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Spermiophagy in the Male Reproductive Tract of Some Passerine Birds

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In order to elucidate the locus and means of spermiophagy in passerine birds, we examined histologically the entire male reproductive tract of sexually mature birds of three passerine species with different forms of sperm competition, namely, the alpine accentor (Prunella collaris), the red-flanked bush robin (Tarsiger cyanurus), and the Bengalese finch (Lonchura striata var. domestica). Spermiophagy occurred consistently and frequently in the epithelial layer of the seminal glomera and ejaculatory duct in each species, which were regularly identified by non-ciliated epithelial cells. The epithelial spermiophagy was occasional or infrequent in other portions of the seminal tract, and spermiophagy by macrophages was uncommon throughout the tract. Quantitative data in the seminal glomera and ejaculatory duct gave no clear answer concerning a possible relationship between the epithelial spermiophagy and different levels of sperm competition among these passerine species. In conclusion, the epithelial lining of the terminal region of the seminal tract is the main site for spermiophagy in the male reproductive tract of these passerine species, which activity serves to maintain the quality of semen by eliminating infertile spermatozoa as well as sperm remaining at the end of the breeding season.

Key words: epithelial cells, passerine birds, seminal glomera, spermiophagy, sperm competition

INTRODUCTION

Spermiophagy (or spermatophagy) denotes the phagocytosis of spermatozoa and their fragments by somatic cells (Holstein, 1978). This activity has been reported to occur widely in various groups of vertebrates, e.g., teleosts (Porawski et al., 2004), amphibians (Sever, 1992), reptiles (Akbarsha et al., 2007), birds (Tingari, 1972; Nakai et al., 1989; Aire, 2000), and mammals (Murakami et al., 1979; 1985; Goyal, 1982; Abou-Elmagd and Wrobel, 1990). In mammals, spermiophagy takes place in various portions of the male reproductive tract, such as the seminiferous tubules (Holstein, 1978), rete testis, efferent duct (Holstein, 1978; Goyal, 1982), ampulla of the vas deferens (Murakami et al., 1985), seminal vesicle (Murakami et al., 1978), and ejaculatory duct (Abou-Elmagd and Wrobel, 1990). In these locations, Sertoli cells, macrophages or epithelial cells lining the seminal tract are involved in this spermiophagy. According to Holstein (1978), spermiophagy is frequently seen in the gonads of older men and in certain cases of oligozoosperma, i.e., generally defined, in man, as a concentration of spermatozoa less than 20 million per ml. Also, in the macaque Macaca fascicularis, spermiophagy increases at the end of the spermatogenic season.

In birds, previous studies on the domestic fowl Gallus domesticus have shown that the epithelial lining of the rete testis and epididymal efferent ducts phagocytose spermatozoa only occasionally (Tingari, 1972; Nakai et al., 1989; Kirby et al., 1990) and that macrophages are also involved in spermiophagy (Nakai et al., 1989). Later, Aire (2000) reported active spermiophagy by the non-ciliated cells in the epithelial lining of the epididymal efferent ducts of the normal chicken. In contrast, studies on wild passerines (Middleton, 1972; Chiba and Nakamura, 2003) suggested spermriophagy in the seminal glomera (or glomus). This organ is known as a characteristic feature of the male reproductive system of passerines (although it has also been reported to exist in the psittacid Melopsittacus undulatus; Samour et al., 1987), and is regarded as an anatomical adaptation that ensures a storage and probable maturation site for spermatozoa that are necessary for sperm competition (Bedford, 1979; Lake, 1981; Birkhead et al., 1993; Aire, 2007). In light of the above, the following subjects should be investigated and clarified in particular concerning the passerines: (1) What is the main site for spermiophagy in their male repro-
ductive system? (2) What types of cell are involved in spermiophagy in the seminal tract? (epithelial cells, macrophages, or cells of other types?), and (3) What is the biological significance of spermiophagy in the normal healthy animal? At present, nearly nothing is known about these aspects of spermiophagy in wild birds (Aire, 2007).

In order to answer these questions, we microscopically examined spermiophagy in the male reproductive system of three species of passerines, namely, the alpine accentor (Prunella collaris), the red-flanked bush robin (Tarsiger cyanurus), and the Bengalese finch (Lonchura striata var. domestica). We also focused on in its possible relation to sperm competition (Birkhead et al., 1993), because there is a background information regarding this possibility; i.e., in passerines with low levels of sperm competition, the incidence of malformed sperm is higher than in species with intense sperm competition, strongly suggesting that spermiophagy may be more intense in species in which sperm competition seems to be less intense (Calhim et al., 2007; Birkhead et al., 2007; Immler et al., 2008). We consider this prediction worth testing in the present study.

MATERIALS AND METHODS

Animals
All treatments complied with the Guidelines for the Care and Use of Laboratory Animals in Nippon Dental University.

Mature adult males of the alpine accentor (four birds, 44–55 g in body weight (b.w.)), red-flanked bush robin (five birds, 13–14 g in b.w.), and Bengalese finch (seven birds, 14–16 g in b.w.) were used in this study. The samples of alpine accentor and red-flanked bush robin were collected (by permission of the Ministry of the Environment of Japan and the Government of Shizuoka Prefecture) during their breeding season, from June to August, on the summit of Mt. Norikura, Nagano Pref., Japan (36°06’N, 137°33’E, 2,600–3,026 m above sea level (a.s.l.)) and in a forest of Mt. Fuji, Shizuoka Pref., Japan (35°26’N, 138°47’E, 1,500–2,000 m a.s.l.), respectively. Those of the Bengalese finch were purchased from a local dealer, but information about their mating conditions in cages was incomplete.

Light and electron microscopy
After measurement, the birds were killed by quick dislocation of their cerviciles, dissected, and immersed in a solution of 5% buffered formaldehyde, 5% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2 or in Bouin’s mixture for a few days. The entire male reproductive system was carefully dissected out and re-fixed in freshly prepared 5% buffered formaldehyde or Bouin’s fixative for light microscopy (LM) or in Karnovsky’s fluid for transmission electron microscopy (TEM). The sexual organ index (SOI: the weight of the entire male reproductive system relative to the body weight) was calculated thereafter. Various sexual organs were examined under a dissection microscope, appropriately trimmed, and processed for routine histology and cytology. Paraffin sections cut at 8-μm thickness were stained with the following stains: Mayer’s hematoxylin-eosin, azan trichrome, and periodic acid Schiff’s (PAS) reagent for demonstration of polysaccharide. The sections were examined under a light microscope. For TEM, the aldehyde-fixed tissue blocks were rinsed with 0.1 M phosphate buffer (pH 7.4), immersed in 1% osmium tetroxide in the same buffer for one hour, dehydrated, and embedded in Spurr resin. Ultra-thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and viewed with a JEOL 1200 EX electron microscope. Semi-thin sections were stained with toluidine blue in borax and observed under a light microscope.

Table 1. Summary of spermiophagy in the male reproductive tract of three species of passerines, namely, the alpine Accentor, red-flanked bush robin, and Bengalese finch.

<table>
<thead>
<tr>
<th>Name of animal</th>
<th>alpine accentor</th>
<th>red-flanked bush robin</th>
<th>bengalese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>n = 4</td>
<td>n = 5</td>
<td>n = 7</td>
</tr>
<tr>
<td>Mean SOI (%)*</td>
<td>6.8</td>
<td>2.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Range</td>
<td>4.6–8.4</td>
<td>1.9–3.6</td>
<td>0.5–0.7</td>
</tr>
<tr>
<td>Type of spermiophagy</td>
<td>SE</td>
<td>SM</td>
<td>SE</td>
</tr>
<tr>
<td>Portions of seminal tract</td>
<td>Ret e testis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Epididymis</td>
<td>++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Seminal glomera</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Ejaculatory duct and papilla</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
</tr>
</tbody>
</table>

*SOI, sexual organ index (weight of the entire male reproductive system relative to the body weight, %). SE, spermiophagy by epithelial cells; SM, spermiophagy by possible macrophages; –, absent; +, rare or uncommon; ++, occasional; ++++, frequent.

Quantitative assessment

Spermiophagy was microscopically examined and its incidence was roughly scored as follows (Table 1): absent (−), rare or uncommon (+), occasional (+++) or frequent (+++). In order to compare the incidence of epithelial spermiophagy (i.e., spermiophagy by seminal duct epithelium) among these three species, we counted the number of absorbed spermatozoa in the epithelium of the posterior region of the seminal tract, namely the seminal glomera (SG) and the ejaculatory duct (ED). For this purpose, three alpine accentors, five red-flanked bushrobins, and three Bengalese finches were examined and the number of absorbed spermatozoa per single section of the seminal tract was counted in 40 randomly selected sections per individual. Furthermore, in order to assess local differences of the epithelial spermiophagy we similarly counted the number of absorbed spermatozoa in three different portions of the seminal tract, i.e., the proximal, middle, and distal portions, respectively (Figs. 1 and 2); the proximal portion includes approximately anterior 2/5 of the SG, the middle one occupies the following (middle) 2/5 of the SG, and the distal one corresponds to the posterior 1/5 of the SG and the whole ejaculatory duct (Fig. 2 for example). In the alpine accentor, remarkably developed SG and ED (Fig. 1) are actually equivalent to the dorsal and ventral lobes of the cloacal protuberance described in a previous paper (Chiba and Nakamura, 2003). In practice, the number of absorbed spermatozoa per single section of the seminal tract was counted in randomly selected 40 sections of each portion per individual. The counting was carried out on one alpine accentor (one of four birds, tentatively named Ac-1), three red-flanked bushrobins (three of five birds: Br-1, -2 and -3), and three Bengalese finches (three of seven birds: Bg-1, -2 and -3) (Fig. 4), due to the limited number and condition of the specimens. We used the median of each individual as a representative value to minimize bias due to pseudoreplication.

RESULTS AND DISCUSSION

The male reproductive system of the birds examined showed considerable inter-specific variation in their SOI values (Table 1), mainly due to differences in the weight of the testis and SG/ED relative to that of the body mass (Fig. 1A–C), probably reflecting the degree of sperm competition related to their mating systems. For example, the polygynandrous alpine accentor, having a higher frequency of copulation than the other two species, has a remarkably voluminous testis and SG/ED (Nakamura, 1990; Chiba and Nakamura, 2003) in comparison with those of the monoga-
mous red-flanked bushrobin, which has occasional extra-pair copulation (about 50% of nests showed the extra-pair fertilization; unpublished data by Morimoto) and of the domesticated monogamous Bengalese finch, in which detailed results on the size of their testes and SG, ejaculating capacity, and sperm depletion were previously documented (Birkhead, 1991).

LM examination for spermiophagy was carried out throughout this system, i.e., the testis, epididymis, vas deferens, SG, ED, and terminal papilla. Spermiophagy was found consistently and frequently in the epithelial layer of the SG and ED in each species examined (Fig. 3A–C; Table 1). There, morphologically intact spermatozoa in the cavity had been ingested by non-ciliated epithelial cells (Fig. 3A–C). However, no clear numerical difference was found in the incidence of the epithelial spermiophagy among the 3 species examined: alpine accentor (20.7 ± 4.6 as mean ± SD, n = 3); red-flanked bushrobin (19.1 ± 10.7, n = 5); and Bengalese finch (21.8 ± 27.9, n = 3). On the other hand, the epithelial spermiophagy in the SG/ED tended to be more active towards the posterior portion of the seminal duct system in the alpine accentor and the red-flanked bushrobin (Fig. 4), although this tendency was not necessarily clear in the Bengalese finch, in which the incidence of epithelial spermiophagy was considerably variable (Fig. 4). The numerical data in Fig. 4 were also shown: 14.6 in the proximal portion, 17.0 in the middle portion, and 25.9 in the distal portion for the alpine accentor (n = 1); 5.3 ± 23.0 (average ± SD), 8.2 ± 12.4, 18.1 ± 12.8 for the red-flanked bushrobin (n = 3); 18.8 ± 25.2, 25.1 ± 32.2, 16.7 ± 15.7 for the Bengalese finch (n = 3), respectively.

TEM examination confirmed the spermiophagy and demonstrated that the epithelial cells had a varied number of microvilli at their apical plasma membrane, empty vesicles or vacuoles of different sizes in their apical cytoplasm, lysosome-like dense bodies, and a moderate number of mitochondria (Fig. 3E), in general accordance with the previous observations made in domestic fowl (Tingari, 1972; Nakai et al., 1989; Aire, 2000). Epithelial spermiophagy was occasional or rare in the epididymal efferent ducts and vas deferens, but not found in the rete testis or ejaculatory duct or its terminal papilla (Table 1). We also observed that free cells, presumptive macrophages, had ingested spermatozoa and fragments of spermatogenetic cells in the cavities of the seminal tract (Fig. 3D, F; Table 1), although this type of spermiophagy was uncommon throughout the seminal tract.

The present study along with previous observations (Middleton, 1972; Chiba and Nakamura, 2003) demonstrated spermiophagy in the seminal glomera and some other locations of the seminal tract in the passerines. Such findings support the view that the seminal glomera, a site for storage and probable maturation of spermatozoa in passerines (Lake, 1981; Bedford, 1979), simultaneously serves as a major site for spermiophagy, contributing to maintenance of the quality of semen by removal of remaining and infertile spermatozoa, i.e., acting as a "physiological filter" (Chiba and Nakamura, 2003) or "sorting apparatus" for selection of healthy fertile sperm as well as for removing immature or aging sperm (Birkhead et al., 1995). Our findings also indicate that this epithelial spermiophagy is the main process for...
sperm absorption in the seminal tract of the passerines under normal healthy conditions. Concerning the site and cell type for spermiophagy in the seminal tract, the present results are inconsistent with those found for chickens, in which spermiophagy by macrophages and/or epithelial cells occurs mainly in the epididymis (Tingari, 1972; Nakai et al., 1989; Kirby et al., 1990; Aire, 2000). In more detail concerning the spermiophagy of the chicken, different authors have reported different results. Tingari (1972) reported that three types of the epithelial cells are responsible for spermiophagy, i.e., the low cuboidal cell in the rete testis, the ciliated cell lining the efferent and connecting ducts in the epididymis, and the non-ciliated type II cell in the vas deferens, whereas Nakai et al. (1989) showed that the luminal macrophages, not epithelial cells, in the epididymis and rete testis showed active spermiophagy. On the other hand, Aire (2000) reported active spermiophagy by the non-ciliated type I epithelial cell in the efferent duct of the epididymis. Thus, the epithelial spermiophagy in the chicken appeared to occur in a wide range of the seminal tract and to be performed by various cell types, being in contrast to the case of the passerine birds studied. We consider that the non-ciliated cells in the passerines responsible for spermiophagy may be equivalent to the non-ciliated type II cell in the chicken. This discrepancy may be due to the differences in the systematic position and breeding habits between the passerines and domestic chickens. The alpine accentor and red-flanked bush robin are seasonal breeders and show clear seasonal changes in their reproductive activities (Nakamura, 1990; Morimoto et al., 2006), although the Bengalese finch as a domesticated passerine can breed throughout the year. Generally, the seminal glomera (and probably the ejaculatory duct) of passerines increases in size in parallel with spermatogenesis and stores plenty of sperm for copulation during the breeding period (Bailey, 1953; Nakamura, 1990); but thereafter it shrinks (Chiba and Nakamura, 2003), and a variable amount of sperm may remain un-ejaculated in the organ, and therefore needs to be absorbed prior to the next breeding season. Thus, it seems reasonable to suggest that the seminal glomera together with the ejaculatory duct also serve as the main site for removing or ingesting the remaining and infertile (including immotile and inactive) sperm.

As mentioned above, the epithelial spermiophagy in the seminal tract tended to be more active towards the posterior portion of the seminal glomera and ejaculate duct, at least in the alpine accentor and the red-flanked bush robin. The results may explain the fact that in the zebra finch (Taeniopygia guttata) the number of morphologically abnormal sperm in the seminal glomera decreases towards the ejaculatory duct, showing a clear pattern of sperm quality across the proximal, mid, and distal regions of the seminal glomera (Birkhead et al., 1995). On the other hand, no clear difference was found in the incidence of epithelial spermiophagy in the seminal tract of the passerines under normal healthy conditions. Concerning the site and cell type for spermiophagy in the seminal tract, the present...
phagy among the three species with different intensities or levels of sperm competition, although it tends to be more active towards the terminal portion of the seminal tract in the alpine accentor and the red-flanked bushrobin in contrast to varied activity in the Bengalese finch. Currently, it is premature to determine whether the present data support the prediction (see Introduction) deduced from the studies on sperm quality and sperm competition (Birkhead et al., 1995, 2007; Cahim et al., 2007; Immler et al., 2008), or not.

In spite of the biological significance of spermiophagy, its physiological mechanism remains obscure and should be analyzed in future studies by experimental approaches as well as from the viewpoints of reproductive and evolutionary biology.

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