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—Review—

Sperm Storage in the Female Reproductive Tract in Birds

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Abstract. The ability to store sperm in the female genital tract is frequently observed in vertebrates as well as in invertebrates. Because of the presence of a system that maintains the ejaculated sperm alive in the female reproductive tract in a variety of animals, this strategy appears to be advantageous for animal reproduction. Although the occurrence and physiological reasons for sperm storage have been reported extensively in many species, the mechanism of sperm storage in the female reproductive tract has been poorly understood until recently. In avian species, the specialized simple tubular invaginations referred to as sperm storage tubules (SSTs) are found in the oviduct as a sperm storage organ. In this review, we summarize the current understanding of the mechanism of sperm uptake into the SSTs, maintenance within it, and controlled release of the sperm from the SSTs. Since sperm storage in avian species occurs at high body temperatures (i.e., 41 C), elucidation of the mechanism for sperm storage may lead to the development of new strategies for sperm preservation at ambient temperatures, and these could be used in a myriad of applications in the field of reproduction.

Key Words: Birds, Fertilization, Japanese quail, Progesterone, Sperm storage tubules, Utero-vaginal junction

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To achieve fertilization, the sperm must encounter the oocytes at the right time and at the right place. It is not thought to be an easy task for the sperm *in vivo* because they must migrate within the female genital tract and reach the site of fertilization when the oocytes are there. Also, the timing of ovulation in the female does not always coincide with the time of insemination by the male. In order to increase the chance of an encounter of the gametes, the female stores the sperm in a reproductive tract. The ability to store sperm in the female reproductive tract is frequently found in a variety of animals including insects, fish, amphibians, reptiles, birds, and mammals [1–3]. In order to store sperm, females possess specialized structures in their genital tracts, such as sperm reservoirs in mammalian species [4], spermathecae in amphibians [5] or spermathecae and seminal receptacles in insects [6], and these structures hold the sperm until the time of ovulation or a time when the oocytes are transported to the site of fertilization. In addition to their function to aid in the success of fertilization, sperm storage organs are also thought to work for sperm selection in the female reproductive tract. This sperm/male selection by the female may maximize the genetic quality of a descendant and the number of offspring by selecting the sperm in the oviduct deposited by different males at different periods (i.e., cryptic female choice [7–9]). From the above, the female reproductive tract acts as not only a path for sperm migration to the site of fertilization, but also provides natural machinery ensuring that eggs are fertilized with favored sperm at the appropriate time and place.

In avian species, specialized simple tubular invaginations referred to as sperm storage tubules (SSTs) are found in the oviduct [10–13]. Because of the presence of this structure, once ejaculated sperm have entered the female reproductive tract, they can survive up to 2–15 weeks in domestic birds, including chickens, turkeys, quails and ducks, depending on the species [14, 15] in contrast to the relatively short life span of mammalian spermatozoa (i.e., several days). SSTs are located in the lamina propria of mucosal folds in the utero-vaginal junction (UVJ) (Fig. 1) and in the infundibulum, although the primary storage site for sperm is the SSTs in the UVJ [13, 16]. Spermatozoa are transported to the infundibulum, which is the site of fertilization and also serves as a secondary sperm storage site [11, 17].

Bakst *et al.* [18] reported that the biological basis of sustained fertility in chicken and turkey hens is their capacity for sperm to reside in the SSTs of the UVJ, and the differences in the duration of fertility between domestic fowl (2 to 3 weeks) and turkeys (10 to 15 weeks) are, in part, related to their respective numbers of SSTs (the mean numbers of SSTs for chickens and turkeys are 4,893 and 30,566, respectively). Although extensive investigation concerning the function of the SSTs in birds has been done since their discovery in the 1960s using ultrastructural analysis [10, 19, 20], we currently know little about the specific mechanisms involved in sperm uptake into the SSTs, maintenance within them, and controlled release of the sperm from them.

In this review, we summarize the current understanding of the mechanism of sperm storage in avian oviducts. In addition, we introduce our recent findings on the mechanism of sperm release from the SSTs in the Japanese quail (*Coturnix japonica*).

Sperm Uptake into the SSTs

Because of a thick and opaque oviductal wall in avian species, it is difficult to observe sperm movement in the oviduct directly. Therefore, we lack basic knowledge of how the sperm are transferred into the SSTs after insemination. After natural mating, the ejaculated sperm are deposited into the vagina; however, it is reported that more than

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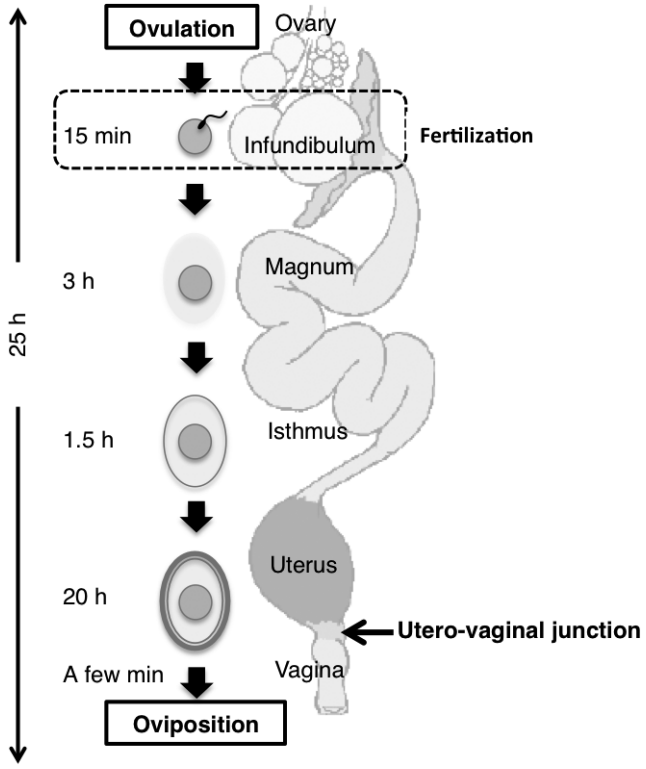

Fig. 1. Schematic drawing of an avian oviduct. After ovulation, the oocyte is incorporated into the infundibulum part of the oviduct, and the sperm ascending the oviduct fertilizes the oocyte within 15 min of ovulation. After fertilization, the surface of the zygote is enveloped by several egg envelopes (i.e., albumen, shell membrane, egg shell and cuticle) through the passage of the oviduct, and is oviposited outside of the body. The position of the utero-vaginal junction is indicated by an arrow. The approximate time of zygote residency within each part of the oviduct is also indicated.

80% of the sperm are egressed from the vagina soon after mating [15]. This suggests the vagina is the primary sperm selection site in avian species. It is also reported that less than 1% of the sperm that inseminate the vagina enter the SSTs [14], and the resident sperm in the SSTs are thought to participate in the subsequent process of fertilization. Das *et al*. observed the time course of the SST filling rate after a single insemination in birds in order to estimate the sperm migration in the oviducts. When the ejaculated sperm were inseminated artificially into the vagina, the sperm reached the SSTs within an hour in the chicken, and the filling rate of the SSTs tended to increase; however, it did not change significantly until 24 h after insemination [21]. Although the mechanisms underlying sperm selection in this process are unknown, the intrinsic motility of the sperm may be an important factor responsible for sperm uptake into the SSTs [22]. Other factors that may influence sperm uptake into the SSTs have also been reported. If vaginal insemination occurs 2 h before or 2 h after oviposition, sperm filling of the SSTs is reduced [23]. This may be at least in part due to the luminal mucosal pH in the vagina, which could affect sperm motility, as it fluctuates during this period [24]. We currently do not know weather a chemotaxis event participates in the process of sperm uptake into the SSTs, but we observed that the filling rate of the SSTs after a single insemination in the quail was approximately 30–50% and that the sperm-filled SST were not uniformly distributed in the UVJ [25]. In addition, when the degree of sperm filling in the SSTs was categorized as being full, partially full, or empty, it differed considerably in each SST in the chicken [26]. These results indicated that unknown mechanisms that affect sperm uptake into the SSTs may be present in avian oviducts, and remain to be elucidated in future studies.

Sperm Maintenance in the SSTs

The period of fertility in birds is correlated with the population of sperm-filled SSTs in the UVJ. It is assumed that the SSTs supply nutrients to the sperm and remove any waste products of sperm metabolism [20]; however, the mechanisms by which the resident sperm are maintained in the SST for extended periods have not yet been elucidated. In the lumen of the SSTs, sperm appear as packed, parallel bundles with their heads directed toward the blind tubular end [19, 25]. In addition, it is reported that the sperm stored in the SSTs are typically immotile [27, 28] and thus thought to be metabolically quiescent as a result of lowered ATP consumption. This is a reasonable strategy because it also leads to reduced production of reactive oxygen species due to sperm respiration, and it may reduce damage to the resident sperm in the SSTs. Thus, the SSTs bringing the stored sperm to a stop may be one of the most important factors in the maintenance of sperm in the SSTs. However, another idea has also been suggested in the chicken: sperm maintain their position against an outward fluid stream in the SSTs via the sperm motility generated by oxidation of exogenous fatty acids released from SST epithelial cells [14]. In this model, sperm efflux is thought to be the result of reduced sperm velocity due to a shortage of energy supplementation from sperm mitochondria. We observed the resident sperm in the UVJ mucosa of the Japanese quail immediately after isolation from birds at 1 h post-mating with non-fixed whole mount specimens. Although we cannot deny the possibility that unexpected changes happened to the sperm due to isolation, we were not able to find any movement of resident sperm in the SSTs.

As suggested by Van Krey *et al*. [29] and Froman and Engel [30], the resident sperm agglutinate head to head in the SSTs of the chicken. We also observed in the quail using light or electron microscopy that most of the sperm in the SSTs attach to each other in a bundle-like agglutination, and single sperm were seldom seen [25]. The tendency of sperm agglutination may be the basis for prolonged *in vivo* storage of spermatozoa because this style of sperm residency is common among domestic birds. In addition, several proteins including carbonic anhydrase [31], avidin [32], aquaporins [33] and alkaline phosphatase [34] have been suggested to have potential roles in sperm maintenance in the SSTs, although no direct implication in sperm storage has been demonstrated. Another important factor supporting sperm storage in the SSTs is defense from anti-sperm immune responses in the oviduct. This is because the immune system in the oviduct is well developed to protect it from infection by various microorganisms. This immune system may also affect the survivability of the sperm in the SSTs when the sperm are recognized as foreign bodies in the oviduct. Das *et al.*

[21] demonstrated that the resident sperm in the SSTs are protected from immune responses by SST structures and transforming growth factor β (TGFb), the expression of which increased when the SSTs were filled with sperm. Since TGFb and the receptors for TGFb are also expressed in sperm, the enhanced expression of TGFb and its receptors may protect sperm in the SSTs by suppressing anti-sperm immunoreactions. Thus, the elimination of anti-sperm immune responses by the TGFb system is one of the factors responsible for sperm maintenance in the SSTs.

We also investigated the mechanism of sperm storage in the Japanese quail. It is reported that sperm at the uterotubal junction (UTJ) in the bovine oviduct bind to the surface of epithelial cells, and that this binding ensures the tethering of the sperm at the UTJ until the time of ovulation [4, 35]. In contrast to the situation in mammalian species, the resident sperm seem to be free from SST epithelial cells in the quail oviduct (Fig. 2). This finding led us to hypothesize that unknown materials in the lumen of the SSTs may affect sperm mobility. In order to confirm this hypothesis, we prepared UVJ extracts, and ejaculated sperm were incubated in the presence or absence of the UVJ extracts. The flagellar movement of the sperm was recorded using a high-speed camera. When the sperm were incubated in the absence of the UVJ extracts, a vigorous flagellar movement was observed (Fig. 3, panel A). However, in the presence of the UVJ extracts, we found that the flagellar movements were relatively quiescent, and that the amplitude of the flagellar movement, as well as the linear velocity of the sperm, decreased (Fig. 3, panel B). More importantly, the addition of UVJ extracts extended the sperm's lifespan *in vitro*. In the presence of UVJ extracts, sperm swam vigorously even after 48 h of incubation, whereas in the absence of the extracts, they usually died within 5 h (data not shown). These results indicate the possibility that unknown molecules responsible for sperm maintenance exist in UVJ extracts. In a previous study, we also observed that the formation of secretory granules in SST epithelial cells fluctuated during the ovulatory cycle [25]. In SST cells, there are well-developed tight junctions among the cells in the apical region, and SST epithelial cells appear to secrete their contents into the SST lumen, where the resident sperm are located. Although we did not elucidate the nature of the secretory granules, it is very likely that the contents of the granules in UVJ extracts affect sperm maintenance in the SSTs.

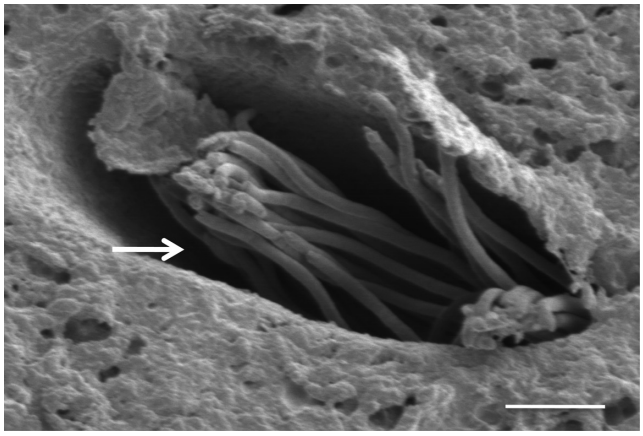

Fig. 2. Ultrastructural observation of the resident sperm in the SSTs. The UVJ was isolated at 1 h after mating, and the tissue was embedded in paraffin wax. Thick sections were prepared, and the surface of the cross section was observed by scanning electron microscopy. The arrow indicates a bundle of the resident sperm in an SST. A representative photograph from those obtained from three different birds is shown. Bar=2.5 μm.

Sperm Release from the SSTs

To achieve fertilization, the resident sperm must be released from the SSTs in order to migrate to the site of fertilization, which is infundibulum part of the oviduct. There are several reports indicating that sperm release from the SSTs is not regulated but occurs in response to the mechanical pressures of a passing ovum, as no contractile elements associated with the SSTs were found [20, 36]. In addition, Burke and Ogasawara [16], who recovered sperm from an inseminated hen oviduct, concluded that sperm release from the SSTs is a slow and continuous event that occurs constitutively during the ovulatory cycle. In contrast, Bobr *et al.* [10], who investigated the distribution of spermatozoa in the hen oviduct after insemination, reported that the resident spermatozoa were discharged from the SSTs close to the times of ovulations and/or ovipositions. In addition, Mero and Ogasawara reported that tubular enlargement of the SSTs is associated with sperm release in the chicken [36]. Moreover, Freedman *et al.* demonstrated the presence of neurons, small ganglia and F-actin in the UVJ of the turkey oviduct and suggested that an unknown neural factor may play a role in the functions related to sperm storage and release from the SSTs [37]. These observations indicated that the timing of the sperm release from the SSTs could be regulated hormonally. To examine whether sperm release from the SSTs is regulated during the ovulatory cycle, a female quail was mated 12 h after oviposition, and the SSTs in the UVJ at 2 or 13 h after mating (corresponding to a time 14 or 25 h after oviposition, respectively) were observed. The percentage of the SSTs filled with sperm at 14 h after oviposition was high (i.e., approximately 50–60%) and decreased significantly to approximately 40% at 25 h. Also, a bundle of sperm extruding into the lumen of the UVJ from the SSTs was frequently seen at 20 h after oviposition, while no such sperm were observed at 8, 14 or 25 h. To test whether hormonal stimulation causes sperm release from the SSTs, birds were injected with various steroid hormones, and the SST filling rate was calculated. As a result, the percentage of SSTs with sperm was only significantly decreased when the animals were treated with more than 0.8 μg/ml progesterone compared with that of control birds injected with a vehicle alone. Scanning electron microscopic observation revealed that the SSTs shrank due to the injection of progesterone, and a bundle of the sperm tail extruded from the SSTs was observed (Fig. 4). This morphological change showed that the SSTs squeezed out the resident sperm into the lumen of the oviduct. These results demonstrated that the release of sperm from the SSTs is a regulated event during the ovulatory cycle, and that progesterone acts as a sperm-releasing factor in birds [25]. If the resident sperm are released from the SSTs without any regulation, most of the sperm ascending the oviduct may be trapped by the descending egg. It is reasonable to suppose that sperm release from the SSTs is stimulated

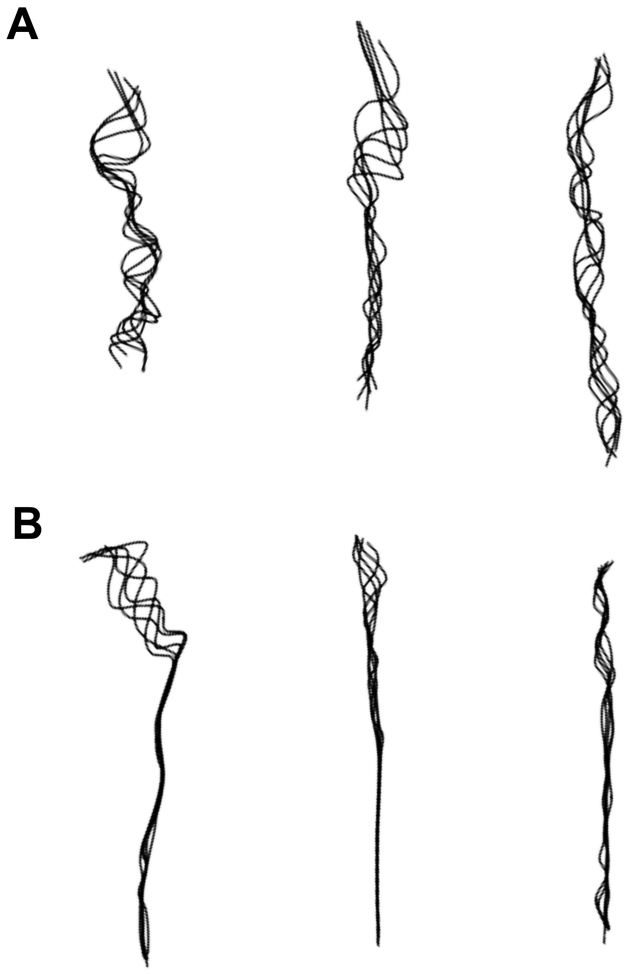

Fig. 3. Effects of UVJ extracts on the motility of the ejaculated sperm. The ejaculated sperm were incubated in the presence (B) or absence (A) of UVJ extracts (300 μg/ml) at 39 C for 10 min. The flagellar movements of the sperm were recorded by a high-speed camera (200 frames per second). The flagella of randomly selected sperm were traced, and 5 images taken every 1/20th of a second were overlaid. Three representative tracing drawings are shown.

by progesterone because there is at least a 5-h grace period before the next ovulation and sperm released from the SSTs can reach the site of fertilization without hindrance from the descending egg. This process may be supported by the lubricant effect of cuticle materials, since the release of cuticle materials from the epithelial cells of the UVJ is also stimulated by progesterone injection [25].

Conclusion

In this review, we reported our understanding of sperm maintenance in the SSTs, as well as the sperm release from the same place. These events are thought to be regulated during the ovulatory cycle. For instance, we demonstrated that progesterone stimulates the release of the resident sperm from the SSTs in the Japanese quail with a contraction-like morphological change in the SSTs. This process may

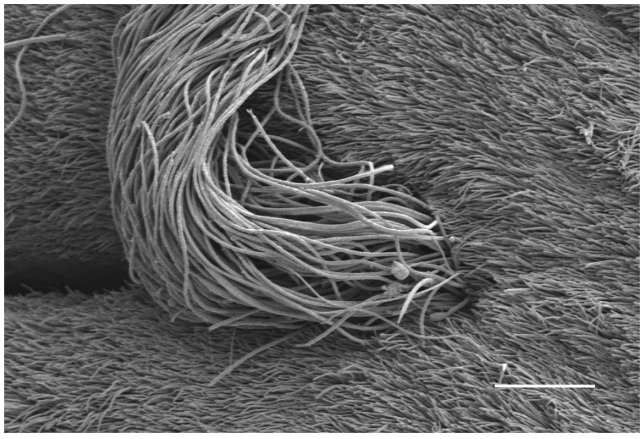

Fig. 4. Ultrastructural observation of the UVJ surface treated with progesterone. After mating, the animals were injected with 0.8 μg/ml progesterone. The UVJ was isolated 1 h after the injection, and the SST entrance area was observed by scanning electron microscopy. A representative photograph from those obtained from three different birds is shown. Bar=10 μm.

be supported by the lubricant effect of cuticle materials secreted from the ciliated cells of the UVJ, as well as unknown materials supplied from SST epithelial cells, in events coincidently triggered under progesterone control. In addition, we observed secretory granules in SST epithelial cells, and the number of these secretory granules fluctuated during the ovulatory cycle, indicating that SSTs epithelial cell derived unknown materials secreted into the lumen of the SST may affect sperm physiology (e.g., motility, respiration, metabolism, etc.) [25]. Although the nature of the molecules responsible for long-term sperm maintenance remains to be clarified, our findings indicated that UVJ extracts possess the ability to reduce avian sperm motility and to extend sperm life span *in vitro*. Because sperm storage in avian species occurs at a high body temperature (i.e., 41 C), elucidation of the mechanism for sperm storage may lead to the development of new strategies for sperm preservation at ambient temperatures, and these could be used for a myriad of applications in the field of reproduction.

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