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Timing associated with oviductal sperm storage and release after artificial insemination in domestic hens

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ABSTRACT

Female birds store sperm in sperm storage tubules (SSTs) in the uterovaginal junction of their reproductive tract for days or weeks (depending on species) before fertilization. Sperm are transported from the SSTs to the infundibulum where fertilization occurs immediately after ovulation of each ovum. The timing of sperm release from the SSTs relative to ovulation is unknown for any bird. Here, we show that, after artificial insemination of domestic fowl *Gallus domesticus*, sperm are not accepted into any region of the oviduct before sexual maturity. Once hens reach maturity, there is a temporal shift in the distribution of sperm throughout the oviduct. Sperm are first accepted into and accumulate in the SSTs 6 to 8 days before ovulation but are at this point significantly less numerous in the infundibulum. From 1 to 6 days before ovulation, approximately 10-fold more sperm (235×10^3 sperm) populate the infundibulum than at 6 to 8 days before ovulation (26×10^3 sperm; $P < 0.001$). Our results suggest that the mechanisms underlying sperm acceptance and release in the oviduct are under fine temporal control, most likely mediated by female hormones.

1. Introduction

Sperm storage in the oviduct is an essential and probably ubiquitous feature of avian reproduction [1]. Sperm are stored in and released from sperm storage tubules (SSTs) located in the uterovaginal junction, from where they travel to and accumulate in the infundibulum where fertilization occurs. Transport of sperm to the infundibulum is rapid, with some sperm reaching the SSTs within less than one hour of insemination in the domestic fowl *Gallus domesticus* and turkey *Meleagris gallopavo* [2,3]. Because avian ova are ovulated sequentially at intervals of 24 hours (or longer in some species) and can be fertilized only during a short window of approximately 15 minutes after ovulation [4], the release of stored sperm from the SSTs ensures a constant supply in the infundibulum [5,6].

The duration of sperm storage varies markedly between the bird species, from about 6 days in pigeons to over 2 months in some seabirds [6,7] and over 3 months in the domestic turkey *M gallopavo* [8]. Because in some bird species, sperm remain viable from inseminations occurring several months before the onset of laying [9], the oviduct in these species must be sufficiently well developed to contain functional SSTs long before egg laying commences. In the fulmar *Fulmarus glacialis*, which routinely stores sperm for at least 3 weeks before fertilization, SSTs are present at least 4 weeks before ovulation [7].

In most temperate breeding birds, the reproductive tract of both sexes regresses outside the breeding season and regrows rapidly the next breeding season [10]. The development of the SSTs and the appearance of sperm in the SSTs relative to the onset of laying are known for three passerine birds: the yellow-headed blackbird *Xanthocephalus xanthocephalus* [11], pied flycatcher *Ficedula hypoleuca* [12], and zebra finch *Taeniopygia guttata* [13]. The first two of these are seasonal

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breeders; the zebra finch (both in the wild and captivity) is an opportunistic breeder and under suitable conditions breeds continuously. When female pied flycatchers and yellow-headed blackbirds first return from migration about 10 days before the onset of egg laying, their oviduct and ovarian follicles are relatively undeveloped but their SSTs are functional [4,10]. Similarly, in the zebra finch, SSTs are present in the oviduct of unpaired females.

Avian SSTs were first discovered and have been studied in most detail in the domestic fowl [3,14,1]. It may therefore seem surprising that the temporal pattern of sperm storage potential relative to the onset of egg laying in the domestic fowl is poorly known. It is known, however, that the domestic fowl, like other birds, can store sperm before the onset of laying and that the uptake of sperm by the SSTs is greater at this time than that when inseminations occur during laying [15–17]. In this respect, the domestic fowl is similar to other birds that copulate most frequently in the days or, in the case of certain seabirds, weeks, before the onset of egg laying and either cease to copulate or reduce the rate of copulation once egg laying has started [18,19].

Sperm storage tubule maturation is at least partially regulated by estradiol and progesterone produced in the ovaries, the levels of which vary with female maturity [20–23]. It therefore follows that female hormones may mediate the uptake and acceptance of sperm into the SSTs.

What remains unknown, for any bird species, is whether sperm accumulate in the SSTs for some time before being released to populate the infundibulum just before ovulation. It seems logical that this is what should occur, especially in seabirds where the interval between the last insemination and fertilization may be several weeks.

The aim of the present study therefore was to establish, using the domestic fowl, (1) the timing of sperm acceptance into the SSTs and (2) the appearance of sperm in the infundibulum relative to the onset of laying.

2. Materials and methods

Immature female domestic fowl from an egg-type strain (ISA Brown; Institut de Selection Animale, Saint-Brieuc, France) were raised collectively in a floor pen (photoperiod: 8L:16D) at Institut National de la Recherche Agronomique research facilities, Nouzilly (France). At 16 weeks of age, the females were caged individually and subjected to a progressively increasing photoperiod (+2 h/wk from 18 weeks of age, reaching 16L:8D at 22 weeks of age) to stimulate gonad development. Food and water were available *ad libitum* throughout the study. All procedures described herein were approved by the Agricultural Agency and the Scientific Research Agency and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Between the ages of 18 and 20 to 22 weeks (the latter is the expected age of laying), each female was artificially inseminated intravaginally, with a single dose of 200×10^6 sperm from the pooled ejaculates of three to four males. Semen was obtained directly from males by dorsoabdominal massage [24]. Semen of 5 μL in 1000-μL NaCl solution (concentration, 9 mM) was analyzed *via* spectrophotometry, and the absorbance reading was used to calculate the volume of semen required for 200×10^6 sperm [25]. The correct volume of semen was then diluted 1:2 with Lake's diluent [26] and inseminated immediately.

Data were collected from 15 females (none of which were visually seen to reject sperm after artificial insemination); all of which were sacrificed 24 hours after artificial insemination by an intravenous administration of sodium pentobarbital (1 mL/kg body weight). Abdomens were immediately excised, ovaries were removed, and the diameter of the largest ovarian follicle (LF) was measured to estimate the stage of follicular development and therefore the number of days before the first ovulation [27,28]. From the LF diameter measurements, females were classified as one of the following using reference data from eight additional mature laying hens (see Electronic Supplementary Material [ESM] 1, Table S1): (1) immature (LF $<$ 5 mm = 8–10 days pre-lay; n = 3), (2) maturing (5 mm $\leq$ LF $<$ 10 mm = 6–8 days pre-lay; n = 3), (3) mature, pre-lay (10 mm $\leq$ LF + nonlaying = 2–6 days pre-lay; n = 3), or (4) mature, in lay (10 mm $\leq$ LF + egg in formation in oviduct = 1 day pre-lay; n = 6). Plasma concentrations of estradiol and progesterone were also measured from blood samples taken from these eight reference hens before, at, and after the onset of egg laying (see ESM 2 for further details).

To assess how many sperm had penetrated the oviduct the following procedure was used. The vaginal region was clamped just distal to the uterovaginal junction (UVJ), injected with 2-mL Dulbecco's Modified Eagle Medium (Sigma, l'Isle d'Abeau Chesnes, France) to distend the mucosal folds, and then agitated for 2 minutes to flush sperm out. The resulting solution was retained for sperm counts. The UVJ was dissected from the tract, cut open, and the mucosa was scraped and digested with Type XI collagenase (Sigma) as described by [29]. After digestion, UVJ tissue preparations were diluted to facilitate sperm counting. Finally, the infundibulum was dissected from the tract at its junction with the magnum, cut open, and the mucosa was rinsed in 2-mL Dulbecco's Modified Eagle Medium. The rinsing solution was retained for sperm counts; then, the infundibula mucosa was digested and diluted as described for the UVJ mucosa. Sperm numbers in the rinsing and digestion solutions were estimated using Thoma hemocytometers (six replicates per solution) under $\times$ 400 phase-contrast microscopy.

Data analysis was conducted using the statistical software R (Version 2.10.0 [30]). Variation in sperm number was analyzed as a function of oviduct region and female maturity status, controlling for female identity as a random effect in a generalized linear mixed model with a Poisson error distribution. The number of sperm found in each region of the oviduct was also analyzed separately with respect to female maturity status, using generalized linear models with a Poisson error distribution. Explanatory terms were considered statistically significant when $P < 0.05$.

3. Results

The number and the distribution of sperm in the different oviduct regions were clearly associated with the degree of sexual maturation. After insemination of 200×10^6 sperm, the oviducts of immature females (8–10 days before laying) were devoid of sperm in all