Original Article

Evaluation of Age-Dependent Sperm Morphometry in Gloster Canaries via CASA System

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Abstract

The canary is a small passerine bird of the finch family. Canary spermatozoa are also morphologically similar to many other songbird species. In this study, the morphological characteristics of Gloster canary spermatozoa of different age ranges were examined, and the length values of the parts forming the spermatozoa were compared. The study used ten active Gloster canaries between the ages of three (Trial-1) and four years (Trial-2). Semen samples from 3-year-old canaries in the study were evaluated, and morphological segment lengths were recorded. In the study, to avoid individual differences and make homogeneous measurements, semen samples were retaken from the same canaries one year later during the breeding period and evaluated. However, since the statistical average result was obtained in the study, the canaries were compared as a whole, not individually. Semen was collected from each bird and fixed in a 5% formaldehyde solution. Collected semen samples were evaluated in terms of head length, total flagellum length, acrosome length, and nucleus size at 20× magnification in the computer-assisted CASA system for morphological examination. As a result, it was observed that the head and flagellum length in domestic canary spermatozoa showed an inverse relationship with age.

Keywords: Canary bird, CASA analyze, morphology, sperm

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Introduction

The reproductive biology of birds is of fundamental importance for the sustainability and evolutionary success of the species. The reproductive system can vary between species and individuals in terms of anatomical and physiological aspects. These differences can cover many areas, from morphological structure to behavioral characteristics. Spermatozoa, as male reproductive cells, are involved in the fertilization process. Sperm morphology varies among species, among populations of the same species, among males within a population, and among ejaculates of a single male (Pitnick *et al.,* 2009). Therefore, studying the morphology of spermatozoa is important to reveal and evaluate interspecific and interindividual differences, to understand reproductive success, and to determine differences between species (Schmoll *et al.,* 2018). Sperm morphology in songbirds generally exhibits a conserved structure. The spermatozoon has a thin filamentous structure in these species, and the head (acrosome and nucleus) is corkscrew-shaped. The middle piece is long and spirally wrapped around the tail (Jamieson 2007). In addition, the sperms of many songbird species have a spiral membrane (acrosome spine) around the acrosome nucleus. In general, the structure of the morphological components of the spermatozoa and the ratio of some sperm parts, such as the head and flagellum length, are thought to affect sperm swimming speed (Lüpold *et al.,* 2009). In addition, it has been observed that the speed of the spermatozoa is related to the flagellum length and that the speed of the spermatozoa increases as this ratio decreases. It has been shown that the length and components of the spermatozoon play a role in determining fertilization capacity (Knief *et al.,* 2017)

Sperm structure and quality may show age-related changes (Abah *et al.,* 2023). Aging leads to the deterioration of physiological functionality and reduced individual fitness and reproductive performance (Partridge & Barton, 1993). Aging is generally associated with changes in physiological and behavioral characteristics such as nutritional efficiency, immune system efficiency, health, or individual condition. However, individual reproductive performance may deteriorate with increasing age. It is well known that increasing male age is associated with a significant decrease in semen parameters such as semen volume, total spermatozoa per ejaculate, sperm concentration, motility, morphology,y, and vitality (Kipper *et al.,* 2017).

Computer-assisted sperm morphometry analysis (CASA-Morph) allows the objective and reliable analysis of the shape and size of reproductive cells in modern research (Santiago-Moreno *et al.,* 2016). The CASA-Morph system has the potential to provide more reliable results by minimizing the risk of error, especially when used in the morphological analysis of bird semen. This system can increase the reliability of the results obtained while shortening the analysis times, thus offering valuable advantages to studies of semen morphology (Hook & Fisher 2020). It is known that morphological defects in bird semen appear, especially during the storage period. In this context, it is noteworthy to emphasize the effectiveness of CASA systems in determining sperm kinematics (Gerzilov & Andreeva 2021). It has been stated that special methods such as CASA-Morph, especially in the morphological evaluation of bird semen, can significantly increase the reliability of comparative studies (García-Herreros 2016). It has been suggested that examining ten spermatozoa in each male bird's semen may be sufficient to evaluate sperm morphology in songbirds (Kleven *et al.,* 2009).

This study aims to evaluate the age-related development of the morphological structure of Gloster canary spermatozoa with the help of Computer Aided Sperm Analyzer (CASA) and to reveal the morphological characteristics of canary sperm in a more detailed and reliable way.

Materials and Methods

Ethics committee permission: Ethics committee approval of this study was obtained from Ondokuz Mayıs University Animal Experiments Local Ethics Committee (Date: 14.08.2023; Acceptance No: 2023/47).

***Animal selection*:** The study used 10 active Gloster canaries aged 3 and 4 years. The canaries were housed in 4-storey cages measuring 60cm × 40cm × 50cm. To induce sexual activation, a photoperiod of 16 hours light and 8 hours dark (Singh *et al.,* 2021) was applied. Necessary care was taken regarding the care and welfare of the animals used in the study. All kinds of stressful environments were avoided while collecting sperm from male birds. Semen was collected by cloacal massage. The care, nutrition, and shelter needs of the female birds were met by observing animal welfare rules.

***Sperm collection and preparation*:** After the photoperiod application, the canaries were trained by taking semen every 4 days for 3 weeks to eliminate the adverse effects that may occur due to stress during the semen collection process. Sperm retrieval was performed using the cloacal massage method (Cramer *et al.,* 2021). The semen samples were immediately diluted 1:1 with Dulbecco's Modified Eagle Medium (DMEM). For each bird, Diluted fresh semen was evaluated under a 38 C heated microscope at 20x magnification. A motility examination was performed twice at different times for each bird. After sperm collection, motility and kinematic parameters of sperm samples were evaluated using a Computer-Aided Sperm Analyzer (CASA) (SCA®, Microptic, Barcelona, Spain). The CASA system is supported by a negative phase-contrast microscope with a heating table (Nikon, Eclipse, Tokyo, Japan). Spermatozoa total motility (%) and motile spermatozoa density parameters were evaluated by reading at least five microscope fields in the software system. After dilution, semen with motility values and active and healthy semen with more than 70% motility were selected for morphological analysis.

Evaluation of spermatozoon morphology: 2µL of a semen sample from each canary was transferred in

20µL Phosphate Buffer Saline (PBS) solution containing 5% formaldehyde. Then, 10µL of sperm sample, spread on the slide, was left to dry at a 45-degree angle. After drying, the slide was dipped in the jar containing SpermBlue dye and left for 2 minutes. After the slide was painted with SpermBlue, it was left to dry at 60-80°C. After drying, the slide dipped twice in a jar containing distilled water and was left to dry again (Cramer *et al.*, 2020). After the staining process, Gloster canary sperm morphology was analyzed using Computer Aided Sperm Analyzer (CASA) at 20x magnification (Nikon, Eclipse, Tokyo, Japan) for at least ten spermatozoa. The morphological structure of the canary spermatozoon was evaluated in terms of parameters such as acrosome length, head length, nucleus length, and total flagellum length, and mean values were calculated (Yang *et al.*, 2020).

Result

Ten active male canaries were used in the study, and ten samples were taken from each canary (Kleven *et al.*, 2009). The findings regarding the spermatozoa morphological values of Gloster canaries are presented in Tables 1-3. The averages of the collected samples were taken and evaluated.

When the spermatozoon morphological part lengths of the two experimental groups used in the study were compared, a statistically significant difference was observed in terms of head length and flagellum length. When the head and flagellum lengths of the Trial-1 (3 years old) group were evaluated, they were longer than those of the Trial-2 (4 years old) group. There is a statistically positive correlation between head and flagellum lengths, regardless of age (P<0.001) (Table 2). There is also a correlation in terms of acrosome length and nucleus length (P<0.05).

When the morphological lengths of age-related spermatozoa in the same sections were evaluated, a correlation was observed between head and flagellum lengths. In addition, the study determined that flagellum lengths had an inverse correlation with age. The study results also note that as age increases, total flagellum lengths shorten.

Fig. 1 shows the lengths of the morphological parts of the Gloster canary semen. These results show that the spermatozoa of the Gloster Canary, a domestic songbird, vary widely in terms of acrosome length, nucleus length, head length, and total flagellum length. When the average statistical data were evaluated, it was seen that the head and flagellum lengths of 3-year-old canary spermatozoa were longer than those of 4-year-old canaries.

These findings are essential to understanding the interaction of genetic and environmental factors in the sperm morphology of Gloster canaries and the intraspecies and interspecies differences in the morphological characteristics of canary semen.

Table 1 Effects of age on spermatozoa morphological parameters in canaries (N=10)

Groups	Acrosome length (µm)	Nucleus length (µm)	Head length (µm)	Flagellum length (µm)
Trial-I	2.020±0.099	2.723±0.084	9.144±0.178	93.066±0.755
Trial-II	1.943±0.111	2.760±0.030	7.973±0.192	86.670±0.744
P	0.613	0.681	<0.001	<0.001

Table 2 Correlations between parts of the spermatozoon (N=20)

Trait*	Acrosome length	Nucleus length	Head length	Flagellum length
Acrosome length	**...**	0.518* (0.019)	0.289 (0.216)	0.028 (0.906)
Nucleus length	0.518* (0.019)	**...**	0.090 (0.707)	-0.219 (0.353)
Head length	0.289 (0.216)	0.090 (0.707)	**...**	0.690** (0.001)
Flagellum length	0.028 (0.906)	-0.219 (0.353)	0.690** (0.001)	**...**

*: The correlation is important.
**: The correlation is very important.

Table 3 Correlation between the same sections of spermatozoa (n=10) according to age

Trait*	Acrosome length (µm) (4 years old)	Nucleus length (µm) (4 years old)	Head length (µm) (4 years old)	Flagellum length (µm) (4 years old)
Acrosome length (µm) (3 years old)	0.249 (0.488)	**...**	**...**	**...**
Nucleus length (µm) (3 years old)	**...**	0.093 (0.799)	**...**	**...**
Head length (µm) (3 years old)	**...**	**...**	0.626 (0.53)	**...**
Flagellum length (µm) (3 years old)	**...**	**...**	**...**	-0.785** (0.007)

*: The correlation is important.
**: The correlation is very important.

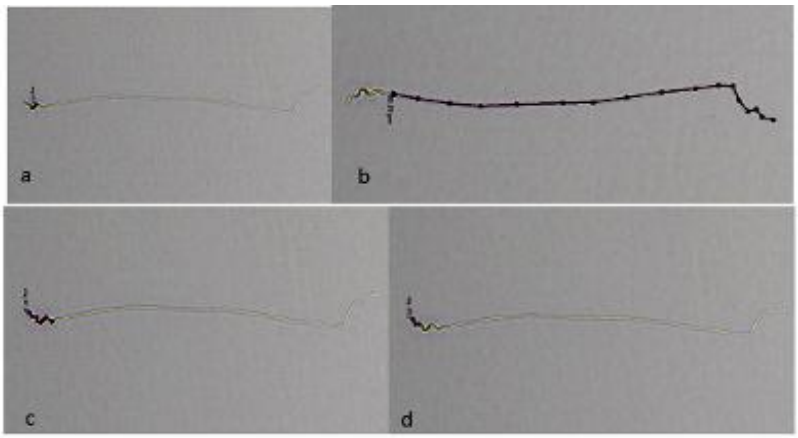

Figure 1 Gloster canary spermatozoon Casa system morphological analysis (**a** nucleus length, **b** flagellum length, **c** head length, **d** acrosome length)

Discussion

The results of this study support the relationship of morphological features of Gloster canary spermatozoa with the ratio of morphological parts of the sperm and reproductive strategies, as in songbirds. In our study, it was observed that the head and flagellum lengths of spermatozoa decreased with age. Additionally, in our study, it was determined that there was a correlation between head and flagellum, in addition to acrosome-nucleus lengths, regardless of age. When age-related correlation data were evaluated, it was found interesting that flagellum length decreased with increasing age. In a study on house sparrows (*Passer domesticus*), the ratio of spermatozoon head length to flagellum length was found to be related to spermatozoa velocity. Fertilization success depends on sperm velocity, which increases the spermatozoon's competitive chances and ability to survive in the female reproductive tract. As the ratio of head length to flagellum length decreases, the swimming speed of the sperm increases. However, this rate is independent of total sperm length. It is also known that short sperm survives longer than long sperm (Helfenstein *et al.*, 2010). In our study, age-related morphological changes in canary spermatozoa were examined. According to the research results, as age increases, total flagellum length and head length decrease. It is thought that this may increase the duration of sperm's stay in the female reproductive tract. Therefore, shortening the head and flagellum may provide an advantage in sperm competition by extending the life of sperm in the female reproductive tract despite some age-related physical disadvantages.

A decrease in total sperm length was observed in house sparrows due to heavy metal toxicity. In addition, it has been reported that spermatozoa with short flagellum length consume less energy (ATP). Additionally, the study concluded that head length reduction negatively affects sperm swimming speed (Yang *et al.*, 2020). The results of this study support our current study. A decrease in sperm head and flagellum length was detected with increasing age. In this case, it can be thought that the competitive ability of spermatozoa, with both increased sperm speed and lifespan, will increase. In our study, no evaluation was made regarding the energy consumption of spermatozoa. The study determined that the amount of energy decreased with the shortening in sperm length. However, we do not have enough information about the reason for this situation, whether it is due to a decrease in sperm size or a special condition related to toxicity.

It is well known that sperm speed is an important factor in increasing fertilization success (Gomendio & Roldan, 2008). When the results obtained in our study were examined, it was revealed that there were significant differences between morphological features such as flagellum length, head length, and acrosome length. These differences are considered an adaptive strategy to increase genetic fertilization success and eliminate unqualified spermatozoa. It has been observed that sperm with long flagella are more durable and structurally stronger (Cramer *et al.*, 2022). However, it has been stated that this does not depend on the total length of the spermatozoa but on the ratio of the components that make up the spermatozoa. In our study, high sperm flagellum length in young birds may increase sperm competition. However, it was concluded that flagellum length alone did not affect swimming speed. We should not forget the effect of other components of sperm. In order to clearly demonstrate the success of fertilization in birds, parameters such as sperm volume, sperm density, and abnormal spermatozoa rate must be considered as a whole, as well as morphological evaluation.

In songbirds, significant differences in the length of spermatozoa and other sperm morphological components of male birds have been reported to affect spermatozoa competition (McCarthy *et al.*, 2021). In this regard, it has been predicted that the diversity between sperm morphological components in masked finches may harm sperm competition. A similar situation in the Gloster Canary highlights the impact of morphological diversity on competition. We think that in male birds, whose sperm competition decreases with age, this deficit is tried to be reduced by some structural differences seen in the spermatozoon.

It is estimated that the sperm of black-throated blue warblers are morphologically longer than that of young birds, and therefore, the fertilization ability of older males is increased (Cramer *et al.*, 2020). However, it is stated that black-throated blue warblers are polygamous birds. Depending on this factor, age may have the expected effect on sperm morphology. However, Helfenstein *et al.* (2010) reported that

reducing the ratio of head to flagella increased swimming speed, but this was not related to the total length of the spermatozoa. Cramer *et al.* (2020) did not provide information on the ratio of head length to flagella of spermatozoa of old male birds. However, only sperm length was considered in the study. Therefore, no inference can be made regarding the determining factor of sperm competition. According to our results, when semen samples were taken from 3 and 4-year-old male canaries, contrary to the study, it was seen that the spermatozoon lengths of older males were shorter. However, a positive correlation was revealed between the flagellum length and the head in older males, independent of age. As the flagellum shortens, the head length also decreases. Therefore, the head/flagellum ratio expressed in the studies may decrease. In addition, when the same parts of the spermatozoon were compared, There was a positive correlation between head lengths and a negative correlation between flagellum lengths in the two experimental groups. Consequently, although head lengths decreased independently of age, flagellum lengths decreased inversely proportional to age.

In conclusion, Gloster canary is a small-bodied domestic songbird, and we think that it can be easily used in semen studies in songbirds because it is easy to find and can be produced in a cage environment. In order to better understand sperm morphology and age-related development in these birds, the lengths of the morphological parts of canary spermatozoa were determined with the help of the computer-aided CASA system. It was examined how the results obtained were affected by age. The results were evaluated and discussed in the light of studies on the subject. It was observed that there was a positive correlation between head length and flagellum length and between nucleus length and acrosome length in canaries, regardless of age. It was also concluded that total flagellum and head length decreased inversely with age. Considering these findings and results, it is predicted that in addition to physical competition, some age-related disadvantages, such as decreased sperm quality in older birds, can be partially compensated by morphological changes in sperm structure over time. Different morphological characteristics affect spermatological competition and reproductive success. Therefore, this study can support future studies on the subject and contribute to a better understanding of the evolutionary dynamics of reproduction in songbirds.

Conflict of interest: The authors do not have any conflict of interest.

Author Contributions: AO, BE, and CK were involved in the systematic search to identify all relevant studies, assessed the eligibility of each selected study, and helped draft the manuscript; AO, BE, and CK visualized the experiments; AO and BE analyzed data and wrote the initial draft; AO participated in performing statistical analysis; All authors read and approved the final manuscript.

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