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Electrostimulation is an effective and safe method for semen collection in medium-sized lizards

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ABSTRACT

The development of safe and consistent semen collection protocols should be ensured to understand basic sperm parameters of a species. Electroejaculation has been hypothesized and tested to be a safe method to evaluate male reproductive potential in wild animals. However, little is known about semen collection protocols in lizards. Adjusting stimulation to species and body mass is important for efficient semen collection as well as for animal welfare. *Tropidurus spinulosus* is a good model to adapt electrostimulation; it is a medium-sized lizard species, males have semen during a long period and operative sex ratio is male-biased. We aimed to provide a thorough and safe method for collecting semen samples from this animal model by means of electrostimulation and characterize basic sperm parameters. Mature males of *T. spinulosus* were captured and their testicular volume was evaluated via portable ultrasound scanning. The lizards were electrostimulated by performing standardized series of stimuli. Semen was obtained successfully in 94% of the males. Samples were contamination-free. Mean sperm number of ejaculates was $2.1 \pm 1.8 \times 10^6$ spermatozoids. The percentage of motile spermatozoa was 78% and sperm dynamic parameters were: VSL 37.26 ± 7.72 μ/s and VCL 84.26 ± 16.27 μ/s. We observed high variability in testicular volume among males; however, almost all the individuals had sperm. Electrostimulation using protocols adjusted to a medium-sized lizard was an effective semen collection method that allowed us to obtain semen samples with high motility (percentage of motile spermatozoa and sperm velocity).

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1. Introduction

Gamete collection is an important laboratory procedure in conservation programs [1,2] and ecological studies [3,4]; however, few studies have evaluated efficiency of semen collection methods. The development of safe and consistent semen collection protocols should be ensured for the development of reproductive assistance programs, since frequent repetition of sampling and viability of the gametes are fundamental factors. An adequate semen collection procedure may contribute to the increase of genetic diversity and reproductive competence of endangered wildlife species [5]. Moreover, understanding gamete biology and basic sperm parameters of a species may help conservation programmers to succeed in fertilization techniques [6]. Likewise, to address ecological questions, such as how environmental factors modulate the performance of gametes [3], or evolutionary questions related to postcopula sexual selection [7,8], morphological and functional sperm comparisons are usually necessary; these comparisons require adjusted and effective techniques to obtain trustworthy semen samples. However, little is known about detailed semen collection protocols for reptiles, and particularly for lizards [5].

Semen collection can be performed in euthanized animals; this procedure has been used to collect semen from the extratesticular storage ducts where spermatozoa undergo maturation in lizards [4,9,10]. However, semen changes over time cannot be tested in longitudinal designs using this technique. Furthermore, even though animal euthanization could be applied in invasive species [10], the sacrifice of animals is usually not desirable [11], and in protected species non-lethal sampling methods instead of lethal collecting are recommended [12]. Semen collection can be alternatively performed in live animals by surgical procedure, massage, or electrostimulation. Although individuals commonly survive the surgical procedure, they undergo the risk of semi-sterilization. The massage technique proposed by Mengden et al. [13] has been

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frequently used with modifications in snakes and geckos [14,15]. Semen collection using massage is a non-invasive technique and may be repeated frequently, with few negative effects on the animal; however, it is not a highly successful technique because often no samples are obtained, samples may be contaminated or small volumes may be collected [16,17].

Electrostimulation has been hypothesized and tested to be a safe method for collection of semen samples to evaluate male reproductive potential and for sperm banking [18–24]. Although some studies have indicated that a small sample volume is obtained by electrostimulation, the technique is effective because semen samples have viable motile spermatozoa and are contamination-free [5]. Moreover, an advantage of this technique is the standardization of the amount and magnitude of the stimulus. To date, electroejaculation has been applied to various reptilian models, including green iguanas (*Iguana iguana*) [5] and snakes [25], as well as to the saltwater crocodile (*Crocodylus porosus*) [16] and chelonians [26–29]. However, the electrostimulation technique has still not been adjusted to medium-sized lizards; this adaptation would require detailed protocols to adjust the equipment and parameters of electrical stimulation to body mass. These are important considerations not only for efficient semen collection but also for animal welfare.

Tropidurus spinulosus is a good model to adapt electrostimulation not only because it is a medium-size lizard (mean snout-vent length SVL = 117 mm), but also because males have semen in the ductus deferens during a long period (similarly to *Tropidurus torquatus*, see Ref. [30]), operative sex ratio is highly biased towards males and since it is a gregarious species, groups of many males are available during the reproductive period. Therefore, we aimed to provide a thorough and safe method for collecting semen samples of this medium-sized lizard by means of electrostimulation and to characterize basic sperm parameters.

2. Materials and methods

2.1. Male collection and handling

Males of *T. spinulosus* were captured (n = 17) in Tanti, Córdoba province, Argentina (31°23′33W, 64°35′48S), located in the phytogeographic region of Chaco mountain forest [31], during the reproductive period [32,33] in October 2016. The capture site was recorded with GPS. The specimens were released at the capture site after semen collection. The government environmental agencies authorized us for scientific capture. This research was approved by the Ethical Committee of the Instituto de Diversidad y Ecología Animal CONICET-UNC (protocol number: 2/2017).

The individuals were acclimated in the laboratory during one week to ensure that epididymides contained sperm before semen collection. The males were kept in individual boxes at 25 °C and under a normal photoperiod (12 h light/12 h dark) with UVB light. The lizards had access to water and were fed ad libitum with live food (crickets and mealworms) dusted with calcium.

2.2. Testicular volume diagnosis

Testicular volume was evaluated by measuring testicle width and length via portable ultrasound scanning (Sonosite 180 Plus, transducer 5–10 MHz). To obtain testis width, the ultrasound scanner was located ventrally 2 cm above the cloaca and transverse scans were performed; testes were identified as a circular paired structure with a well-defined hyperechoic membrane and a homogeneous hyperechoic echographic consistency in the interior [34,35]. To obtain testicle length, the ultrasound scanner was located ventrally parallel to the spine and longitudinal scans were performed; a testis was identified as an oval structure with the same echogenic characteristics mentioned previously. Testicular volume was calculated using the equation for the volume of an ellipsoid, according to [36] (Fig. 1).

2.3. Electrostimulation and semen collection

The electrostimulation equipment consists of an output signal of monophasic type modulated in amplitude (adapted from Ref. [37]). The metal cloacal probe is 3.5 mm in diameter and 14 mm in length, and longitudinal electrodes are separated by 2 mm (Fig. 2). The lizards were electrostimulated by performing three series, each one consisting of five stimuli and each stimulus lasting 5 s (in the first second the stimulus ramps up from 0 to 1 V and in the other 4 s the stimulus is stable at 1 V). A period of a minimum of 5 s was allowed between each stimulus and between each series during the electrostimulation procedure to allow muscles to relax and sperm to flow. All the semen expelled in this three series was collected.

Before beginning semen collection and to reduce semen contamination, the cloaca was rinsed with sterile saline solution (0.9% NaCl) to eliminate any pasty urates and feces [38,39]. Initially, a ventro-lateral stroking was performed to achieve relaxation of the musculature of the animal and later the anal plate was massaged to produce distention of the cloacal musculature [13]. After that, the base of the hemipenes was rubbed with a metal probe (which was inserted 1 cm into the cloacal aperture) by performing circular movements, occasionally leading to the eversion of the hemipenes. Using this procedure, the semen was expelled from the ductus deferens and emptied into the cloacal ampulla, where semen samples were obtained (Fig. 2).

Since semen of *Tropidurus spinulosus* was usually too viscous to be aspired, collection was facilitated by putting 10 μl of the saline solution on the semen drops expelled from the ductus deferens; immediatly the semen was aspired with a micropipette and introduced into an Eppendorf tube that contained 10 μl of saline solution, where the micropipette was rinsed off. The semen was expelled in small aliquots (less than 1 μl), so this procedure was repeated several times over the three electrostimulation series. All samples were completed to a fixed volume of 20 μl of semen solution (semen + saline solution) in the Eppendorf tube. It was not possible to determine the exact volume of the extracted semen, but the described procedure was standardized for all males and the constant volume of 20 μl semen solution was considered as “an ejaculate”.

To estimate the total sperm number (see Ref. [40]), an aliquot of 5 μl of semen solution was fixed and diluted in 0.1% formaldehyde. Sperm concentration was estimated by counting cells using a Neubauer chamber in phase contrast optics at 40× magnification. Total sperm number was calculated as follows: sperm concentration x dilution factor x volume of the semen solution (20 μl).

Anesthesia was not used because the applied procedures were considered non-traumatizing for the specimens and because whether anesthesia may affect semen quantity or quality in reptiles is unknown [39]. The specimens were restrained by manual immobilization of the head and cloacal region in order to avoid any reaction to manipulation. After semen collection, all sampled lizards were observed during one week and appeared to be in good health, with no known ill-effects or complications from the electrostimulation procedure or any perceptible alterations in behaviour after handling. Lizards were daily checked for normal behaviour, not only natural behaviours, but also their appropriate range and context, such as normal/relaxed alertness, calm smell or taste of objects or air, subtle changes in body posture and orientation, unhurried body movements and locomotion, relaxed drinking and feeding and relaxed breathing [41].

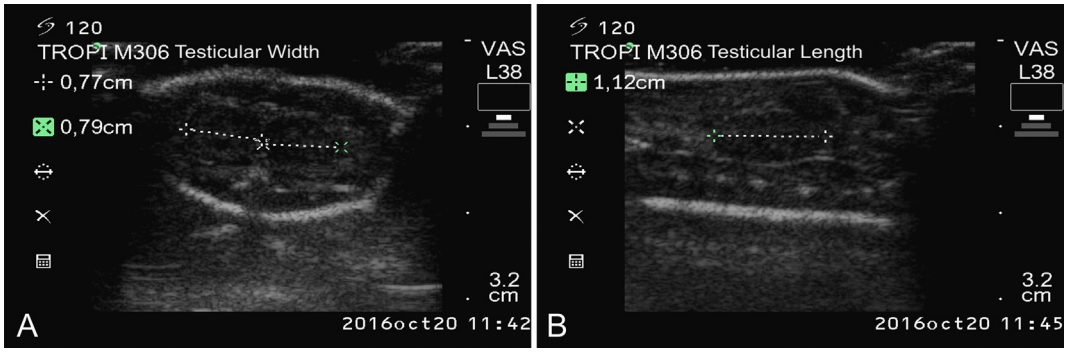

Fig. 1. Ultrasound scanning images of testes. a) Testicular width b) Testicular length.

2.4. Sperm dynamic traits

Immediately after collection, spermatozoa were motile but movement was not translational. Therefore, the spermatozoa were incubated using Ham's F-10 culture medium (Ham's F-10. Gibco, New York, USA) [5,42–44], supplemented with 1% bovine serum albumin. 45 μl of the culture medium were put in an Eppendorf tube and 5 μl of the semen solution were added. Samples were incubated in the thermal bath at 25 °C for 10 min (Thermo Scientific™ Precision™).

Dynamic parameters were measured at room temperature (25 °C) using a video microscopy system composed of a phase contrast microscope (Eclipse 50i; Nikon Microscope, Tokyo, Japan) equipped with a video camera (Nikon Digital Sight DS-Fi2). Digital videos were captured using NIS-Elements D 4.00.03 software. The samples were recorded at 40× magnification for 3 min with a

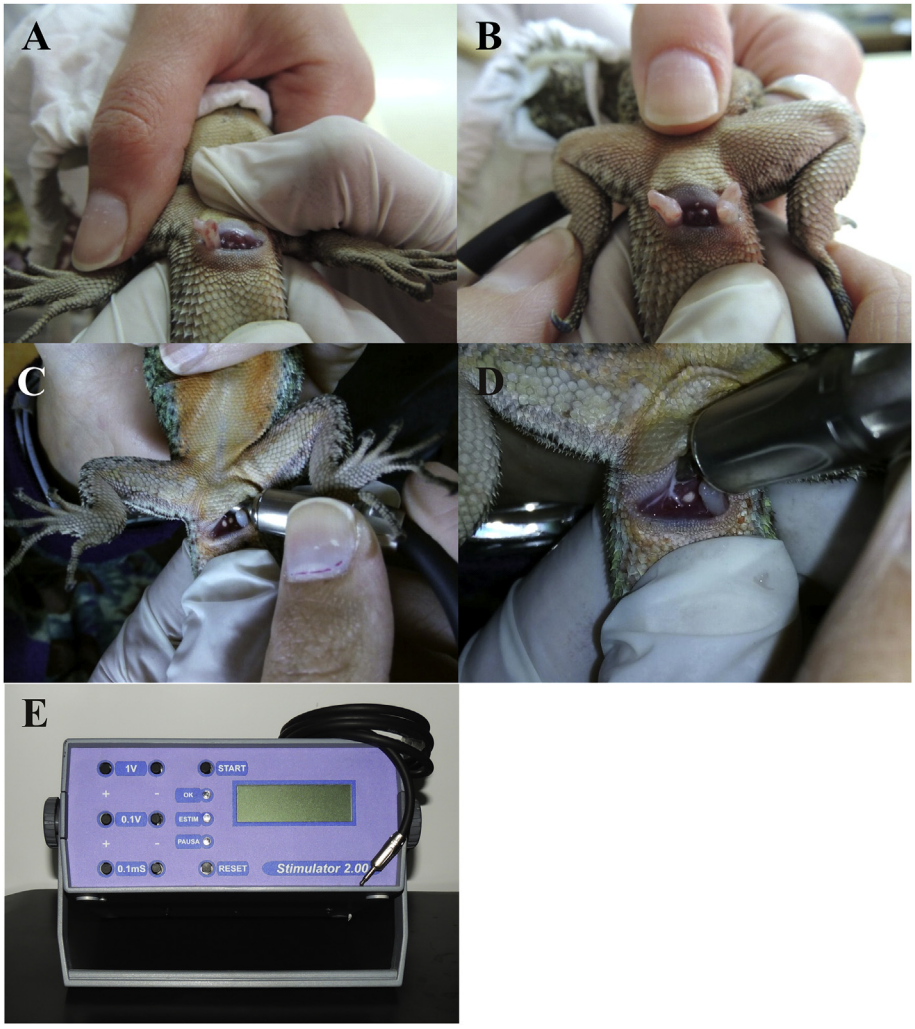

Fig. 2. Electrostimulation and semen collection in *Tropidurus spinulosus*. A and B: Ventro-lateral stroking to achieve relaxation of the musculature and hemipenes eversion. C and D: Electrostimulation and semen expelled. E: Electrostimulation equipment with a metal cloacal probe.

random change of the microscope field every 3 s. Subsequently, individual sperm tracks were followed for 3 s in 45 cells/sample and transformed to a matrix of Cartesian coordinates using ImageJ version 1.43u (NIH) and its plug-in MtrackJ v. 1.1.0 (Eric Meijering). The following sperm dynamic parameters were calculated from this matrix using Spermtrack v. 4.2 (Universidad Nacional de Cordoba, Argentina): straight line velocity (VSL; μm/sec), curvilinear velocity (VCL; μm/sec), and linearity (LIN; LIN = VSL/VCL) [8].

2.5. Statistical analyses

The mean ± sd and range of SVL and of testicular volume were calculated for each individual. The percentage of males in which the technique was successful was calculated. Measurements of semen samples included mean ± sd of sperm number and of sperm velocity variables, and percentage of motile spermatozoa. All statistical analyses were performed using the software INFOSTAT, 2015 version (Universidad Nacional de Córdoba) [45]. The significance level was set at 0.05.

3. Results

Mean SVL of the study males was 117.85 ± 8.58 mm (Range: 98.69 mm–131.93 mm). Mean testicular volume was 0.31 ± 0.09 mm^3 (Range: 0.18 mm^3–0.49 mm^3). Although there was a relationship between testicular volume and SVL ($R^2 = 0.47$; $p = 0.0025$), a high variability in testicular volume was observed (Fig. 3).

Semen was obtained successfully in 94% of the males. Mean sperm number of ejaculates was $2.1 \pm 1.8 \times 10^6$ (Range: 4.8×10^4 to 5.9×10^6) spermatozoa. Percentage of motile spermatozoa was 78.25 ± 9.26% (Range: 61%–95.7%). Sperm dynamic parameters were: VSL 37.26 ± 7.72 μ/s, VCL 84.26 ± 16.27 μ/s and LIN 0.45 ± 0.06.

4. Discussion

Electrostimulation is an effective and safe method for semen collection in wild animals. However, to obtain reliable semen samples it is necessary to establish specific procedures. This study evidenced that electrostimulation was an effective semen collection method using protocols adjusted to a medium-sized lizard. In squamates, spermatozoa generally complete their maturity in the vas deferens [46]. This condition highlights the importance of ejaculation as a method for semen collection to obtain semen samples without morphological abnormalities due to spermatozoa immaturity [38].

According to our results, the electrostimulation technique was highly effective (94% of the individuals). The semen samples were of good quality, contamination-free, and were obtained rapidly. Additionally, since the anal glands are not subjected to pressure, as in the massage stimulation method, the contamination of the ejaculate is reduced [38]. The semen samples showed high sperm number, presence of live spermatozoa, and a high percentage of viable motile spermatozoa with translational movement. In one individual it was not possible to obtain viable semen sample because although spermatozoa were present in the sample, they were immobile even after the addition of HAM's F-10. Another important advantage of this method is that it also allowed standardization of the stimulus, i.e., all males were stimulated by performing three series, each series consisting of five stimuli and each stimulus lasting 5 s. The results of this study will contribute to the optimization of protocols for semen collection and the establishment of basic parameter values, which would be useful to evaluate the reproductive potential of the individuals. Such protocols might be applied to answer questions about sexual selection, sperm competition, sperm storage, reproductive cycles, mating systems and fertilization. Moreover, from a biological conservation perspective, although *Tropidurus spinulosus* is categorized as 'least concern' by the IUCN [12] it will be a good model in species conservation programs, since the methods and the results obtained might be useful for other endangered species, mainly for many closely related ones [12,47–49].

The rubbing with a metal cloacal probe by performing circular movements at the base of the hemipenes occasionally led to the eversion of the hemipenes for a short time. However, semen was collected without hemipenis eversion, i.e. the ejaculation of semen did not require eversion of a hemipenes. Moreover, sometimes semen was obtained without further electrical stimulation; however, the amount was so small that it was necessary to apply electrical stimulation. This observation is important because although hemipenis eversion takes a long time or rarely occurs, semen is collected successfully.

When we obtained the semen sample, the first step was to

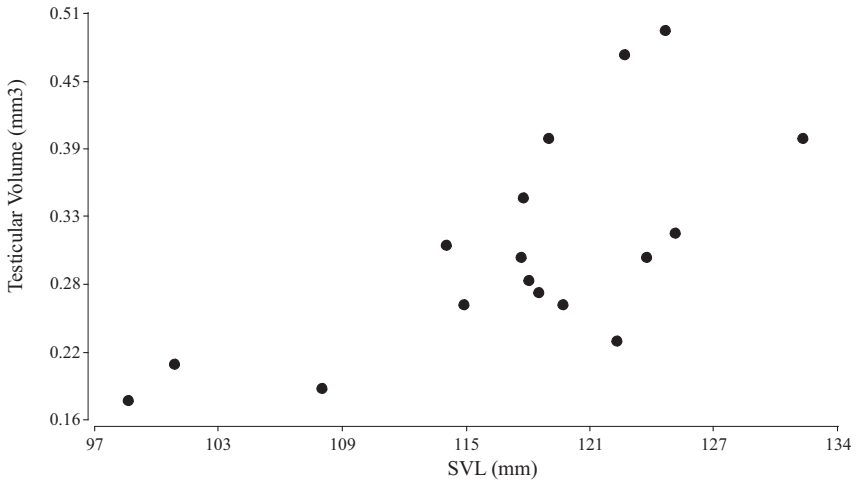

Fig. 3. Relationship between testicular volume and snout vent length (SVL) in *Tropidurus spinulosus*. Testicular volume was calculated using the equation for the volume of an ellipsoid (TV = 4/3 π (a^2b), TV = testicular volume, a = 1/2 smaller testicular diameter and b = 1/2 greater testicular diameter) [33], for which width and length of the testes were obtained through ultrasound scanning.

characterize the quality of the sample by macroscopic examination. Although we did not perform statistics, visual color assessment of semen samples was helpful for characterizing macroscopically sample quality, since semen samples that had a white milky appearance were found to have a high spermatozoa number. More concentrated semen would have a whiter appearance than less concentrated semen because of the increased number of sperm cells [50]. In this study the semen sample volume obtained was generally small but enough to perform all the analyses of sperm dynamic parameters. Accordingly, Zimmerman et al. [5] suggested that the volume of a semen sample may not be predictive of quality, since semen sample volume would not be correlated with spermatozoa concentrations.

Regard less of macroscopic traits, semen samples should be evaluated microscopically (morphology and performance) to determine semen quality. We did not observe morphological abnormalities in spermatozoa of *T. spinulosus*. Particularly, the absence of cytoplasmic droplets might indicate that electrostimulation is a suitable technique because it allows us to obtain spermatozoa that have completed their maturity in the ductus deferens [16]. However, this interpretation is limited because no comparisons to other technique were made and because absence of abnormalities was tested only by phase contrast microscopy and not by other methods such as ultrastructure or physiological evaluations.

Previous studies indicate that the number of motile spermatozoa and their movement speed is directly correlated with fertilization success [42,51,52]. As these variables characterize cell viability, they indirectly determine seminal quality, providing invaluable information for the study of sperm physiology and semen conservation [53]. The mean percentage of motile spermatozoa at the time of semen sample collection was high (78%) and similar to that obtained for green iguanas in semen samples collected also by electrostimulation [5]. Furthermore, the sperm velocity obtained for *T. spinulosus* was higher than that obtained for *Salvator merianae* and *S.rufescens* in semen samples collected from the terminal portion of the epididymides [4]. Sperm velocity is positively correlated to competitive fertilization success in several species [52,54,55]. Our results suggest that electroestimulation is an effective method for collection of semen samples with high motility (percentage of motile spermatozoa and sperm velocity).

Effective semen collection methods allow the evaluation of male reproductive potential, since the presence of semen indicates that the male has the real potential to fertilize eggs. Especially in lizards that have seasonal reproduction, it is important to determine the period when sperm is present in ductus deferens to disentangle reproductive cycles and variability of reproductive strategies among males. Accordingly, high variability in testicular volume was observed among males in *T. spinulosus*; however, nearly all the individuals presented sperm, suggesting different reproductive strategies. This result suggests that males may have sperm regardless variability in testicular volume.

5. Conclusion

In conclusion, the results of the present study suggest that electrostimulation is an effective and safe method for semen collection in *Tropidurus spinulosus* and probably for lizards of similar size, as well for other endangered species. The sample had viable motile spermatozoa and was contamination-free, which makes it useful for evaluating male reproductive potential.

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References

[1] Zacariotti RL, Grego KF, Fernandes W, Sant'Anna SS, de Barros Vaz Guimarães MA. Semen collection and evaluation in free-ranging Brazilian rattlesnakes (Crotalus durissus terrificus). Zoo Biol 2007;26:155–60.
[2] Molina FC, Bell T, Norbury G, Cree A, Gleeson DM. Assisted breeding of skinks or how to teach a lizard old tricks! Herpetol Conserv Biol 2010;52:311–9.
[3] Tourmente M, Giojalas LC, Chiaraviglio M. Sperm parameters associated with reproductive ecology in two snakes species. Herpetologica 2011;67:58–70.
[4] Blengini CS, Sergio N, Gabriela C, Giojalas LC, Margarita C. Variability in sperm form and function in the context of sperm competition risk in two Tupinambis lizards. Ecol. Evol. 2014;4:4080–92.
[5] Zimmerman DM, Mitchell MA, Perry BH. Collection and characterization of semen from green iguanas (Iguana iguana). A. J. Vet. Res. 2013;74:1536–41.
[6] Busso JM, Ponzio MF, Chiaraviglio M, De Cuneo MF, Ruiz RD. Electroejaculation in the Chinchilla (Chinchilla lanigera): effects of anesthesia on seminal characteristics. Res Vet Sci 2005;78:93–7.
[7] Blengini CS, Naretto S, Cardozo G, Giojalas LC, Chiaraviglio M. Relationship between pre-and post-copulatory traits in Salvator rufescens (Squamata: teiidae). Biol J Linn Soc 2016;119:932–42.
[8] Blengini CS, Teves ME, Uñates DR, Guidobaldi HA, Gatica LV, Giojalas LC. Human sperm pattern of movement during chemotactic re-orientation towards a progesterone source. Asian J Androl 2011;13:769–73.
[9] Depeiges A, Dacheux JL. Acquisition of sperm motility and its maintenance during storage in the lizard, Lacerta vivipara. J Reprod Fertil 1985;74:23–7.
[10] Young C, Ravida N, Curtis M, Mazzotti F, Durrant B. Development of a sperm cryopreservation protocol for the Argentine black and white tegu (Tupinambis merianae). Theriogenology 2017;87:55–63.
[11] Waugh CA, Monamy V. Opposing lethal wildlife research when nonlethal methods exist: scientific whaling as a case study. J. Fish Wildl. Manag 2016;7:231–6.
[12] IUCN. Guidelines for appropriate uses of IUCN red list data. Version 3.0. 2016 [Adopted by the IUCN Red List Committee].
[13] Mengden GA, Platz CG, Hubbard R, Quinn H. Semen collection, freezing and artificial insemination in snakes. In: Murphy JB, Collins JT, editors. Reproductive biology and diseases of captive reptiles. Society for the study of Amphibians and reptiles; 1980. p. 71–8. Oxford, Connecticut, USA.
[14] Tourmente M, Cardozo G, Bertona M, Guidobaldi A, Giojalas L, Chiaraviglio M. The ultrastructure of the spermatozoa of Boa constrictor occidentalis, with considerations on its mating system and sperm competition theories. Acta Zool 2006;87:25–32.
[15] Todd AC. Mating strategies and sperm competition in New Zealand geckos (family Gekkonidae). 2003.
[16] Johnston SD, Lever J, McLeod R, Oishi M, Qualischefski E, Omanga C, Leitner M, Price R, Barker L, Kamau K, Gaughan J, D'Occhio M. Semen collection and seminal characteristics of the Australian saltwater crocodile (Crocodylus porosus). Aquaculture 2014;422–423:25–35.
[17] Clulow J, Clulow S. Cryopreservation and other assisted reproductive technologies for the conservation of threatened amphibians and reptiles: bringing the ARTs up to speed. Reprod Fertil Dev 2016;28:1116–32.
[18] Ball L. Electroejaculation. In: Klemm WR, editor. Applied electronics for veterinary medicine and animal physiology; 1976. p. 394–440. Springfield, USA.
[19] Rodger JC, Pollitt CC. Radiographic examination of electroejaculation in marsupials. Biol Reprod 1981;24:1125–34.
[20] Carter KK, Chakraborty PK, Bush M, Wildt DE. Effects of electroejaculation and ketamine-HCl on serum cortisol, progesterone and testosterone in the male cat. J Androl 1984;5:431–7.
[21] Wildt DE, Meltzer D, Chakraborty PK, Bush M. Adrenal–testicular–pituitary relationship in the cheetah subjected to anaesthesia/electroejaculation. Biol Reprod 1984;30:665–72.
[22] Holt W, Bennett PM, Volobouev V. Genetic resource banks in wildlife conservation. J Zool 1996;238:531–44.
[23] Holt W, Pickard AR. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod 1999;4:143–50.
[24] Keeley T, Harris M, McGreevy PD, Hudson D, O'Brien JK. Development and evaluation of electroejaculation techniques in the Tasmanian devil (Sarcophilus harrisii). Reprod Fertil Dev 2012;24:1008–18.
[25] Quinn H, Blasedel T, Platz CC. Successful artificial insemination in the checkered garter snake Thamnophis marcianus. Int Zoo Yearbk 1989;28:177–83.
[26] Platz CC, Mengden G, Quinn H, Wood F, Wood J. Semen collection, evaluation and freezing in the green sea turtle, Galapagos tortoise, and red-eared pond

turtle. Proceedings. Am. Assoc. Zoo. Vet 1980:18–24.
[27] Wood F, Platz C, Critchley K, Wood J. Semen collection by electroejaculation of the green turtle, Chelonia mydas. Br J Herpetol 1982;6:200–2.
[28] Millar JD, Watson PF. Cryopreservation of gametes and embryos in reptiles and amphibians. In: Watson PF, Holt WV, editors. Cryobanking the genetic resource: wildlife conservation for the future? London: Taylor and Francis; 2001. p. 171–7.
[29] Sirinarumitr K, Patthong Y, Jaimjaturong P, Woonwong Y, Petchsamut W, Limpasuntisin P, Manawatthana S, Sirinarumitr T, Sanyathitiseree P, Kornkaewrat K, Suithunmapinunta P. Extender for sperm dilution in olive ridley turtle (Lepidochelys olivacea) and hawksbill turtle (Eretmochelys imbricata) semen. Kyoto University Research Information Repository; 2010.
[30] Ortiz MA, Boretto JM, Piantoni C, Álvarez BB, Ibargüengoytía NR. Reproductive biology of the amazon lava lizard (Tropidurus torquatus) from the wet Chaco of corrientes (Argentina): congeneric comparisons of ecotypic and interspecific variations. Can J Zool 2014;92:643–55.
[31] Zak MR, Cabido M, Hodgson JG. Do subtropical seasonal forests in the Gran Chaco, Argentina, have a future? Biol Conserv 2004;120:589–98.
[32] Martori R, Aun L. Aspects of the ecology of a population of Tropidurus spinulosus. Amphib-Reptil 1994;15:317–21.
[33] Cruz FB, Teisaire E, Nieto L. Reproductive biology of the lizard Tropidurus spinulosus in the Chaco of Salta, Argentina. Stud Neotrop Fauna Environ 1997;32:28–32.
[34] Bertona M, Chiaraviglio M. Reproductive biology, mating aggregations, and sexual dimorphism of the Argentine Boa Constrictor (Boa constrictor occidentalis). J Herpetol 2003;37:510–6.
[35] Cardozo G, Chiaraviglio M. Landscape changes influence the reproductive behaviour of a key 'capital breeder'snake (Boa constrictor occidentalis) in the Gran Chaco region, Argentina. Biol Conserv 2008;141:3050–8.
[36] Méndez FR, Villagrán M. Reproducción asincrónica de Sceloporus palaciosi (Sauria: phrynosomatidae) en México, con comentarios sobre sus ventajas y regulación. Rev Biol Trop 1998;46:1159–61.
[37] Ponce AA, Carrascosa RE, Aires VA, de Cuneo MF, Ruiz RD, Ponzio MF, Lacuara JL. Activity of Chinchilla laniger spermatozoa collected by electroejaculation and cryopreserved. Theriogenology 1998;50:1239–49.
[38] Tourmente M, Cardozo GA, Guidobaldi HA, Giojalas LC, Bertona M, Chiaraviglio M. Sperm motility parameters to evaluate the seminal quality of Boa constrictor occidentalis, a threatened snake species. Res Vet Sci 2007;82:93–8.
[39] Zimmerman DM, Mitchell MA. Semen collection and ejaculate characteristics of the Leopard Tortoise (Stigmochelys pardalis). Conserv. Physiol 2017;5.
[40] Gómez-Montoto L, Magaña C, Tourmente M, Martín-Coello J, Crespo C, Luque-Larena, Gomendio M, Roldan ER. Sperm competition, sperm numbers and sperm quality in muroid rodents. PLoS One 2011;6, e18173.
[41] Warwick C, Arena P, Lindley S, Jessop M, Steedman C. Assessing reptile welfare using behavioural criteria. In Pract 2013;35:123–31.
[42] Gist D, Turner T, Congdon J. Chemical and thermal effects on the viability and motility of spermatozoa from the turtle epididimys. J Reprod Fertil 2000;119:271–7.
[43] Howard JG, Lynch C, Santymire RM, Marinari PE, Wildt DE. Recovery of gene diversity using long-term cryopreserved spermatozoa and artificial insemination in the endangered black-footed ferret. Anim Conserv 2016;19:102–11.
[44] Rosati L, Prisco M, Di Fiore MM, Santillo A, Sciarrillo R, Valiante S, Laforgia V, Coraggio F, Andreuccetti P, Agnese M. Sex steroid hormone secretion in the wall lizard Podarcis sicula testis: the involvement of VIP. J Exp Zool Part A 2015;323:714–21.
[45] Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. InfoStat versión 2015. Universidad Nacional de Córdoba; 2015.
[46] Jones R. Evolution of the vertebrate epididymis. J Reprod Fertil Suppl 1998;53:163–81.
[47] Lima AFB. Tropidurus erythrocephalus. In: IUCN 2012. IUCN red list of threatened species. Version 2012.1; 2010. Available from: http://www.iucnredlist.org. [Accessed 25 June 2012].
[48] Lima AFB, Rocha PLB. Tropidurus psammonastes. In: IUCN 2012. IUCN red list of threatened species. Version 2012.1; 2010. Available from: http://www.iucnredlist.org. [Accessed 25 June 2012].
[49] Carvalho AL. On the distribution and conservation of the south american lizard genus Tropidurus wied-neuwied, 1825 (squamata: tropiduridae). Zootaxa 2013;3640:42–56.
[50] Fahrig BM, Mitchell MA, Eilts BE, Paccamonti DL. Characterization and cooled storage of semen from corn snakes (Elaphe guttata). J Zoo Wildl Med 2007;38:7–12.
[51] Froman D, Feltmann A, Rhoads M, Kirby J. Sperm mobility: a primary determinant of fertility in the domestic fowl (Gallus domesticus). Biol Reprod 1999;61:400–5.
[52] Birkhead T, Martínez J, Burke T, Froman D. Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc Roy Soc Lond 1999;266:1759–64.
[53] Gadea J. La evaluación de la capacidad fecundante de los espermatozoides porcinos mediante la fecundación in vitro (Revisión). Investig Agrar Prod Sanid Anim 2001;16:63–77.
[54] Burness G, Casselman SJ, Schulte-Hostedde AI, Moyes CD, Montgomerie R. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (Lepomis macrochirus). Behav Ecol Sociobiol 2004;56:65–70.
[55] Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr Biol 2004;14:44–7.