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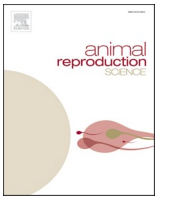

Collection of epididymal semen in the tomcat (*Felix catus*) by stereomicroscope-aided retrograde flushing (SARF) improves sample quality

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ABSTRACT

In small and laboratory animals epididymal spermatozoa can be collected only by the mincing technique however, blood and cell debris contamination may adversely affect the quality of the samples. This study aimed at performing the retrograde flushing technique, for the first time, in the tomcat. In the first experiment attempts to insert a blunted needle into tomcats' *ductus deferentes* were made and the cannulation time and the success rate were recorded. In the second experiment, insertion of the needle into the *ductus* was carried out under a stereomicroscope for one testis, while the other was submitted to mincing for sperm recovery and the samples obtained from paired testes were compared. In the first study, only twenty-eight percent of the cannulation attempts were successful whereas in the second experiment, 91% of the *deferentes* were successfully cannulated: 80% of the *ducts* were cannulated in less than three minutes. The stereomicroscope-aided retrograde flushing samples showed significantly higher sperm viability, motility and velocities, as compared to those resulted from mincing. The domestic cat serves as a model for more than 36 species of wild felids; the developed stereomicroscope-aided retrograde flushing technique has the potential to become the gold standard method for harvesting spermatozoa of endangered species that have a small *ductus deferens*.

1. Introduction

Good quality ejaculates in the tomcat (*Felix catus*) can be collected in vivo by urethral catheterization, by electroejaculation, or by copulation with an artificial vagina (Zambelli and Cunto, 2006; Pisu et al., 2017). Since testes are available in large number after routinary castration procedures, the use of epididymal spermatozoa (ES) could be advantageous when needed for research purposes on assisted reproductive technologies (ARTs) (sperm freezing, artificial insemination, in vitro embryo production). In addition, the collection of ES is of particular importance in case of sudden death of valuable individuals and, more often, for gametes' conservation of endangered species (Filliers et al., 2008).

The techniques used to recover epididymal spermatozoa *post-mortem* are: i) squeezing; ii) mincing; and iii) retrograde flushing. To perform the squeezing technique, the epididymis is clamped with forceps at the transition between the *cauda epididymis* and the *ductus*

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deferens, the latter is then "squeezed" from the *cauda* towards its free-end and the ES are collected in a Petri dish containing an extender (Martins et al., 2012). This technique is the least used, however, due to the small amounts of sperm that could be retrieved.

The mincing and the retrograde flushing methods require the isolation of *epididymis* and *vas deferens* from surrounding structures (Luvoni and Morselli, 2017). Mincing is performed by making a series of cuts to the *ductus deferens* and to the *cauda epididymis*, after placing them in a Petri dish containing a semen extender (Hewitt et al., 2001); this is the only technique that is currently used for ES collection in the tomcat, but the obtained samples become contaminated with blood and cell debris, which may adversely affect the sperm quality and freezability (Rijsselaere et al., 2004).

Retrograde flushing is performed by inserting a cannula or a blunted needle (connected to a syringe containing an extender) into the *ductus deferens* and, after cutting at the level of the *cauda epididymis*, by pushing the extender opposite to the physiological sperm direction (i.e. retrograde) (Ponglowhapan et al., 2006). With this technique, the collected solution is rarely contaminated (Hori et al., 2015; Mogheiseh et al., 2022).

Several studies report the effective use of retrograde flushing in different animals: stallion (Bruemmer, 2006), jackass (Podico and Canisso, 2022), bull (Turri et al., 2012), deer (Martinez-Pastor et al., 2006), buck (Turri et al., 2016, 2014), dromedary camel (Desantis et al., 2021; Monaco et al., 2020), and dog (Hori et al., 2015). In small dogs, due to the reduced diameter of the *ductus deferens*, the retrograde flushing could be tricky, laborious, and with little chance of success whereas mincing allows the retrieval of ES in animals of any size (Hori et al., 2015).

After comparing retrograde flushing and mincing in the dog, Hori et al. (2015) suggested the use of the former as the gold standard technique and resorting to mincing only if the retrograde flushing attempts fails. On the other hand, in the tomcat (*Felix catus*), attempts to collect ES by retrograde flushing, have never been reported.

The domestic cat serves as a model for more than 36 species of wild felids five of which are classified as endangered (Pope, 2000; Hassan et al., 2021). A new technique for performing retrograde flushing in the tomcat could reduce the detrimental factors and improve the application of ARTs but could be also useful for ES recovery in endangered animals whose small size of the *ductus deferens* makes the retrograde flushing application difficult. The first aim of the present study was, therefore, to perform the retrograde flushing in the tomcat and to evaluate the outcomes of its application. Following the development of a stereomicroscope-aided retrograde flushing (SARF) technique, the second aim of the study was to compare the parameters of tomcat ES obtained by SARF and by mincing, after collection, and following their storage at 4 °C for 24 h.

2. Materials and methods

The study was carried out at the Department of Veterinary Medicine (DiMeV) of the University of Bari Aldo Moro from September 2021 to September 2022. In compliance with welfare guidelines and with the regulations for the use of animals for research purposes, the experimental protocol was previously evaluated, and approved, by the ethics committee of the DiMeV (approval number 6/2019).

Testes of 28 tomcats that underwent bilateral orchidectomy were used. Neuterings were performed for birth control in feline colonies or upon owners' requests, to prevent undesirable marking and spraying behavior. The day before the surgery a clinical examination was performed to evaluate the animal's suitability for the anesthesia and surgery procedures (ASA Physical Status Classification System).

2.1. Anesthesia and surgical approach

Dexmedetomidine hydrochloride (Dexdomitor Orion Pharma Milan MI Italy; 1 μg/kg), Methadone hydrochloride, (Semfortan, Dechra, Turin, Italy; 0.15 mg/kg), and Tiletamine and Zolazepam (Zoletil, Virbac S.r.l., Milan, Italy; 0.35/0.35 mg/kg) were used as sedative, analgesic and anesthetic, respectively. After anesthesia induction, the cephalic vein was catheterized, and ringer lactate fluid therapy was provided (3 mL/kg/h) until the awakening of the animal.

The tomcats underwent bilateral orchidectomy *more solito*; nevertheless, particular care was taken to avoid damage to the *caudae epididymides* with the scalpel, during the incisions of the *tunicae*. In addition, ligation of the spermatic cord was performed more proximally to facilitate, later, the *ductus deferens* cannulation.

Each *testis* (with attached spermatic chord) was placed in a 60 mm Petri dish among saline-moistened gauzes, to prevent dehydration; the dish was covered with its lid and kept at room temperature until the contralateral testis was removed. Both *testes* were then transported to the laboratory and processed as soon as possible.

2.2. Experimental design

Two separate experiments were made; in the first experiment, each testis was considered as an experimental unit, and attempts to insert a blunted needle into the *ductus deferens* were made. The "cannulation time" was recorded, starting from the first insertion attempt and ending at confirmation of the correct insertion. A maximal time of 20 min was set and attempts were discontinued thereafter. Mincing was performed if: i) the maximal time was exceeded; ii) *ductus deferens* became too short (see below) or irreparably damaged. The success rate and the average time for *ductus deferens* cannulation were evaluated, in addition to the parameters of the samples collected by retrograde flushing and mincing.

In the second experiment, the insertion of the needle was carried out following the same technique but the *ductus deferentes* were placed under a stereomicroscope and the success rate and cannulation timing were again evaluated. Testes were considered as paired experimental units: if retrograde flushing was performed on one *cauda epididymis*, the contralateral was processed by mincing and the

parameters of the the tqwo techniques were compared soon after collection, and after storage at 4 °C for 24 h.

2.3. Experiment 1

2.3.1. Preparation of the samples

After dissection of the vascular cone, each testis was weighted with its attached *epididymis* and *ductus deferens*; the *epididymis* and *ductus deferens* were then removed, by careful dissection, and weighted (Fig. 1a). The free end of the *ductus deferens* was held with Spencer well artery forceps, and the surrounding tissues (*mesorchium,* deferential artery, and *tunica adventitia*) were torn off in the direction of the *cauda epididymis*, using a Halsted mosquito; the procedure was repeated until *ductus deferens* resulted clean (Fig. 1b-1c). The free end of the *ductus deferens* was then cut, perpendicularly to its major axis, with a scalpel blade n°21, to remove the part (3–5 mm), irreparably crushed during the previous operation (Fig. 1d).

2.3.2. Time evaluation, insertion of the needle, retrograde flushing

After preparation, the free end of the *ductus deferens* was kept suspended from its outer margin by means of a 12.5 cm Halstead mosquito leaning against the edge of the Petri dish (Fig. 2a-2b). A 30 G, rounded tip, side port needle (KerrHawe, Switzerland) was used for cannulation. The needle was held with the right hand while the left hand helped positioning of the *ductus deferens* end, through an atraumatic dissecting forceps (Fig. 2c).

The cannulation time recording started at first needle insertion attempt and proceeded until i) confirmation of the correct insertion; ii) when maximal time (20 min) was exceeded; iii) *ductus deferens* became too short (see below) or irreparably damaged. In the latest two circumstances, attempts were discontinued, and the samples processed by mincing.

In the event that the free end of *ductus deferens* was damaged during the attempts (i.e. incorrect insertion), another cut was made before a new attempt, and more cuts were made, if needed, until reaching the *ductus deferens* part close to the *cauda epididymis* where the smaller diameter and the tortuous shape made further attempts impossible.

Once the needle was inserted, it was secured in situ using a straight Klemmer forceps (Fig. 2d) then, a 5 mL syringe with a plastic plunger containing a tris-citrate-fructose diluent (TCF) (3.028 g Tris-hydroxymethyl-aminomethane, 1.78 g citric acid monohydrate; 1.25 g D-fructose; 100 mL distilled water (modified from Villaverde et al., 2013), kept at room temperature, was connected. The correct insertion of the needle was verified by applying light pressure on the syringe plunger and observing the engorgement of the *ductus deferens* and *cauda epididymis,* in absence of TCF leakage.

The *cauda epididymis* was then separated from the *corpus* with a scalpel, the residual blood at the cut surface was removed with a saline solution-soaked gauze, and retrograde flushing was performed by applying a continuous but delicate pressure on the syringe plunger while keeping the *cauda epididymis* on the edge of an Eppendorf tube. After flushing about 0.5–1 mL, the change of the recovered TCF, from opaque (containing sperm) to transparent, indicated the absence of spermatozoa and thus the end of the procedure.

2.3.3. Mincing

The *caudae epididymides* and *ductus deferentes* were placed in a 30 mm Petri dish with 1–1.5 mL of TCF solution and subjected to several cuts until minced; the dish was incubated at 38 °C for 30 min and then the TCF-ES solution was transferred into an Eppendorf

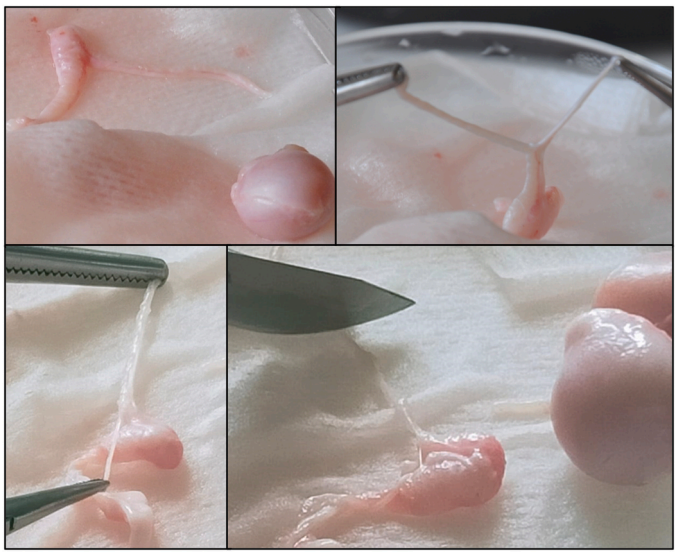

Fig. 1. Tomcat isolated epididymis (1a), removal of the *deferens duct's* surrounding tissues (1b−1c), scalpel cut for removal of the crushed free end (1d).

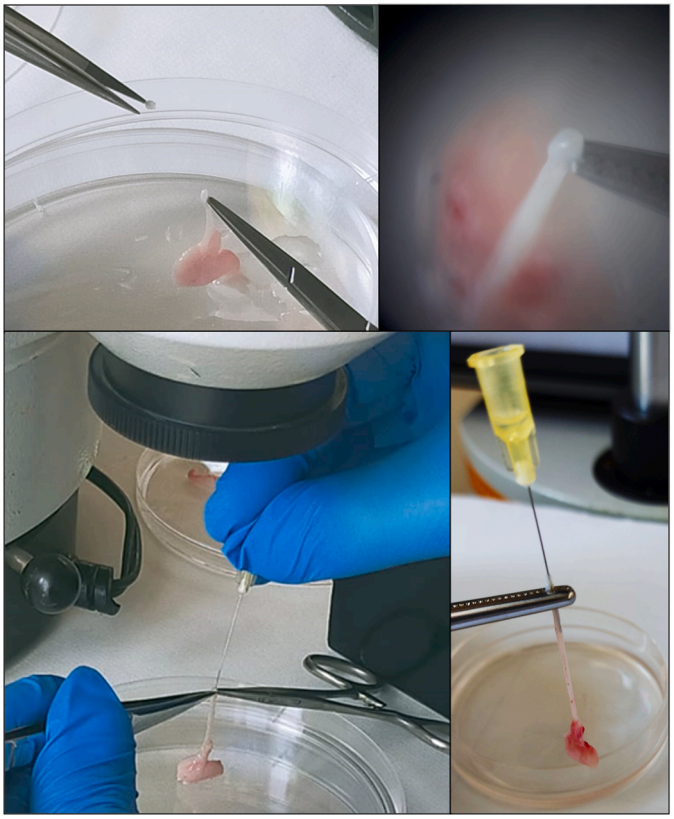

Fig. 2. *Deferens duct* hold by its outer margin and 120 X magnification detail (2a−2b), stereomicroscope aided cannulation technique (2c), needle kept in situ after successful cannulation (2d).

tube for the evaluation of the semen parameters.

2.3.4. Evaluation of epididymal semen parameters

The volume of the obtained samples was assessed using a graduated Eppendorf tube. A sample aliquot was collected, kept in an incubator at 38 °C, and used for the evaluation of the following seminal parameters: total number of collected ES, viability, morphology, motility, and sperm kinematic parameters.

The sperm concentration was calculated using an Improved Neubauer counting chamber. The membrane permeability (viability) and the morphology of the spermatozoa were assessed, by the same experienced operator, after staining (eosin/nigrosin solution), smearing, and evaluation under a light microscope (Nikon Eclipse Ci-E), at 200 and 1000X magnification, respectively. The viability and normal spermatozoa (Normal sperm and those with head, midpiece, and tail abnormalities as well as sperm with proximal and distal cytoplasmic droplets) were evaluated counting at least 200 and 100 spermatozoa respectively (Monaco et al., 2020).

The sperm motility was assessed through a computer-assisted analysis system (CASA) (IVOS 12, Hamilton Thorne, USA); when needed, the sperm were diluted to a concentration between 25 and 40 $\times$ 10^6 spz/mL. The following parameters were evaluated: average trajectory velocity (μm/s) (VAP); straight-line velocity (μm/s) (VSL); curvilinear velocity in (μm/s) (VCL); straightness (STR: VSL/VAP); amplitude of lateral head displacement in μm (ALH); linearity (LIN: VSL/VCL); crossing frequency in Hz (BCF). In addition, the percentages of: motile spermatozoa (VAP>10 μm/s); progressively motile spermatozoa (VAP>65 μm/s and STR>75%), rapid spermatozoa (>65 μm/s); medium spermatozoa (>30 μm/s, <65 μm/s); slow spermatozoa (>10 μm/s, <30 μm/s) and static spermatozoa (VAP<10 μm/s), were evaluated (Villaverde et al., 2009).

2.4. Experiment 2

2.4.1. Preparation of samples

The samples were prepared as described in 1st experiment but, in addition to the weight of the *testis* and *epididymis*, also the weight of *caudae epididymides,* with their *ductus deferentes*, were measured; before mincing or after retrograde flushing, respectively.

2.4.2. Time evaluation, insertion of the needle, retrograde flushing

In the 2nd experiment, the needle insertion and the retrograde flushing were performed following the same procedure as in

experiment 1 but the Petri dish was placed under a stereomicroscope (Wild Heerbrugg, Germany) and the insertion of the needle was carried out at 120 X magnification. The success rate and the time for the insertion were evaluated as in the first experiment.

2.4.3. Evaluation of epididymal semen parameters

Semen parameters were evaluated as in experiment 1, except for the volume of the ES-TCF which was assessed by weighing the Eppendorf, before and after the ES collection, and considering 1 g as equal to 1 mL.

The samples collected by the two techniques were processed under the same described conditions and the seminal parameters (total sperm concentration, membrane permeability, morphology, motility, and sperm kinematics) were evaluated using the same methods of the first experiment.

2.4.4. Epididymal sperm storage

Epididymal spermatozoa were stored at 4 °C after the addition of TCF with 10% clarified egg yolk: an aliquot of 450 μl of ES was added with 50 μl of TCF clarified egg yolk, prepared according to (Fernández-Santos et al., 2009). After 24 h of refrigeration, the semen vials were placed in an incubator at 38 °C for 30 min and the sperm's viability, motility, and the kinematic parameters, were then assessed as described before.

2.5. Statistical Analysis

2.5.1. Experiment 1

Each testis was considered as an experimental unit.

The data were inserted into an Excel spreadsheet and data analysis was performed with R Studio software (version 2022 02 0). Continuous variables were described as mean ± standard deviation (SD). The Shapiro-Wilk test and Bartlett's test were used to assess the distributional normality. Student's t-test was performed to compare the weights of the right and left testes and epididymis.

2.5.2. Experiment 2

Paired testes were considered as an experimental unit. The data were inserted into an Excel spreadsheet and their analysis was performed with the Minitab® statistical software (version 21). Data distribution was assessed by the Anderson Darling test, thereafter variables were described as mean ± standard deviation or as median (1st quartile – 3rd quartile) when data were normally distributed or not normally distributed, respectively. Similarly, differences were evaluated either by Student's paired t-test or by Wilcoxon signed rank test for paired data, as appropriate depending on data distribution. Statistical significance was set for $P < 0.05$.

3. Results

3.1. Experiment 1

In the first experiment, 14 testes of 7 tomcats (mean age 15.4 ± 6.5 months and mean weight 3.7 ± 0.82 kg), were used. Differences were not identified between right and left testes (supplementary table 1).

From the 14 attempts: 4 *ductus deferentes* (28.6%) cannulations were successfully performed in 2, 7.4, 14 and 5.4 min, respectively; the remaining 10 *caudae epididymides* and *ductus deferentes* were processed by mincing: six (42.8%), because of exceeding the time limit and four (28.6%) due to inability to continue the cannulation attempts. The total number of spermatozoa, the sperms' morphology, the viability, motility percentages and the sperm kinematic parameters of samples collected by the two techniques are reported in supplementary Table 2.

3.2. Experiment 2

In the second experiment testes of 21 tomcats, aged between 12 and 48 months (mean 22.4 ± 11.1 SD) and weighing between 2.9 and 6 kg (Mean 3.8 kg ± 0.80 SD), were used. Testes and *caudae epididymides* average weights, grouped for the retrograde flushing and mincing techniques, did not show significant differences (supplementary Table 3).

Among the 21 stereomicroscope aided attempts, 19 (90.5%) *ductus deferentes* were successfully cannulated while two (which were already too short at the beginning of the procedure) were irreparably damaged and were therefore processed by mincing. In order to keep samples paired, the contralateral specimens were subjected to retrograde flushing. Since these two attempts were successfully completed, the overall success rate was calculated on a total of 23 samples: 21/23 (91.3%). The median time needed for the cannulation procedure is shown in Table 1. Of the 21 successful cannulations, 11 (52.4%) were completed in less than 60 s, 6 (28.6%) between 61 and 180 s, and 4 (19%) between 181 and 480 s

Table 1
Time parameters of tomcat *ductus deferentes* ($n = 21$) cannulations, performed by a stereomicroscope-aided procedure.

	Mean	Median	Min	Max	Q1	Q3
Cannulation time (sec)	131.42	60	20	480	40	180

In five of the 21 paired samples, no sperm were recovered, neither by flushing nor by mincing technique. Four of these tomcats were one year old while one of them was 18 months old. The morphology and the total number of sperm, collected by the two methodologies in the 16 remaining tomcats, are reported in Table 2. The number of spermatozoa recovered by the SARF technique was higher than the number of sperm collected by mincing ($P < 0.001$).

Viability, motility, and kinematic parameters of ES collected by the two techniques, and evaluated before chilling, are reported in Table 3.

Significant differences were found for live, total, and progressively motile sperms ($P < 0.01$) as well as for path, straight, and curvilinear sperm velocities (VAP, VSL, VCL; $P < 0.05$) and for the lateral head amplitude (ALH; $P < 0.05$); higher values were observed in the samples collected by retrograde flushing. Differences were found also after 24 h of storage at 4 °C with retrograde flushing technique showing significantly higher percentages of viable and motile sperm (Table 4).

4. Discussion

The epididymis contains a valuable source of sperm that might be recovered in cases of sudden death or after castration (elective or in emergency) of a high genetic value male. Since there is only one opportunity to obtain sperm from a testis, the most efficient technique must be used for optimizing the gametes' recovery, preservation (germplasm banking) and use, through assisted reproductive technologies. In small and laboratory animals, because of the reduced diameter of the *ductus deferens*, the only technique used for collecting the ES is mincing. Epididymal spermatozoa collected by mincing, however, are exposed to blood cells and to the interstitial fluid and it would be preferable to minimize this kind of contamination. In samples collected by retrograde flushing, instead, the contact with blood and other fluids is limited because only one cut is performed (Martinez-Pastor et al., 2006).

The conservation strategies for endangered wild small felids would benefit from a methodology alternative to mincing. To the best of our knowledge, the Beagle (9–14 kg) is the smallest domestic animal where the RF has been successfully applied (Hori et al., 2015; Batista et al., 2016; Mogheiseh et al., 2022). A stereomicroscope aided procedure was developed for the cannulation of the mice *ductus deferentes* (Baker et al., 2014) but such procedure is not commonly used or reported in literature beside being more difficult and time consuming. The aim of this study was, therefore, the application and success rate of the retrograde flushing technique in the tomcat, for the first time. Initially, it was thought that the cannulation of the small *ductus deferens* could have been easily performed, by the naked eye, using the 30 G rounded tip, side port needle. The results of the first experiment, however, did not confirm such hypothesis: only four (28%) of the 14 attempts were successful. It is possible that, without a time limit of 20 min, more *ductus deferentes* could have been cannulated by continuing the attempts for 30 min or more; the naked-eye procedure, however, was found to be tricky and time-consuming as previously observed by other authors (Hori et al., 2015).

On the contrary, with the aid of the 120 X stereomicroscope magnification, the narrow and irregular lumen of the *ductus deferens*, characterized by numerous mucosal folds, was easily recognized and the cannulation procedure was simplified. The success rate improved considerably and the time to perform the procedure was shortened: 80% of the samples were cannulated in less than three minutes as compared with an average of 7 min needed in the first experiment.

The total number of sperm recovered by mincing, in the present study, is similar to the number of spermatozoa obtained with the same technique in 34 cats aged between 12 and 48 months by Jelinkova et al. (2018) (13.6 ± 11.5 spz x 10^6) and lower than the values observed by Prochowska et al. (2015) in 132 cats between 8 months and 6 years of age (52.9 ± 45.0 spz x 10^6). In the present study the retrograde flushing technique improved the recovered spermatozoa, similarly to previous observations in the stallion and in the *Capra pyrenaica* (Santiago-Moreno et al., 2009; Talluri et al., 2023). A slight upward trend of the total number of recovered spermatozoa was observed, in the bull, in the deer, and in the dog species, by using the RF collection technique, as compared to mincing (Martinez-Pastor et al., 2006; Turri et al., 2012; Hori et al., 2015).

The samples collected with the SARF also showed higher viability and motility values as compared to mincing. Similar results were observed in bulls where samples collected by retrograde flushing showed 12% higher motility and 9% higher viability, even if the sperm kinematics were not affected by the two collection techniques (Turri et al., 2012). In the present study however, the retrograde flushing samples showed higher VAP, VCL, VSL and ALH in agreement with the findings of Martinez-Pastor et al. (2006), who observed higher VAP and ALH in in pre-freezing and post-thawing RF collected samples.

The motility and kinematic parameters of the mincing-collected ES of the present study were lower than the results obtained by Prochowska et al. (2016) but, in the latter work, the sperm kinematic parameters were assessed only in samples with a total sperm count and motility higher than 40×10^6 and 60%, respectively. The kinematic values of SARF collected spermatozoa were instead similar to the values observed by Prochowska et al. (2016) and higher than the mincing sample sperm values observed by Barbosa et al. (2020). It should be noted, however, that a true comparison with the other studies is difficult because of differences in the number, age

Table 2
Puberal tomcat epididymal semen samples sperm count (10^6/mL) and morphology (%) (Mean ± SD or *Median, Q1 and Q3) obtained by mincing ($n = 16$) and by retrograde flushing ($n = 16$).

	§ Tot Spz 10^6/mL	# Normal morph. %	# Head abnorm %	# Midpiece abnorm %	# Tail abnorm %	§ Proximal droplets %	§ Distal droplets %
Mincing	* 6.1 (3.2–12.9)^{C}	67.0 ± 11.2	3.4 ± 2.4 ^{A}	8.5 ± 6.0	4.8 ± 3.8	* 8.3 (2.8–18.7)	* 3.8 (1.8–6.7)
Flushing	* 22.2 (12.6–49.1)^{D}	73.5 ± 12.2	2.1 ± 1.6^{B}	6.6 ± 4.1	3.3 ± 3.2	* 5.2 (3.6–11.2)	* 3.0 (0.9–7.1)

§ Wilcoxon matched pairs signed rank test or # Paired sample t.test: **A‡B P < 0.01 C‡D P < 0.001**

Table 3
Parameters of tomcat epididymal semen samples (Median, Q1 and Q3) obtained by mincing ($n = 16$) and by retrograde flushing ($n = 16$).

	Mincing		Flushing	
Live %	73.4	(70.3–79.6)^{C}	83.7	(75.6–90.3)^{D}
Motile %	32.5	(12.0–49.7)^{C}	65.5	(22.0–80.0)^{D}
Progressive %	5.5	(0.0–6.7)^{C}	19.0	(6.2–44.2)^{D}
Rapid %	7.0	(0.5–10.7)^{C}	30.0	(8.5–52.5)^{D}
Medium %	23.0	(5.2–38.2)	23.5	(9.0–37.7)
Slow %	12.5	(5.0–20.7)	8.0	(3.5–14.5)
VAP (mm/sec)	36.5	(24.6–68.1)^{A}	82.6	(64.6–108.5)^{B}
VSL (mm/sec)	26.2	(21.7–46.1)^{A}	64.0	(47.0–87.3)^{B}
VCL (mm/sec)	79.2	(50.6–121.7)^{A}	152.0	(124.6–191.9)^{B}
ALH (mm)	6.2	(4.3–6.5)^{A}	6.8	(6.3–7.7)^{B}
BCF (Hz)	36.3	(29.8–40.7)	37.0	(29.7–39.7)
STR (%)	58.0	(54.0–75.0)	66.0	(62.0–78.0)
LIN (%)	31.0	(27.0–44.0)	37.0	(34.0–47.0)

Wilcoxon matched pairs signed rank test: **A,B**: $P < 0{,}05$ **C,D: *P*< 0,01**
VAP: average path velocity; **VSL**: straight line velocity; **VCL**: curvilinear velocity; **BCF**: beat cross frequency; **STR**: straightness; **LIN**: linearity

Table 4
Parameters of tomcat epididymal semen samples (Median, Q1 and Q3) obtained by mincing ($n = 16$) and by retrograde flushing ($n = 16$) after 24 h of storage at 4 °C.

		Mincing		Flushing
Live %	69.8	(61.2–72.5)^{A}	76.6	(68.8–81.5)^{B}
Motile %	12.5	(0.0–22.0)^{C}	37.5	(4.7–60.7)^{D}
Progressive %	1.0	(0.0–5.7)^{C}	5.5	(0.7–22.2)^{D}
Rapid %	1.0	(0.0–6.25)^{C}	7.5	(1.0–29.2)^{D}
Medium %	10.0	(0.0–16.7)^{A}	22.5	(1.2–30.7)^{B}
Slow %	6.5	(0.0–24.2)	5.0	(0.2–22.0)
VAP (mm/sec)	34.7	(21.3–55.5)	54.7	(38.3–84.2)
VSL (mm/sec)	22.8	(14.1–43.5)	39.2	(26.2–69.0)
VCL (mm/sec)	67.8	(43.9–100.7)	108.7	(80.5–146.9)
ALH (mm)	8.1	(5.0–8.9)	7.2	(6.6–8.3)
BCF (Hz)	31.7	(20.9–35.7)	32.5	(26.8–35.1)
STR (%)	56.0	(54.0–63.0)	63.5	(56.7–67.5)
LIN (%)	31.0	(27.0–37.0)	35.0	(29.5–37.7)

Wilcoxon matched pairs signed rank test: **A,B**: $P < 0{,}05$ **C,D: *P*< 0,01**
VAP: average path velocity; **VSL**: straight line velocity; **VCL**: curvilinear velocity; **BCF**: beat cross frequency; **STR**: straightness; **LIN**: linearity

and source (owned vs colony tomcats) of the animals, anesthetic protocols, used extender, and samples' selection procedures (Filliers et al., 2008; Jiménez et al., 2011; Nuñez-Favre et al., 2012; Prochowska et al., 2016; Barbosa et al., 2020).

After 24 h of cooled preservation, viability and motility in SARF samples were higher than mincing samples, even do the kinematics values were not affected by the collection technique. Indeed, in chilled canine spermatozoa, the blood cells' contamination was found not to be detrimental to the sperm parameters; the negative effects were observed only after freezing-thawing, due to the RBC hemolysis and to the release of hemoglobin (Rijsselaere et al., 2004). Since this is the first successful application of the SARF in the tomcat, more comprehensive studies will be needed to evaluate the benefits of using this technique.

5. Conclusions

The epididymal sperm can be successfully collected, in the tomcat, by the retrograde flushing technique through a stereomicroscope aided cannulation procedure. Compared to mincing the retrograde flushing improved the total number, the viability, and the motility of the collected ES. The stereomicroscope-aided retrograde flushing technique, therefore, has the potential to become the gold standard method for harvesting ES of endangered species that have small *ductus deferentes*. More comprehensive studies will be needed to assess the biological impact that SARF could have on the assisted reproductive technologies (AI and IVF) currently used for conservation strategies.

CRediT authorship contribution statement

Conceptualization: DM; Methodology: DM, ARizzo; Formal analysis: DM, ARota; AC; Investigation: DM, AC, EL; Resources: GML, ARizzo; Data Curation: DM; Writing - Original Draft: DM; Writing - Review & Editing: DM, ARota; Visualization: DM; Supervision: ARizzo, GML.

Declaration of Competing Interest

Authors have no conflicts to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2023.107388.

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