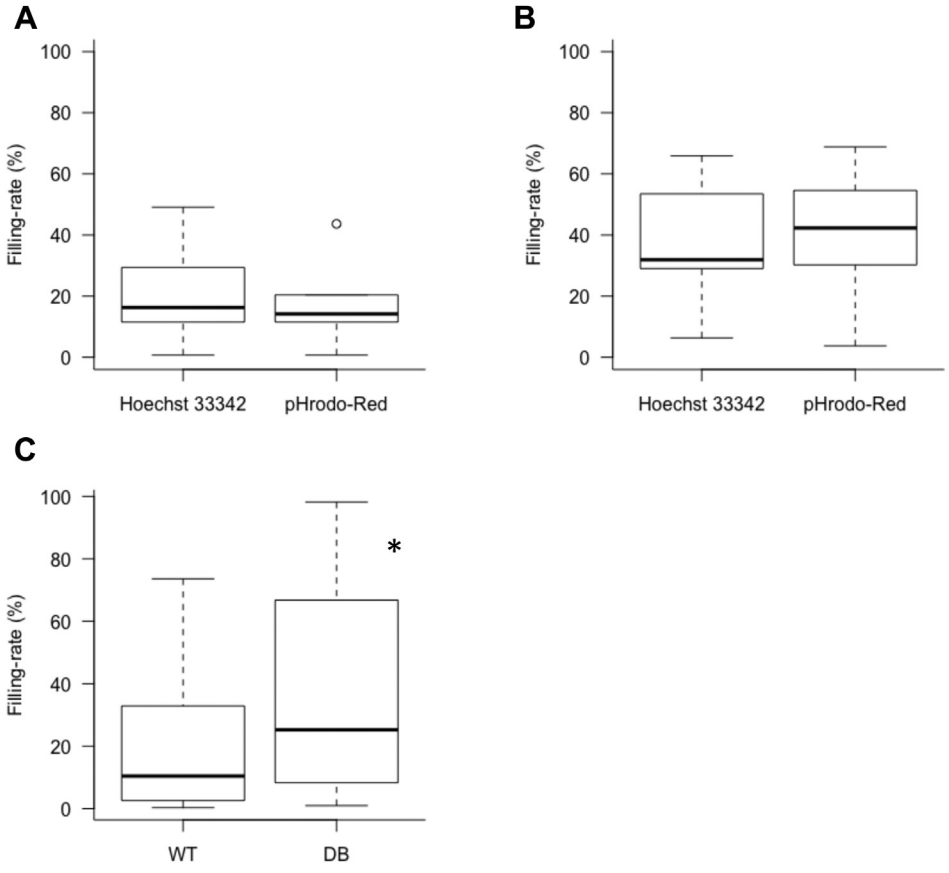

Figure 4. Dominant black sperm were more predominantly stored in the sperm storage tubules than wild-type sperm. Sperm-filled SST were counted, and the sperm filling rate with each dye was calculated. (A) and (B) were under a noncompetitive mode using (A) wild-type sperm (n = 5) or (B) dominant black sperm (n = 5). There was no statistically significant difference ($P = 0.25$ and 0.38 for wild-type and dominant black, respectively). Panel (C) shows the wild-type and dominant black sperm filling rates under a competitive situation following simultaneous insemination of a mixture of wild-type sperm and dominant black sperm. *Denoted statistically significant ($P = 1.3 \times 10^{-2}$). All the data are expressed as a boxplot and the median is shown as a bold line between the first and third quartiles. Error bar indicates the smallest and the largest data point excluding outliers (open circle). Abbreviations: DB, dominant black; SST, sperm storage tubule; WT, wild-type.

Table 3. Computer-assisted sperm motility analysis of ejaculated sperm obtained from wild-type or DB males[1].

	Motility (%)	VSL (μm/s)	VCL (μm/s)	VAP (μm/s)	ALH (μm)	BCF (Hz)
Wild-type	92.8 ± 9.2	24.0 ± 10	52.1 ± 13	33.7 ± 11	1.1 ± 0.2	9.3 ± 0.8
DB	89.9 ± 12	20.0 ± 10	45.6 ± 14	28.5 ± 13	0.9 ± 0.3	9.1 ± 0.5

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; DB, dominant black; VSL, linear velocity; VCL, curvilinear velocity; VAP, average velocity.

[1]7 wild-type and 7 DB males were used for sperm collection. All the data presented here are expressed as means ± SD. There is no statistically significant difference between wild-type and DB sperm.

low viscosity medium (i.e., HBSS without MC). However, when the medium viscosity was increased by the addition of MC, the VCL, VSL, VAP, and ALH, but not BCF, of both groups' sperm decreased in an MC concentration–dependent manner (Figures 5A–5E). The best model explaining the decrement of sperm motility (VCL, VSL, VAP, and ALH) by the increasing concentration of MC included the fixed effects of MC, type and MC*type (Table 4). When likelihood ratio tests with and without MC*type were conducted, *P*-values for all parameters (VCL, VSL, VAP, and ALH) showed statistical significance (Table 5). These results suggested that wild-type sperm motility decreased significantly more than DB-type sperm when medium viscosity was increased.

We found that overall flagellar length was significantly longer in DB sperm than in wild-type sperm (Figure 6C). We found that the variation observed in overall flagellar length was associated with the midpiece length but not with the tail length (Figures 4A and 4B).

DISCUSSION

As far as we know, our study is the first to report the fate of sperm from different male quails simultaneously inseminated into a female's vagina. The results presented here indicated that DB males sired more embryos when their sperm competed with wild-type sperm. Our novel method showed that DB male-biased fertilization success is because of the superior ability of DB sperm to enter the SST after AI, which may be explained by the longer flagellum and midpiece.

In this study, we developed a new method to investigate the competitive mode of sperm storage in the female reproductive tract of birds by staining ejaculates with different fluorescent dyes. There are several reports investigating the fate of spermatozoa in the female reproductive tract after insemination in birds. For instance, Bakst (1994) stained turkey hen spermatozoa with a nuclear fluorescent dye, *bis*benzimide, before insemination. They found that sperm entering SST

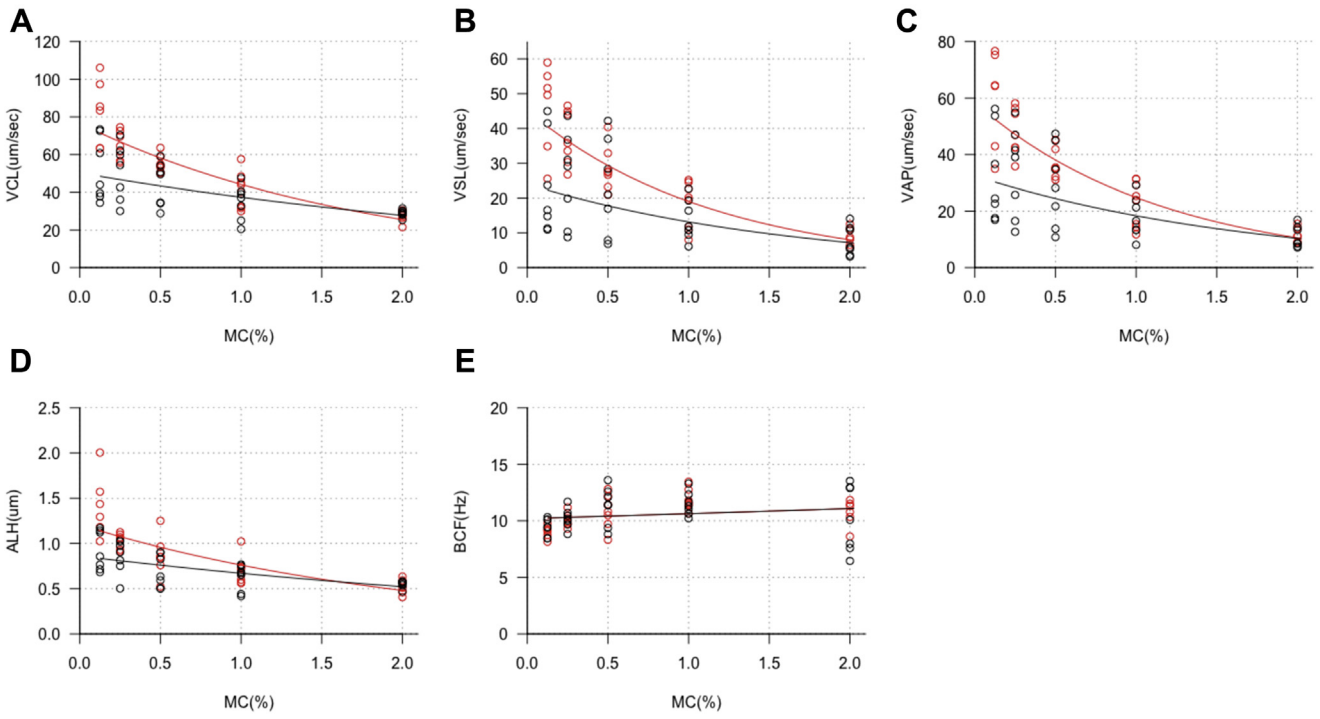

Figure 5. Dominant black sperm showed superior ability to swim faster in a viscous solution. (A–E) Comparison of the decrement in VCL (A), VSL (B), VAP (C), ALH (D), and BCF (E) in a medium containing increasing concentrations of methyl cellulose. The regression curves of wild-type (red line, n = 7) and dominant black sperm (black line, n = 7) are made by the formula of best fit model shown in Table 4. Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; MC, methyl cellulose; VSL, linear velocity; VCL, curvilinear velocity; VAP, average velocity.

Table 4. Comparison of various models for evaluating sperm motility parameters.

Terms	Model	k[1]	AIC	ΔAIC[2]
VCL	**MC + type + MC*type**	**4**	**493.22**	**0**
	MC + type	3	509.44	16.22
	MC	2	510.38	17.16
	Type	1	586.71	93.49
	Null	1	588.19	94.97
VSL	**MC + type + MC*type**	**4**	**457.3**	**0**
	MC + type	3	461.79	4.49
	MC	2	462.28	4.98
	Type	1	542.17	84.87
	Null	1	543.37	86.07
VAP	**MC + type + MC*type**	**4**	**474.44**	**0**
	MC + type	3	482.82	8.38
	MC	2	483.42	8.98
	Type	1	574.3	99.86
	Null	1	575.58	101.14
ALH	**MC + type + MC*type**	**4**	**−46.217**	**0**
	MC + type	3	−38.941	7.276
	MC	2	−38.148	8.069
	Type	1	14.862	61.079
	Null	1	16.228	62.445
BCF	MC + type + MC*type	4	262.76	2.7
	MC + type	3	262.05	1.99
	MC	**2**	**260.06**	**0**
	Type	1	262.77	2.71
	Null	1	260.78	0.72

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; MC, methyl cellulose; VSL, linear velocity; VCL, curvilinear velocity; VAP, average velocity.

Best models are shown in bold.

[1]k represents number of parameters.

[2]ΔAIC is calculated from subtracting a particular model's AIC value from the best-fitting model for that comparison.

were associated with the onset of egg production (Bakst, 1994). McDaniel et al. (1997) developed this technique in a more quantitative way, directly determining the sperm number in a chicken SST homogenate under a fluorescent microscope. From this study, they estimated that as little as 0.22% of the sperm population inseminated was found in the UVJ at 24 h after AI. More recently, King et al. (2002) investigated the relative distribution of sperm in the SST after successive inseminations by different males. They sequentially inseminated the hens with *bis*benzimide-stained and unstained sperm, and the sperm residing in the SSTwere observed under a fluorescent microscope (King et al., 2002). They found that sperm from 2 males generally segregated into different SST in both chicken and turkey hens, indicating that the mechanism of LMSP in birds may not be because of the stratification of sperm within the SST. These studies contributed to an understanding of avian SST physiology. However, none of them were able to investigate sperm storage in a competitive situation in the female reproductive tract. This is because they used a single fluorescent dye for visualization of the sperm nuclei, so it was impossible to distinguish the spermatozoa obtained from different males after simultaneous insemination. We previously found that a commercially available fluorescent pH indicator dye, pHrodo-red-AM, successfully labeled quail sperm under acidic conditions (Matsuzaki et al., 2015). In addition, we found that the lumens of quail SST were acidic because of the accumulation of a large quantity of lactic acid under hypoxic conditions (Matsuzaki et al., 2015), indicating that this fluorescent dye may be the most suitable for visualizing the resident sperm in the SST. As expected, we were able to distinguish the 2 populations of sperm obtained from different male ejaculates without affecting the process of sperm entrance into the SST.

In zebra finch (*Taeniopygia guttata*), it was found that longer sperm won the competitive fertilization race and fertilized more eggs (Bennison et al., 2015). In that study, sperm competition experiments were performed using mate-switching in which the female zebra finch copulated twice; first with a male producing long sperm and then after a 3-d interval with a male producing shorter sperm. They further reported that longer sperm's superior fertilization success was because of the fact that longer sperm swim faster than shorter ones. This assumption fits with previous findings showing that sperm swimming velocity has been found to be a major determinant of sperm competition in fish (Gage et al., 2004), birds (Birkhead et al., 1999; Donoghue et al., 1999), and mammals (Tourmente et al., 2019).

In our study, we also found that DB sperm possess a significantly longer flagellum than wild-type sperm, but we failed to detect any differences in the swimming speed between wild-type and DB sperm in a low viscous medium. However, we found that DB sperm swam faster than wild-type sperm when the medium viscosity was increased to a higher concentration. We have no information about the actual viscosity of the fluid in the female reproductive tract in birds because the fluid present in the lumen of the female reproductive tract is too small to analyze; however, cervical mucous in humans was reported to be 200 to 680 cP (Smith et al., 2009). Hence, we assumed that longer sperm swim faster in the oviduct because of greater propulsive (total shear) forces in a viscous environment. In our

Table 5. Targeted likelihood ratio tests with and without MC*type.

Terms	Best model	vs. model	Df[1]	χ^2	*P* value
VCL	MC + type + MC*type	MC + type	1	18.2	1.97×10^{-5}
VSL	MC + type + MC*type	MC + type	1	6.49	1.08×10^{-2}
VAP	MC + type + MC*type	MC + type	1	10.4	1.28×10^{-3}
ALH	MC + type + MC*type	MC + type	1	9.28	2.32×10^{-3}
BCF	MC	Null	1	2.72	9.90×10^{-2}

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; MC, methyl cellulose; VSL, linear velocity; VCL, curvilinear velocity; VAP, average velocity.

[1]Degree of freedom.

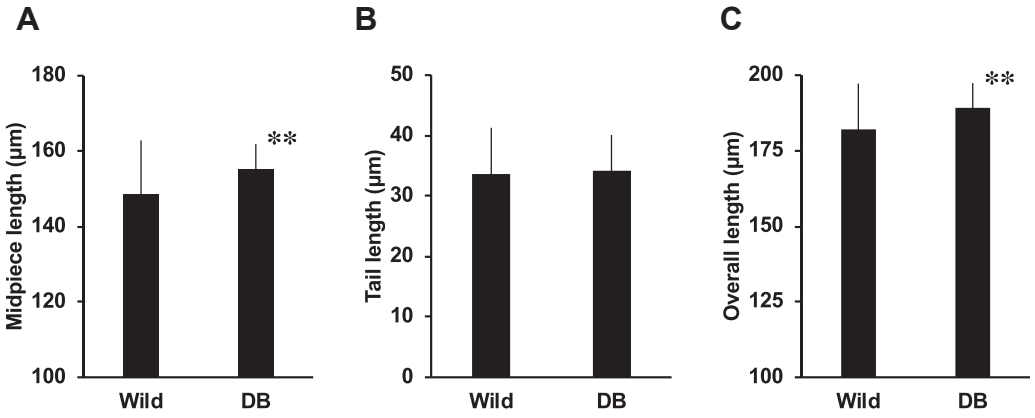

Figure 6. Comparison of flagellar length in wild-type and dominant black sperm. Ejaculated sperm were stained with amide black solution, and the midpiece (A) and tail length (B) were measured. Overall flagellum length was calculated by summing the midpiece and tail length (C). Abbreviations: Wild, wild-type sperm (mean ± SD, n = 127 from 5 birds); DB, dominant black sperm (mean ± SD, n = 125 from five birds). ** denotes statistically significant ($P < 4 \times 10^{-6}$).

preliminary observation, we found that the fluid flow in the UVJ lumen occurs from the upper (infundibulum) to the lower (vagina) orientation produced by cilia movements on the epithelium (our unpublished data). These results indicate that sperm migrating in the UVJ require a strong propulsive force against the reverse flow in the viscous fluid to ensure entry into the SST. Moreover, we found that the sperm flagellar length was associated with the midpiece length. Because the midpiece contains mitochondria that generate ATP, the fuel for flagellar movement, we think that sperm with a longer midpiece have superior ability to produce ATP.

The results from the "sperm filling rate" experiments suggest that DB sperm are much more likely to enter the SST than wild-type sperm, and paternity bias was observed between the 2 strains; the sperm from DB males were predominantly used for fertilization. These results demonstrated that sperm filling of the SST directly reflects subsequent fertilization success in Japanese quail. In other words, it is conceivable that males can increase their reproductive success if the SST are prevalently filled with their sperm.

In this study, we have developed a new method to visualize competitive sperm storage in birds, and by using this technique, we demonstrated that spermatozoa with a longer flagellum have an advantage in terms of fertilization success. Although we cannot deny the possibility that the fertility advantage of DB sperm is because of another unknown factors arised strain differences, these findings agree with a previous finding in Zebra finch sperm. We further demonstrated that the longer sperm advantage is related to the superior ability of sperm to enter the SST and increase sperm filling. Therefore, we conclude that sperm competition does occur in birds competing for residency in the SST, and filling the SST with their own spermatozoa is a very important factor to achieve better fertilization success for male birds.

ACKNOWLEDGMENTS

This work was supported, in part, by a Grant-in-Aid for Young Scientist (20K15648 to MM), a Grant-in-Aid for Scientific Research (B) (General) (17H03902 to TS), and a Grant-in-Aid for Challenging Exploratory Research (20K21368 to TS). All authors approved the final manuscript.

DISCLOSURES

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

REFERENCES

Bakst, M. R. 1994. Fate of fluorescent stained sperm following fnsemination: new light on oviducal sperm transport and storage in the Turkey. Biol. Reprod. 50:987–992.

Bennison, C., N. Hemmings, J. Slate, and T. Birkhead. 2015. Long sperm fertilize more eggs in a bird. Proc. R. Soc. B Biol. Sci. 282: 20141897.

Berger, T. 1995. Proportion of males with lower fertility spermatozoa estimated from heterospermic insemination. Theriogenology 43:769–775.

Birkhead, T. 1987. Sperm competition in birds. Trends Ecol. Evol. 2:268–272.

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

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

Evans, J. P., and A. E. Magurran. 2001. Patterns of sperm precedence and predictors of paternity in the Trinidadian guppy. Proc. R. Soc. Lond. Ser. B Biol. Sci. 268:719–724.

Firman, R. C. 2018. Postmating sexual conflict and female control over fertilization during gamete interaction. Ann. N. Y. Acad. Sci. 1422:48–64.

Firman, R. C., C. Gasparini, M. K. Manier, and T. Pizzari. 2017. Postmating female control: 20 years of cryptic female choice. Trends Ecol. Evol. 32:368–382.

Fisher, H. S., E. Jacobs-Palmer, J.-M. Lassance, and H. E. Hoekstra. 2016. The genetic basis and fitness consequences of sperm midpiece size in deer mice. Nat. Commun. 7:13652.

Florman, H., and T. Ducibella. 2006. Fertilization in mammals. Pages 55–112 in Knobil and Neill's Physiology of Reproduction. J. D. Neill and M. O. Louis, eds. 3rd ed. Elsevier, St Louis, MO.

Flowers, W. L., F. Deller, and K. R. Stewart. 2016. Use of heterospermic inseminations and paternity testing to evaluate the

relative contributions of common sperm traits and seminal plasma proteins in boar fertility. Anim. Reprod. Sci. 174:123–131.
Gage, M. J. G., C. P. Macfarlane, S. Yeates, R. G. Ward, J. B. Searle, and G. A. Parker. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. 14:44–47.
Gasparini, C., E. Daymond, and J. P. Evans. 2018. Extreme fertilization bias towards freshly inseminated sperm in a species exhibiting prolonged female sperm storage. R. Soc. Open Sci. 5:172195.
Ito, T., N. Yoshizaki, T. Tokumoto, H. Ono, T. Yoshimura, A. Tsukada, N. Kansaku, and T. Sasanami. 2011. Progesterone is a sperm-releasing factor from the sperm-storage tubules in birds. Endocrinology 152:3952–3962.
Karr, T. L., and S. Pitnick. 1999. Sperm competition: Defining the rules of engagement. Curr. Biol. 9:R787–R790.
Kekäläinen, J., and J. P. Evans. 2018. Gamete-mediated mate choice: towards a more inclusive view of sexual selection. Proc. R. Soc. B Biol. Sci. 285:20180836.
Kempenaers, B., K. Foerster, S. Questiau, B. C. Robertson, and E. L. M. Vermeirssen. 2000. Distinguishing between female sperm choice versus male sperm competition: a comment on Birkhead. Evolution 54:1050–1052.
King, L., J. Brillard, W. Garrett, M. Bakst, and A. Donoghue. 2002. Segregation of spermatozoa within sperm storage tubules of fowl and Turkey hens. Reproduction 123:79–86.
Korn, N., R. J. Thurston, B. P. Pooser, and T. R. Scott. 2000. Ultrastructure of spermatozoa from Japanese quail. Poult. Sci. 79:407–414.
Kuroki, M., and M. Mori. 1997. Binding of spermatozoa to the perivitelline layer in the presence of a protease inhibitor. Poult. Sci. 76:748–752.
Laturney, M., R. van Eijk, and J. Billeter. 2018. Last male sperm precedence is modulated by female remating rate in *Drosophila melanogaster*. Evol. Lett. 2:180–189.
Lupold, S., S. Pitnick, K. S. Berben, C. S. Blengini, J. M. Belote, and M. K. Manier. 2013. Female mediation of competitive fertilization success in Drosophila melanogaster. Proc. Natl. Acad. Sci. 110:10693–10698.
Manier, M. K., J. M. Belote, K. S. Berben, D. Novikov, W. T. Stuart, and S. Pitnick. 2010. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. Science 328:354–357.
Martin, P. A., and P. J. Dziuk. 1977. Assessment of relative fertility of males (cockerels and boars) by competitive mating. J. Reprod. Fertil. 49:323–329.
Martin, P. A., T. J. Reimers, J. R. Lodge, and P. J. Dziuk. 1974. The effect of ratios and numbers of spermatozoa mixed from two males on proportions of offspring. Reproduction 39:251–258.
Matsuzaki, M., S. Mizushima, G. Hiyama, N. Hirohashi, K. Shiba, K. Inaba, T. Suzuki, H. Dohra, T. Ohnishi, Y. Sato, T. Kohsaka, Y. Ichikawa, Y. Atsumi, T. Yoshimura, and T. Sasanami. 2015. Lactic acid is a sperm motility inactivation factor in the sperm storage tubules. Sci. Rep. 5:17643.
Matsuzaki, M., and T. Sasanami. 2017. Sperm storage in the female reproductive tract: a conserved reproductive strategy for better fertilization success. Pages 173–186 in Avian Reproduction. T. Sasanami, ed. 1st ed. Springer Singapore, Singapore.
McDaniel, C. D., R. K. Bramwell, and B. Howarth. 1997. Development of a novel fluorescence technique for quantifying the total number of spermatozoa stored in the uterovaginal junction of hens. J. Reprod. Fertil. 109:173–179.
Nadeau, N. J., F. Minvielle, and N. I. Mundy. 2006. Association of a Glu92Lys substitution in MC1R with extended brown in Japanese quail (*Coturnix japonica*). Anim. Genet. 37:287–289.
Olsson, M., M. Pagel, R. Shine, T. Madsen, C. Doums, A. Gullberg, H. Tegelström, and H. Tegelstrom. 1999. Sperm choice and sperm competition: suggestions for field and laboratory studies. Oikos 84:172.
Parker, G. A. 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45:525–567.
Pitnick, S., and W. D. Brown. 2000. Criteria for demonstrating female sperm choice. Evolution 54:1052–1056.
Presgraves, D. C. 2005. Recombination enhances protein adaptation in *Drosophila melanogaster*. Curr. Biol. 15:1651–1656.
Sasanami, T., S. Izumi, N. Sakurai, T. Hirata, S. Mizushima, M. Matsuzaki, G. Hiyama, E. Yorinaga, T. Yoshimura, K. Ukena, and K. Tsutsui. 2015. A unique mechanism of successful fertilization in a domestic bird. Sci. Rep. 5:7700.
Sasanami, T., M. Matsuzaki, S. Mizushima, and G. Hiyama. 2013. Sperm storage in the female reproductive tract in birds. J. Reprod. Dev. 59:334–338.
Smith, D. J., E. A. Gaffney, H. Gadêlha, N. Kapur, and J. C. Kirkman-Brown. 2009. Bend propagation in the flagella of migrating human sperm, and its modulation by viscosity. Cell Motil. Cytoskeleton 66:220–236.
Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. 122:765–788.
Tourmente, M., M. Varea-Sánchez, and E. R. S. Roldan. 2019. Faster and more efficient swimming: energy consumption of murine spermatozoa under sperm competition. Biol. Reprod. 100:420–428.
Tsudzuki, M. 2008. Mutations of Japanese quail (*Coturnix japonica*) and recent advances of molecular genetics for this species. J. Poult. Sci. 45:159–179.