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COMPARISON OF CHARACTERIZATION OF FIGHTING ROOSTER (Gallus gallus) SEMEN EJACULATES RECOVERED BY ELECTROEJACULATION AND DORSAL MASSAGE TECHNIQUES

Comparación de características de eyaculado de semen de gallo de pelea (gallus gallus) recuperados por electroeyaculación y técnicas de masaje dorsal

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ABSTRACT

Implementing alternatives methods to dorsal massage (e.g., electroejaculation) for recovering semen from fighting rooster, known to be very stressful due to its aggressiveness, has become a priority for breeders of this cock breed in Ecuador. Therefore, the objective of the present study was to evaluate two semen collection techniques in fighting roosters, one by electroejaculation (EE) and another by dorsal massage (DM) on seminal quality parameters. For this purpose, thirty attempts of semen recovery from six adult Spanish fighting roosters were carried out using DM (n = 12) and EE (n = 18). Electroejaculation was performed previous sedation, applying five stimulation cycles (of 2 s) generated from a handmade electroejaculation probe (9 to 12 V). The results showed that the EE produced lower response ($P < 0.01$) to semen ejaculation than the DM (44.4 % vs. 100.0 %, respectively). However, semen samples obtained by EE had better ($P < 0.05$) spermatic kinetic with greater values of straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), and beat-cross frequency (BCF, Hz) as well as higher percentages ($P < 0.01$) of wobble and linearity compared to DM, irrespective of sperm viability. In addition, the number of urates present in the ejaculates obtained by EE was lower ($P < 0.05$) than those obtained by DM. In conclusion, electrical stimulation with prior sedation produced a low semen ejaculation response in fighting cocks. However, EE yielded semen ejaculates with better spermatic kinetic compared with the conventional dorsal massage technique.

Keywords: rooster spermatozoa, electroejaculation, dorsal massage, kinetic sperm.

RESUMEN

Implementar métodos alternativos al masaje dorsal (ej. electroeyaculación) para recuperar semen de gallo de pelea, conocido por ser muy estresante debido a su agresividad, se ha convertido en una prioridad para los criadores de esta raza de gallos en Ecuador. Por lo tanto, el objetivo del presente estudio fue evaluar dos técnicas de recolección de semen en gallos de pelea, una por electroeyaculación (EE) con sedación previa y otra por masaje dorsal (DM) sobre parámetros de calidad seminal. Para ello, se desarrollaron treinta intentos de recuperación de semen de seis gallos de pelea españoles adultos utilizando las técnicas de DM (n = 12) y EE (n = 18). La electroeyaculación se llevó a cabo mediante sedación previa, aplicando cinco ciclos de estimulación (de 2 s) generados a partir de una sonda de electroeyaculación artesanal (9 a 12 V). Los resultados mostraron que la técnica de EE produjo una respuesta baja ($P < 0,01$) en la eyaculación de semen comparado con la de DM (44,4% vs. 100,0%, respectivamente). Sin embargo, las muestras de eyaculado obtenidas por EE produjeron una mejor cinética espermática ($P < 0,05$) con valores más altos de velocidad rectilínea (VSL, µm/s), velocidad promedio (VAP, µm/s) y frecuencia de batida de flagelo (BCF, Hz), así como porcentajes más altos ($P < 0,01$) de oscilación y linealidad en comparación con las obtenidas por DM, independientemente de la viabilidad espermática. Además, el número de uratos presentes en los eyaculados obtenidos por EE fue menor ($P < 0,05$) que los obtenidos con la DM. Se concluye que la estimulación eléctrica con sedación previa produjo una baja respuesta a la eyaculación de semen en gallos de pelea. Sin embargo, el procedimiento de EE produjo eyaculaciones de semen con cinética de espermatozoides mejorada en comparación con la técnica de masaje dorsal.

Palabras clave: espermatozoide de gallo, electroeyaculación, masaje dorsal, cinética espermática.

INTRODUCTION

At Ecuador´s south region, the breeding of fighting cocks has gained importance among breeders and hobbyists of this discipline. The crossbreeding and genetic improvement programs for these roosters have been performed by natural mating, using local and introduced breeds with diverse phenotypes and genotypes. The crossbreeding programs have allowed to improve fighting skills of this rooster, irrespective of social controversies about rooster breeding for these purposes. This conditions have promoted the interest of researchers and breeders to preserving genetic material (e.g., spermatozoa), for genetic improvement, as well as to finding different methods of semen collection. Nevertheless, to our knowledge, scarce studies has been reported on semen collection techniques (none in Ecuador) or sperm quality from ejaculates of fighting cocks (Kanatiyanont et al., 2012; Álvarez-Gallardo et al., 2016).

The first reports about successful sperm collections in domestic birds such as roosters, turkey, and other poultry species were published in the '30s. These reports included the use of artificial vagina, electroejaculation (EE) and dorsal massage (DM) techniques (Kono and Hiura, 1983). The conventional DM method has been used efficiently for recovery semen of rooster. Nevertheless, the DM has limitations for sperm recovery from wild species due to the high stress that implicates its manipulation (Pereira and Blank, 2017). This technique also causes stress on domesticated species (e.g., rooster or turkey) that cannot be trained to yield fertile semen even that many of them prove to be fertile in natural mating (Lake, 1957). The fighting rooster is known for its high aggressiveness during manipulation, which is directly dependent on testosterone level (Ungerfeld, 2020). Likewise, high levels of testosterone affect the seminal quality of the rooster (Castaño et al., 2015). Captivity conditions or high manipulation can cause increased stress in fight roosters, and consequently, affect the process of semen recovery. Also, it has been reported that these events may alter the central nervous system, reducing sexual behavior, because fear is a potent inhibitor of the ejaculatory response (Betzen, 1985). We speculated that in case DM technique is not applied with previous training, semen collection of rooster might be compromised. That is why alternative methods for semen collection may be useful to recover spermatozoa from fighting roosters.

Electroejaculation (EE) is a semen collection method that does not require cooperation of male birds. With this technique a lot of problems associated with DM can be overcome (Almquist, 1968). Some investigators already claim advantages of its use in domestic ducks, geese (Serebrovski and Sokolovskaja, 1934; Watanabe, 1957), pigeons (Betzen, 1985), and large parrots (Harrison and Wasmund, 1983). Certainly, EE is the most practical semen collection method for use in most avian species, but several difficulties need yet to overcome (e.g., artificially induced erection of the avian phallus using electrical stimuli)., Contractions of the cloacal muscles during application of electrical current remains to be a challenge because the same anatomic structure interferes with erection and ejaculation. Additionally, at the same time that electrical stimuli cause an erection, it also causes feces and urates to expel.

Electroejaculation with low voltage has previously been reported as a safe procedure for aggressive male birds (e.g., Siamese fighting cocks) (Kanatiyanont et al., 2012; Álvarez-Gallardo et al., 2016). Thus, it was hypothesized that using of a craft probe 6.0 cm long and 0.5 mL in diameter with three copper wire electrodes might generate short repetitive electrical stimuli (pulses of 2 x 1 s) from 9 to 12 V and yield an efficient ejaculation. It is likely that this tool may induce the erection of the avian phallus and cause the ejaculation of the rooster's semen after the use of an anesthesia protocol (to avoids stress). The aim of this study was to assess two semen collection techniques of fighting roosters, one by electroejaculation with prior sedation and another by dorsal massage on sperm kinetics and seminal quality.

MATERIALS AND METHODS

Animals

Six adult Spanish fighting rooster, 1.5 to 2 year of age, weighted 1.74 ± 0.76 kg, and clinically healthy were used in this study. All roosters were housed individually in a 0.42 m3 outdoor cage (0.60 x 0.70 x 1.0 m of length, width and height respectively) in a farm located in Tañiloma, Cuenca, Ecuador (3°00'05.6"S 79°01'13.8"W). All animals received the same management and feed conditions, based in a commercial diet (16% protein, 3% fat, 4% raw fiber; 120 g daily for each rooster) and free access to water. All animals were handled according to procedures approved by the Veterinary Science Faculty Committee, of the Agricultural Sciences Department from "Universidad Católica de Cuenca", and the research was performed in accordance with the chapter 7.8 of the Terrestrial Animal Health Code -2019© OIE (07/08/2019), regarding the protection of animals used in scientific experiments.

Semen collection

Before starting the experiment, roosters were trained for four weeks in both methods of semen collection. Thirty attempts of semen collection in three sessions weekly were carried out using both techniques: dorsal massage (DM, n = 12) and electroejaculation (EE, n = 18). After two or three attempts of semen recovery per rooster in each procedure, twenty semen ejaculates were successfully recovered from DM (n = 12) and EE (n = 8), according to the following procedure. Briefly, to avoid stress all the roosters were restrained as was described (Álvarez-Gallardo et al., 2016). Feathers were removed from the area around the cloacal opening and cleaned with sterile gauze and physiological saline; gentamicin sulfate (50 µg/mL) was also applied. In addition, to avoid contamination of semen samples with fecal material, 8 hours fasting period was performed prior collection. Each sample was obtained with not time restrictions and total care, avoiding excessive emission of transparent fluid or external contamination.

Dorsal massage technique

This method was performed following the procedures reported by Burrows and Quinn, (1937). This method of semen collection consisted in apply repetitive dorsal massages with the operator hand. At the same time, with the other hand, a direct pressure over the cloaca was made. Seminal fluid was collected into a sterile 1 mL syringe, and then placed into a 1.5 mL microtube. Immediately after collection, each semen sample was assessed for determining macroscopic and microscopic features.

Electroejaculation technique

Previous electroejaculation, a physical examination was performed and a dose of anesthesia was administered. The anesthesia protocol consisted in 0.5 mg/kg intramuscular (IM) midazolam (Dormicum®; Roche Farma, S.A., 28914, Madrid, Spain), and five minutes later 10 mg/kg of ketamine hydrochloride (Ketamina 50; Holliday, 1643, Buenos Aires, Argentina) and 0.5 mg/kg xylazine IM (Dormi-Xyl® 2; AgrovetMerket, 15021, Lima, Peru). The electroejaculation procedure was performed as described by Álvarez-Gallardo et al., (2016).

An electroejaculation device was crafted for a continuous electrical current input of 110 V. The device was constructed with a 1 mL insulin syringe (6.0 cm in length and 0.5 cm in diameter) and with three electrodes (two positive sides and a negative central one) of copper wire adapted to the syringe and sealed with varnish. A rocker switch, a current transformer for 110 to 9 V and a conductor for an intensity of 1 ampere were used. The device converted direct current into alternating current, attenuating it to a range of 9 to 12 V. This transformation into alternating current was necessary to make the cloacal probe functional (Figure 1).

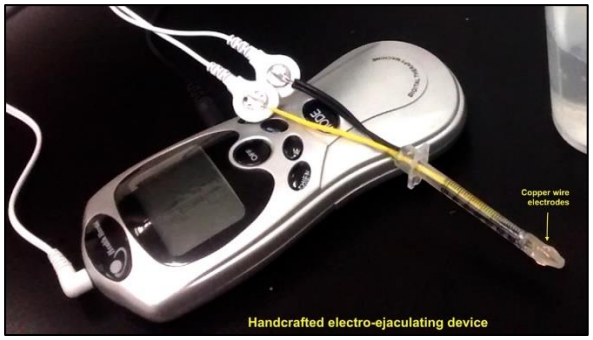

Figure 1. Illustration of the manufacture of the handcrafted electro-ejaculating device

The stimulation was produced by placing the electrodes of the probe on the dorsal region of the cloaca. Electroejaculation was attained after stimulation with pulses of two seconds followed by one second of rest (approximately five cycles of stimulation) as described by Kanatiyanont et al., (2012).The semen ejaculated was collected into a sterile 1 mL syringe, and afterward placed into a 1.5 mL microtube. Each semen ejaculated was assessed for determining macroscopic and microscopic features.

Sperm analysis

The volume of each semen ejaculates was measured by pipetting all the content of the 1.5 mL microtube. The sperm concentration was estimated with a Neubauer chamber (Marienfeld, Lauda- Königshofen, Germany). The percentages of sperm viability (V, %; equivalent to plasma membrane integrity) and total morphological abnormalities (MA, %) were assessed using eosin/nigrosin staining as described by Quintero-Moreno et al., (2017). The plasma membrane functionality percentage was evaluated by hypoosmotic swelling tests (HOST+) according to Jeyendran et al., (1984). Additionally, the presence of urates and coccidia was assessed for each semen sample.

Subjective analysis for individual progressive motility was assessed using light-field light microscopy. The sperm kinetic parameters were determined using CASA system (Sperm Class Analyzer, SCA® 2018, v.6.4, software. Microptic S.L., Barcelona, Spain) coupled to a phase-contrast microscope (Nikon Eclipse model 50i; negative contrast). Briefly, semen samples were diluted with a commercial extender (Extendyl®; Ref. 025880- 017059, IMV technologies, France) at an approximated concentration of 30 x 10^6 spermatozoa/mL. An aliquot of 5 µL of fresh-extended samples was placed on a warmed (37°C) slide and covered with a cover slide. A minimum of 3 fields and 200 sperm tracks were evaluated for each sample at 100 X magnification (image acquisition rate 25 frames/s). The following sperm kinetic parameters were assessed, as previously described by Galarza et al., (2018): percentage motile sperm (SM), percentage progressive sperm (PSM), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), straightness (STR, %), linearity (LIN, %), wobble (WOB, %), the amplitude of lateral head displacement (ALH, µm) and beat-cross frequency (BCF, Hz).

Statistical analysis

Statistical analyses was performed using statistical software for windows V.12 (StatSoft Inc. Tulsa, OK, USA). Sperm variables that showed non-normal distributions, as determined by the Shapiro-Wilk test, were transformed to arcsine (percentages values) or log10 (numeric values) prior analysis. The effects of collection methods on fresh sperm quality were then compared by one-way ANOVA using the General Linear Model procedure. In addition, due to variability between some roosters, male factor was included as covariable in the statistical model. Significance was set at $P < 0.05$. All results are presented by the mean ± SEM.

RESULTS

The results of this study showed that according to the number of attempts performed to semen collection and the number (and percentage) of ejaculates segregated the DM technique was more effective ($P < 0.01$) in recovering semen from fighting roosters compared to EE (100.0 vs. 44.4% of effectiveness for DM and EE respectively) (Table 1).

Table 1. Number (n) of attempts and response of semen ejaculation of fighting rooster subjected to the technique of electroejaculation or dorsal massage.

	Electroejaculation		Dorsal massage	
Rooster	Sessions (n)	Attempts applied / ejaculates segregated (n / n)	Sessions (n)	Attempts applied / ejaculates segregated (n / n)
1	3	3 / 2	2	2 / 2
2	3	3 / 1	2	2 / 2
3	3	3 / 1	2	2 / 2
4	3	3 / 1	2	2 / 2
5	3	3 / 1	2	2 / 2
6	3	3 / 2	2	2 / 2
Total		18 / 8	2	12 / 12
(%)		$(44,4)^b$		$(100,0)^a$

$^{a\text{-}b}$ Different superscripts in the same row indicate significant differences. $^{a\text{-}b}$ $P < 0.01$.

The percentage of sperm viability was greater ($P < 0.05$) in semen ejaculates obtained by DM than those obtained by EE. However, the number of urates found in semen samples collected with EE was lower ($P < 0.05$) than with DM. There were no significant differences ($P > 0.05$) between semen collection techniques concerning to semen features: volume, concentration, subjective motility, coccidia present in ejaculates, HOST+ and MA (Table 2).

Table 2. Sperm variables evaluation of fighting rooster semen obtained by both electroejaculation (EE) or dorsal massage (DM) techniques.

Variable	Semen collection techniques	
	EE (n = 8)	DM (n = 12)
Volume (µL)	75.0 ± 10.00	74.2 ± 7.92
Concentration (x 10^6 sperm/mL)	2682.0 ± 1036.3	2735.0 ± 672.9
Urates (n)	0.9 ± 0.33[b]	2.1 ± 0.19[a]
Coccidias (n)	1.0 ± 0.37	2.0 ± 0.39
Subjective total sperm motility (%)	74.0 ± 10,3	81.3 ± 8.3
Viability – eosine / nigrosine stain (%)	69.8 ± 2.80[b]	78.8 ± 1.84[a]
Total morphological abnormalities (%)	3.5 ± 0.95	7.7 ± 1.52
HOST+ (%)	32.3 ± 6.25	52.2 ± 4.97

Different superscripts in the same row indicate significant differences. [a-b] $P < 0.05$

Regarding to sperm kinetic parameters (Figure 2), the VSL, VAP, and BCF values of rooster sperm recovered by EE was greater ($P < 0.05$) than those obtained by DM. Parameters of progression ratio such as the percentage of LIN ($P < 0.05$) and WOB ($P < 0.01$) were greater in samples recovered by EE than DM.

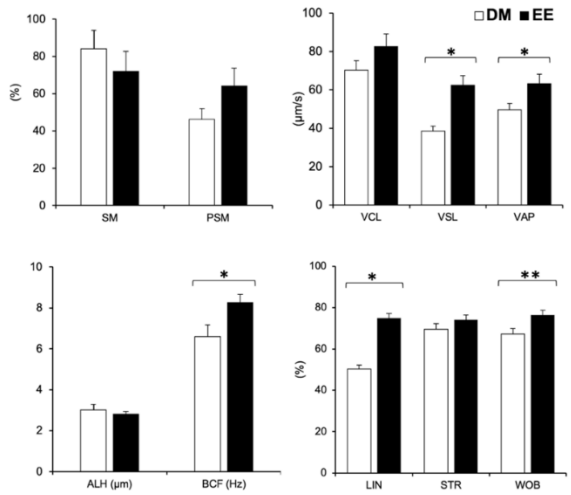

Figure 2. Sperm kinetic parameters of fighting rooster measured by CASA system after semen collection by dorsal massage (DM) or electroejaculation (EE) techniques. Asterisk indicate significant differences between sperm collection techniques in each parameter (*$P < 0.05$ and **$P < 0.01$). SM (%): motile sperm; PSM (%): progressive sperm; VCL (µm/s): curvilinear velocity; VSL (µm/s): straight-line velocity; VAP (µm/s): average path velocity; ALH (µm): amplitude of lateral head displacement; BCF (Hz): beat-cross frequency; LIN (%): linearity; STR (%): straightness; WOB (%): wobble.

DISCUSSION

The results of this study showed a low response of semen ejaculates segregated by the electrical stimulation technique. However, those ejaculates obtained by EE yielded better sperm kinetic (e.g., VCL, VAP, LIN, WOB, and BCF) than those by DM. Also, the number of urates present in semen samples after electroejaculation was fewer. Electrical stimulation applied with the handcrafted probe after sedation allowed the ejaculation of the cocks without aggression or stress, however, the response to EE was lower than DM, as only 44% of semen collection attempts were successful.

The volume and sperm concentration values from rooster semen recovered by DM technique have been reported between 0.35 to 0.9 mL, and 2.4 to 8.0 x 10^9 sperm/mL, respectively (Stephens, 2020; Barna et al., 2020). Kanatiyanont et al., (2012) compared the EE and DM techniques in Siamese fighting cocks and found no difference in ejaculate volume; nevertheless, the sperm concentration was lower in ejaculates obtained by EE. Similar results of semen volume and concentration in ejaculates from domestic drakes were obtained by either electrical stimulation and DM (Watanabe, 1957) have previously been reported. Even, similar fertility was achieved after artificial insemination with fresh semen samples obtained by both methods (Watanabe and Sugimoru, 1957). The results of volume and sperm concentration of this study are consistent with those reports aforementioned.

The electroejaculation technique has not been used routinely in domestic birds due to some drawbacks (e.g., use of anesthesia and contamination). In fact, the most frequent technique used to recover cock semen is the DM (Barna et al., 2020). However, EE has been used efficiently in Anseriformes, Columbiformes, and Psittaciformes (Gee and Temple, 1978; Gee, 1995; Watson, 1998; Blanco et al., 2009). Semen samples have been successfully collected in Psittaciformes (even without anesthesia; Gee et al., 2004) or birds of prey [e.g., monkey hawk (Spizaetus tyrannus), Novaes et al., 2018] by electroejaculation with adequate sperm quality and little impact on bird welfare. However, collection of semen samples in fighting rooster by electroejaculation have been scarce. Kanatiyanont et al., (2012) and Álvarez-Gallardo et al., (2016) used electroejaculation for semen collection in fighting cocks and obtained semen samples with similar quality to the DM technique. These authors controlled the aggressiveness and stress in the fighting roosters through sedation before the EE procedure. The results of the current study are consistent with those previously cited, both in the quality of the semen recovered and in the sedation prior to the EE procedure, which allowed to yield semen segregation despite the ejaculation response was low compared to DM.

On the other hand, the use of EE in duck and goose (Samour et al.,1985), pigeons (Betzen, 1985), and psittacines (Harrison and Wasmund, 1983) caused contamination of semen with urine and injuries on tissues. Another research, however, found no urates in ejaculates of fighting cocks obtained by electrical

stimulation (Álvarez-Gallardo et al., 2016). It is known that contamination of the semen reduces sperm quality (Haines, 2020), and avian semen contaminated with water, blood, feces, urates, or cell debris should not be used for cryopreservation and artificial insemination (Barna et al., 2020). In this study the number of urates was lower in semen samples retrieved by EE than DM. We believe that the electrical current generated by the handcrafted probe as well as the frequency of electrical pulses, provoked an adequate stimulation to induce erection of the avian phallus and ejaculation without affecting neither somatic nerves controlling elimination of urates nor muscles of the rectum which may cause feces to be voided (Betzen, 1985). Accordingly, the use of EE in fighting roosters is a useful and less contaminant procedure for recovering semen samples in these birds than the DM technique.

The sperm kinetic parameters of avian semen indicating better quality are the PSM (>75%), VCL and VSL (10 - 100 µm/s), LIN, and STR (Santiago-Moreno et al., 2014). Indeed, a linear relationship has been observed between sperm mobility and the number of spermatozoa with high VSL values (Froman, 2007; Froman and Feltmann, 2000). Average path velocity has also been correlated with sperm mobility (Pizzari, 2007). Santiago-Moreno et al., (2014) suggested that VSL and VAP on the rapid sperm subpopulation (>50 µm/s) may play an important role in Spanish chicken breeds fertility. Traditionally, SM has been understood as an important quantitative trait related to fertility (Froman et al., 1999). Whereas mobile sperm must be motile, not all motile sperms are mobile. Indeed, VSL must be >30 µm/s for sperm from an overlaid sperm suspension to penetrate an Accudenz solution (Froman, 2007). In the study carried out by Kanatiyanont et al., (2012) no differences between EE and DM were found regarding to sperm kinetic parameters. The present data revealed the VSL and VAP values as well as LIN and WOB percentages of fresh fighting rooster sperm were greater (as objectively determined by CASA) after semen collection by EE than by DM. Production of ATP is required for motility (Miki, 2007). Differences in motility kinematic parameters of rooster sperm might be due to a gradual loss of energy (ATP) and the ability to undertake straight and progressive movements necessary for fertilization (Santiago-Moreno et al., 2012). It has been suggested that production of ATP might be poorer in rooster sperm collected by DM technique and then frozen-thawed, due to sensitivity of mitochondria membrane (Long, 2006).

Plasma membrane integrity have been established to be the best variable for predicting the fertilization potential of rooster ejaculates obtained by DM technique (Santiago-Moreno et al., 2009). Previous studies have shown that Spanish rooster sperm has relatively poor membrane integrity (Prieto et al., 2011). However, Santiago-Moreno et al., (2014) showed that compensation is attained by a high VSL and VAP and that this affords an advantage in sperm competition scenarios under in vitro or in vivo (fertility) conditions. Our results are consistent with these studies when semen was obtained with electroejaculation. High VSL and VAP values obtained in this study after electrical stimulation allow us to speculate that is enough to compensate the low plasma membrane integrity (measured as viability) compared with semen obtained by DM technique. Moreover, the greater BCF value from sperm obtained by EE than DM supports this standpoint. This suggesting that the kinetic parameters of sperm samples obtained by electroejaculation might be useful to assess semen samples when this collection technique is implemented in roosters, or even in other domestic or wild avian species, as was already recently reported in fighting cocks and other aggressive birds (Kanatiyanont et al., 2012; Alvarez-Gallardo et al., 2016).

CONCLUSION

In conclusion, the electroejaculation technique with previous sedation had low ejaculation response in fighting rooster. However, EE yielded semen ejaculates with better spermatic kinetic compared with the DM technique. Thus, the electrical stimulation technique might become a successful alternative for collecting semen samples in fighting cocks and other domestic or wild bird species.

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Authors' contributions
All authors contributed to the study conception and design of the study. Material preparation, collection and assessment of semen samples, data collection and analysis were performed by [Andrés Moscoso Piedra], [Marco Muñoz], [Daniel Argudo Garzón], [Jorge Samaniego] and [Diego Galarza]. Funding acquisition y project administration were performed by [Andrés Moscoso Piedra], [Marco Muñoz], [Manuel Maldonado], [Bolívar Cabrera] and [Juan Carlos Alvarado]. The first draft of the manuscript was written by [Diego Galarza] and all authors commented on previous versions of the manuscript. All authors read and approved the final version of manuscript.

Conflict of Interest Statement
The authors declare that they have no conflict of interest.

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