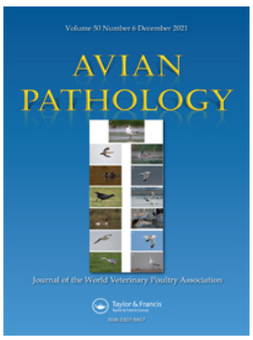

Avian Pathology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/cavp20

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To cite this article: Barbara Végi, Enikő Bíró, Dénes Grózner, Árpád Drobnyák, Zsuzsa Kreizinger, Miklós Gyuranecz & Judit Barna (2021) *Mycoplasma* species in the male reproductive organs and the fresh and frozen semen of the Hungarian native goose, Avian Pathology, 50:6, 458-464, DOI: 10.1080/03079457.2021.1978391

To link to this article: https://doi.org/10.1080/03079457.2021.1978391

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AVIAN PATHOLOGY
2021, VOL. 50, NO. 6, 458–464
https://doi.org/10.1080/03079457.2021.1978391

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ORIGINAL ARTICLE

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Mycoplasma species in the male reproductive organs and the fresh and frozen semen of the Hungarian native goose

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ABSTRACT

The objective of this study was to clarify whether the most common species of *Mycoplasma* can be detected in the reproductive organs and the cloaca, as well as in the semen of asymptomatic native Hungarian male geese. As it is necessary for the semen of that breed to be preserved pathogen-free in an *in vitro* gene-conservation programme, the presence of and sources of infection, as well as prevention of the survival of pathogens following semen cryopreservation, are key issues. Ten asymptomatic, 2-year-old ganders were tested. For the detection of mycoplasmas, samples were taken from both fresh and frozen/thawed semen, cloaca, phallus lymph, testes and vas deferens; that is five samples from each of the 10 ganders. The semen was statically frozen using dimethyl-formamide as a cryoprotectant and stored in liquid nitrogen at −196°C. Species-specific PCR systems targeting *M. anserisalpingitidis*, *M. anseris* and *M. cloacale* were used for screening and identification. Results of this study have shown, for the first time, that (1) among the three *Mycoplasma* species examined, all were detectable in the indigenous Hungarian ganders, with no clinical signs; (2) the pathogens could be detected in the cloaca, in both fresh and cryopreserved semen samples, but remained undetected within the inner reproductive organs; and (3) as pathogens were able to survive the freezing/storing/thawing procedures, the possibility of vertical transmission of the pathogens during artificial inseminations does exist, which causes problems in the *in vitro* gene-conservation programmes for this breed.

ARTICLE HISTORY
Received 14 April 2021
Accepted 3 September 2021

KEYWORDS
Mycoplasma; gander reproductive organs; semen; cryopreservation; gene conservation; geese

Introduction

The presence of various species of *Mycoplasma* in most goose breeds is a common problem, not only in Hungary, but all over the world (Stipkovits, 2012). Kosovac and Djurisic (1970) first demonstrated the effects of *Mycoplasma* infections in geese. Since then, a number of *Mycoplasma* species have been identified in multiple organs of most waterfowl breeds, such as *M. anserisalpingitidis* (earlier: *Mycoplasma* sp. 1220; Stipkovits, Varga *et al.*, 1984; Stipkovits, 2012; Volokhov *et al.*, 2020), *M. anseris* (Stipkovits, Varga *et al.*, 1984; Bradbury *et al.*, 1988; Stipkovits, 2012), *M. cloacale* (Stipkovits, Varga *et al.*, 1984; Varga *et al.*, 1989; Stipkovits, 2012). Diseases caused by any of the *Mycoplasma* species can generate significant economic losses to the stocks concerned (Szép *et al.*, 1974, Dobos-Kovács *et al.*, 2009; Stipkovits *et al.*, 2012). It is widely recognized that pathogens are able to spread not only in a horizontal manner, but vertically as well, and thus appear in both the male and female reproductive organs in a multitude of poultry species (Fabricant & Levine, 1963; Yoder & Hofstad, 1964; Stipkovits, Bove *et al.*, 1984; 1986, Dobos-Kovács, 2009). It has been demonstrated that mycoplasma-free female turkeys are susceptible to infection with mycoplasmas through artificial insemination with contaminated semen (Kleven *et al.*, 1971). Benčina *et al.* (1988) were the first to demonstrate, also in turkeys, the natural transmission of *M. gallisepticum* and *M. synoviae* to their embryos. However, numerous questions remain unanswered regarding the pathogenesis and the transmission of mycoplasmosis (Berčič *et al.*, 2008; Carnaccini, 2016), and only a handful of studies have addressed the presence of *Mycoplasma* species in semen (Buntz *et al.*, 1986; Grózner *et al.*, 2019). Currently, no data exist on exactly how mycoplasmas find their way into the semen. It is presumed that mycoplasmas may similarly be detected in birds without them showing specific clinical signs (Stipkovits *et al.*, 1986; Hinz *et al.*, 1994).

The long-term preservation of gander semen has proven to be an important tool both for programmes relating to genetic-diversity management and for the

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conservation of endangered breeds. With the intensive spread of the goose-breeding industry, the number of genotypes has decreased significantly, greatly emphisizing the importance of gene-bank protection for the traditional and local goose breeds. In the case of avian species, neither oocytes nor embryos can be, in essence, frozen due to their high content of yolk, which is non-freezable. For now, the most effective and widely-used method of *in vitro* preservation is the deep freezing of semen (Gee, 1995; Hammerstedt, 1995; Reedy *et al.*, 1995).

In order to create and maintain a reliable sperm bank, it is crucial to know the complete and accurate health status of the donor birds. It is necessary to be certain that the conserved germplasm does not transmit pathogens along with its genetic information; that is, donor birds must be free from infectious diseases which may be transmitted via semen (FAO, 2012). This is likewise an essential prerequisite for the quality assurance of a cryobank (Blesbois, 2007). Consequently, new methods are required for the elimination of the pathogens from the semen in situations when the donor birds carrying rare and valuable genes are all infected. For mammalian species, sanitary considerations have been well established and are widely implemented for the long-term preservation of spermatozoa, oocytes and/or embryos, while similar requirements for poultry are unfortunately still lacking or incomplete (FAO, 2012).

With this in mind, we made the decision to focus solely on *Mycoplasma*, one of the pathogens which may appear in the semen and, in a vertical manner, may infect the females via artificial insemination.

The primary aims of our preliminary study were to clarify whether native Hungarian geese, the samples from which are to be *in vitro* preserved long term in liquid nitrogen, are infected with mycoplasmas, and if so, in the case of ganders, which reproductive organs are affected. In addition, the effect of the semen cryopreservation protocol on the survival of the pathogens was also examined.

Materials and methods

Birds

Ten asymptomatic, 2-year-old, native Hungarian ganders having frizzled-like feathers *(Anser anser domestica)* were used as sperm donors. The ganders originated from the protected flock of the National Centre for Biodiversity and Gene Conservation, Hungary. The birds were placed individually into pens on a base of deep litter. They were kept under a 9 L:15 D (light-to-dark) photoperiod and fed with commercial goose feed (300 g/gander/day) with access to water provided *ad libitum*. To avoid any contamination of the sperm with faeces, the feed was removed from the pens in the afternoon before the day of sperm collection.

Chemicals

Unless otherwise stated, chemicals used in this study were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary) and Reanal Laboratory Chemicals LLC (Budapest, Hungary).

Semen collection and freezing

The ganders were accustomed and trained to semen collection beginning 2 weeks prior to sperm-qualification testing. Semen was collected from individual specimens through a modified version of abdominal massage technique (Burrows & Quinn, 1937) into a special pre-heated glass container. The semen samples were diluted in a 1:1 ratio in Eppendorf tubes with LK-1 diluent (Lukaszewicz, 2001). The samples were equilibrated at 5°C for 20 min and then 10% of dimethylformamide (DMF) cryoprotectant was added. The prepared samples were then drawn into 500 µl straws (IMV Technologies Inc., L'Aigle, France) and statically frozen at 1 cm above liquid nitrogen (LN) for 15 min. They were stored at −196°C in LN for 6 months prior to thawing. Thawing was performed in a water bath at 40°C for 20 s.

Semen qualification

For semen qualification, traditional single-function tests were used. Samples were evaluated prior to freezing and after thawing. Sperm motility was examined and recorded via subjective scoring on a scale of 0–5 (1 = 5–20%, 2 = 20–40%, 3 = 50–70%, 4 = 75–85%, 5 = 85–95% motile spermatozoa), and sperm concentration by spectrophotometer (Accucell, IMV, L'Aigle, France). Morphological abnormalities and live/dead sperm ratio were determined using aniline blue-eosin staining (Certistain, 115935 Eosin Y, Merck Ltd., Budapest, Hungary). Slides were evaluated microscopically (Zeiss, Axioscope; Carl Zeiss Microscopy GmbH, Germany) using an oil immersion objective at ×1000 magnification. A total of 200 spermatozoa were counted per slide (Váradi *et al.* 2019).

Mycoplasma detection and culture

For the purpose of detecting the various species of *Mycoplasma,* swab samples were taken from the cloaca, while phallus lymph samples were taken with a syringe and injection needle. In addition, 100 µl of the diluted fresh and 100 µl frozen/thawed semen samples of the same birds were also taken and measured in Eppendorf tubes. Samples were also taken from the testes and vas deferens following

euthanasia. Euthanasia was performed by intravenous administration of phenobarbital 100 mg/kg of bodyweight. Semen samples were investigated by PCR and culture whilst all other clinical materials were examined by PCR only.

The QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany) was used for DNA extraction directly from all the clinical materials according to the manufacturer's instructions. Species-specific PCR systems targeting *M. anserisalpingitidis, M. anseris* and *M. cloacale* were used for screening and identification (Grózner *et al.*, 2019).

Mycoplasma culture was performed from the fresh and cryopreserved semen samples. The samples were washed in 2 ml of *Mycoplasma* broth medium (pH 7.8) (ThermoFisher Scientific Inc./Oxoid Inc., Waltham, MA) supplemented with 0.5% (w/v) sodium pyruvate, 0.5% (w/v) glucose, 0.15% (w/v) L-arginine hydrochloride and 0.005% (w/v) phenol red, filtered through a 0.65 µm pore-sized syringe filter (Sartorius GmbH, Goettingen, Germany), then transported to the laboratory immediately. Broth cultures were incubated at 37°C until colour change (1–3 days) then inoculated onto solid *Mycoplasma* media (Thermo Fisher Scientific Inc./Oxoid Inc.), supplemented with 0.15% (w/v) L-arginine hydrochloride and incubated at 37°C and 5% CO_2 until visible colonies appeared (1–3 days). The culture of mycoplasmas was ascertained by the colour change of the broth and the morphology of the colonies on the solid media. Further isolation and identification of the mycoplasma colonies to the species level was not performed.

Ethics statement

All geese were kept and exterminated in conformity with the provisions for animal welfare as specified in the Hungarian Animal Protection Law (Act no. XXVIII of 1998). Permission for any and all experimental animal research by the research institution was obtained from the National Food Chain Safety Office, Animal Health and Animal Welfare Directorate, Budapest, Hungary (permission number: 13/2015). All experimental methods applied were approved by the Institutional Ethical Review Board (No. 2/2016).

Table 1. Mean data ± SD from the qualification of fresh and frozen semen of 10 ganders.

		Fresh semen	Frozen semen
Concentration million/ml		640.9 ± 191.5	
Motility scores (0–5)		3.5 ± 1.5	1.5 ± 1.5*
Live spermatozoa %	**intact membrane with normal morphology**	71.3 ± 13.3	57.9 ± 23.9*
	intact membrane with abnormal morphology	15.8 ± 7.6	10.9 ± 12.1
Dead spermatozoa with damaged membrane %		12.9 ± 8.1	31.1 ± 27.6*

*statistically significant differences ($P \leq 0.05$).

Statistical analysis

Percentage data were arcsine-transformed and analysed (Reiczigel, Harnos, & Solymosi, 2006). Statistical analysis was performed using Statistica software version 12.0 (StatSoft Ltd., Budapest, Hungary). Semen-parameter results were analysed via one-way ANOVA using the Tukey *posthoc* test.

Results

Qualification of the fresh semen of the 10 ganders produced results showing typical data for the goose species in general. Mean concentrations of spermatozoa were found to be at 640.9, ranging between 494.8 and 966.1 million/ml, while average motility was around 3.5 on a scale of 0–5. The mean proportion of live, intact spermatozoa was 71.3%, while the proportions of live abnormal cells and dead cells were 15.8% and 12.9%, respectively. In cryopreserved samples, sperm parameters decreased as follows: average motility data were found to be around 1.5, while the proportion of live, intact cells was 57.9%. The ratios of live abnormal cells did not change significantly (10.9%), while the proportions of dead cells increased to 31.1%. The data showed high individual variances, similar to other avian species, both in terms of fresh semen quality and cryopreservability (Table 1).

According to the species-specific PCR assays used in this study, none of the three *Mycoplasma* species examined was found in the testes or in the vas deferens. The phallus lymph of one bird in particular showed positive PCR results for *M. anserisalpingitidis* and *M. anseris*, and that of another bird was positive for *M. anseris* and *M. cloacale*. Concerning the examination of the cloaca swab samples, 10/10 were positive for *M. anserisalpingitidis* and *M. anseris*, and 9/10 samples for *M. cloacale*. All three *Mycoplasma* species were detected within the fresh semen samples of the 10 birds, with the exception of one case where *M. anseris* was not detected (note that, in this case, the cloacal sample was positive for *M. anseris* in the PCR test). Upon freezing and thawing, all *Mycoplasma* species were detected in the majority of samples based on the species-specific PCR results. Samples 4, 9 and 10 were evaluated as positive with the *M. cloacale*-specific PCR in the fresh semen samples; however, their positivity was observably weak and their results became negative after the freezing–thawing process (Table 2; Figure 1).

Table 2. The detected *Mycoplasma* species in different organs, regions, and in fresh and frozen semen.

	Testis			Ductus deferens			Phallus lymph			Cloaca			Fresh semen			Frozen semen		
Sample	Mp AS	Mp A	Mp C	Mp AS	Mp A	Mp C	Mp AS	Mp A	Mp C	Mp AS	Mp A	Mp C	Mp AS	Mp A	Mp C	Mp AS	Mp A	Mp C
1	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
2	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
3	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
4	–	–	–	–	–	–	+	+	–	+	+	–	+	+	+	+	+	–
5	–	–	–	–	–	–	–	–	–	+	+	+	+	–	+	+	–	+
6	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
7	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
8	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
9	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–
10	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	–

Note: Mp AS = *Mycoplasma anserisalpingitidis*; Mp A = *Mycoplasma anseris*; Mp C = *Mycoplasma cloacae*.

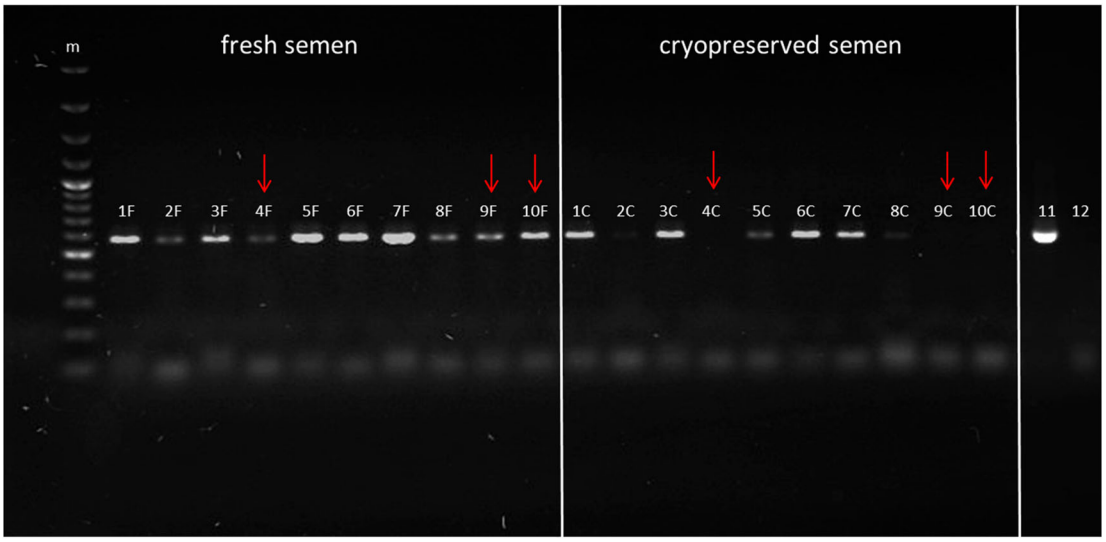

Figure 1. Gel electrophoretic analyses of *Mycoplasma cloacale* in fresh and cryopreserved gander semen. Notes: Lane m: molecular weight marker (GeneRuler 100 bp Plus DNA Ladder, Thermo Fisher Scientific, Waltham, USA); Lanes 1F-10F: PCR products of *M. cloacale*–specific assay in fresh semen from 10 ganders; Lanes 1C-10C: PCR products of *M. cloacale*–specific assay in cryopreserved semen from the same individuals (the same numbers indicate the same ganders); Lane 11: positive control; Lane 12: negative control. The vertical red arrows show the differences between the fresh and cryopreserved semen samples.

Mycoplasma culture was successful from all 10 fresh and all 10 post-thaw semen samples as the broths changed colour and the colonies had "fried-egg" morphology, typical of the waterfowl *Mycoplasma* species (Stipkovits, Varga *et al*, 1984; Kleven, 2008).

Discussion

We have confirmed in the study that the native Hungarian goose breed is susceptible to colonization by *M. anserisalpingitidis*, *M. anseris* and *M. cloacale* mycoplasmas, all of which could be detected in asymptomatic ganders. Furthermore, it was proven that the pathogens in the semen were derived from the cloaca, while the inner reproductive organs did not contain mycoplasmas. The positive PCR results of the DNA samples extracted directly from the fresh and frozen-thawed semen samples and the successful culture of mycoplasmas before and after freezing clearly indicate that the mycoplasmas managed to survive the cryopreservation and storage in liquid nitrogen. This study is the first of its kind to demonstrate that mycoplasmas are capable of surviving the cryopreservation protocol of gander semen, as well as its long-term storage at −196°C in liquid nitrogen.

The mere presence of the tested pathogens in an indigenous goose breed is not particularly surprising in itself, since nearly all commercial goose breeds in most parts of the world are similarly contaminated with various types of mycoplasmas (Stipkovits & Kempf, 1996; Stipkovits & Szatmáry, 2012; Carnaccini, 2016; Gyuranecz, 2020, Kovács, 2020). The main factors contributing to the widespread distribution of mycoplasmas can be primarily attributed to the prevalence of multi-age goose farms, where vertical transmission of the pathogens is inevitable, and the basic underlying difficulties involved in implementing programmes towards establishing pathogen-free waterfowl farms.

An important consideration in the field of gene-conservation is that a given pathogen may also be present in asymptomatic birds. Without a doubt,

excessive stress and/or inappropriate hygienic conditions can exacerbate mycoplasma infections and may also lead to clinical and pathological signs of a variable nature. *M. anserisalpingitidis* is capable of causing cloaca and phallus inflammation, salpingitis, airsacculitis, peritonitis and embryo mortality (Stipkovits *et al.*, 1986; Stipkovits & Kempf, 1996; Dobos-Kovács *et al.* 2009). *M. anseris* has been likewise associated with cloaca and phallus inflammation of ganders (Stipkovits & Szatmáry, 2012), in addition to airsacculitis, peritonitis and embryo mortality (Stipkovits & Kempf, 1996). *M. cloacale* has been noted to cause egg infertility in geese (Stipkovits *et al.*, 1986).

In this study, *M. anserisalpingitidis*, *M. anseris* and *M. cloacale* were detected in all cloaca swabs and in the semen of each bird with slight variations based on PCR results, while no *Mycoplasma* species were observed in the testes nor in the ductus deferens. The pathogens detected were presumably acquired from the cloaca by direct contact, when the semen was running down the phallus during sperm collection. From a technical standpoint, it is unfortunate that contact between the semen and the inner/outer part of the cloaca and/or the surface of the phallus is inevitable, as this makes it impossible to avoid contamination completely. Interestingly, in this study, the phallus lymph was also shown to contain mycoplasmas, although only in two individuals. The lymph-like fluid is actually a vascular transudate, produced in the capsular network within the vascular bodies of the wall of the cloaca, near to the base of the phallus (Bakst, 1992). Based on the anatomical and physiological processes mentioned above, we hypothesize that these pathogens may have been derived either from the blood of the infected birds or from the surface of the contaminated phallus due to spoiled sampling in the case of these two specific ganders. The fact that the *Mycoplasma* species examined were undetected in the testes and ductus deferens leads us to suppose that the pathogens were not present in the bloodstream, and consequently, contamination of the phallus lymph from the surface of the phallus is more probable in these cases.

It is common knowledge that for bacteria which lack cell walls, such as those of the genus *Mycoplasma,* the major source of carbon and energy is glycerol, derived from phospholipids of their human or bird hosts (Blötz & Stülke, 2017). Due to the fact that sperm membranes contain high levels of phospholipids, it may be assumed that semen provides an extremely habitable medium for *Mycoplasma* species.

In regard to the quality of the fresh and cryopreserved semen examined, the parameters observed did not differ significantly from the results of earlier studies on other goose breeds (Marvan *et al.*, 1981, Scheller, 1989, Ferdinand, 1992; Łukaszewicz, 2002; Barna *et al.*, 2010). Thus, the presence of pathogens did not impair the average quality of the fresh or cryopreserved semen derived from asymptomatic individuals. However, a latent infection may be easily passed on to offspring, which is significant, as this is equivalent to sitting on a biological timebomb which could explode at any time.

The cryopreservation may have decreased the number of viable mycoplasmas as the results from certain samples having an apparently low quantity of *M. cloacale* DNA (weak positive PCR results in the fresh semen) were negative after freezing/thawing. This could be verified with a quantitative real-time PCR method which is unfortunately not available for waterfowl *Mycoplasma* species. Although mycoplasma colonies cultured from frozen/thawed samples were not identified to the species level in this study, our investigation proved that at least one (if not all) *Mycoplasma* species survived the cryopreservation procedure of the gander semen. Similar observations were observed with regard to *Salmonella* in a study by Iaffaldano *et al.* (2010), who demonstrated in turkeys that the semen cryopreservation process was capable of reducing, but not completely eliminating, the bacteria. An earlier study demonstrated that *Mycoplasma hominis* in human sperm, together with other microorganisms, also survived the freezing process (Mazzilli *et al.*, 2006).

In conclusion, it was demonstrated for the first time that *M. anserisalpingitidis, M anseris* and *M. cloacale* are capable of colonizing indigenous Hungarian geese without producing any clinical signs, although according to literature data the mentioned *Mycoplasma* species can generate clinical signs depending on the environmental circumstances (Stipkovits *et al.*, 1986; Stipkovits & Kempf, 1996; Dobos-Kovács *et al.*, 2009; Stipkovits & Szatmáry, 2012). These bacteria could not be found in the inner reproductive organs, but were detected in the cloaca in the case of both fresh and cryopreserved semen. Possibly, the pathogens found in the semen could have been acquired by the digestive route from the cloaca. These cryo-resistant mycoplasmas could viably be a source of vertical transmission of the pathogens during subsequent artificial inseminations.

In the future, in accordance with the standards for *in vitro* gene-conservation, development of new and innovative procedures will be required for the purpose of eliminating pathogens from fresh or frozen samples without damaging the spermatozoa.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the Szechenyi 2020 Operational Strategical R&D Excellence Program [VEKOP-2.3.2-16-2016-00012], the Lendület program of the

Hungarian Academy of Sciences [LP2012-22], and the K_16 (119594), FK_17 (124019) and KKP19 (129751) grants of the National Research, Development and Innovation Office, Hungary. M. Gyuranecz and Z. Kreizinger were supported by the Bolyai János Fellowship of the Hungarian Academy of Sciences, while M. Gyuranecz and D. Grozner were supported by the Bolyai+ Fellowship [ÚNKP-19-4-ÁTE-1] and the [ÚNKP-19-3-1-ÁTE-3] of the New National Excellence Program of the Ministry of Innovation and Technology. The funders had no role in the design of this study, its data collection and interpretation, nor the decision to submit the work for publication.

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