

1 **Cover page**

2

3 **Title**

4 **Sperm Parameters in *Pristidactylus achalensis* (Squamata: Leiosauridae), an Endemic Lizard to the**
5 **Highest Mountain Areas in Central Argentina.**

6

7 **Author names:** Cecilia Soledad Blengini^{1,2}, Guadalupe López Juri^{1,2}, Margarita Chiaraviglio^{1,2}, Diego Rafael
8 Uñates^{1,2}, Sergio Naretto^{1,2}

9

10 **Running head**

11 Sperm traits of *Pristidactylus achalensis*

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15

16 **Affiliation and address of authors:**

17 1. Universidad Nacional de Córdoba, Facultad de Ciencias Exactas, Físicas y Naturales. Laboratorio de

18 Biología del Comportamiento. Córdoba, Argentina.

19 2. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto de Diversidad y

20 Ecología Animal (IDEA). Córdoba, Argentina.

21 Postal address: Av. Velez Sarsfield 299, CP:5000, Cordoba City, Province of Cordoba, Argentina.

22

23 **Email address:**

24 ceciliablengini@gmail.com, lopezjguada@hotmail.com, mchiaraviglio@gmail.com, dunates@yahoo.com.ar,

25 narettosergio@gmail.com

26

27 Send reprint requests to this address: narettosergio@gmail.com

28 **Twitter:** @S_Naretto

29 Reproductive physiology is influenced by environmental pressures, particularly in ectothermic species
30 living in harsh climatic conditions. Studying the period of time in which males exhibit semen
31 availability and their sperm traits, constitutes a powerful tool to evaluate the fertility potential of males
32 because sperm size and sperm velocity have been proposed as a determinant in fertilization success.
33 *Pristidactylus achalensis* is an endemic lizard to the highest mountain areas in Central Argentina and
34 shows a pattern of seasonal activity. Here, we study the period of time when males possess sperm
35 during the active season, and characterize the spermatozoa based on sperm morphometric and
36 dynamic traits among males and within ejaculate. Males possess sperm in their ejaculates only during
37 the spring months of the active season suggesting that reproductive activity is restricted to this time.
38 Spermatozoa of *Pristidactylus achalensis* shows intra-specific variation in sperm traits among males and
39 within ejaculate, suggesting different reproductive potential among males. Since this species only
40 occupies the highest areas of the mountains and is categorized as vulnerable, basic information about
41 reproduction and sperm physiology could be useful for potential population conservation strategies.

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56 REPRODUCTIVE physiology studies in rare species are relevant because they provide useful data to wildlife
57 conservation and also to generate ecology and evolutionary questions on post copulatory sexual selection
58 (Tourmente et al., 2007; Friesen et al., 2017; Goblet et al., 2018; Caldas and Godoy, 2019). Particularly,
59 sperm analysis constitutes a powerful tool to evaluate the fertility potential of males (Goeritz et al., 2003).
60 The application of advanced reproductive technologies to improve breeding programs needs basic information
61 on rare or endangered species (O'Brien et al., 2009; Browne et al., 2019). For that, research on species-
62 specific variations in cellular physiology of spermatozoa of endangered species is needed. Moreover,
63 reproductive studies of ectotherm species living in harsh climate conditions can help to understand the
64 relationship between environment and reproductive physiology (Friesen et al., 2017; Boretto et al., 2018).
65 Even though reproductive studies on lizards in temperate zones typically reveals seasonal timing of
66 reproduction (Fitch, 1970; James and Shine, 1985; Brown and Shine, 2009), reproductive traits have evolved
67 in response to changing environments. Mountains are isolated areas where lizards face a challenge in hostile
68 conditions related to reproduction and climate seasonality (Shine 2004; Esquerré et al., 2018).

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70 Several sperm traits have been proposed as determinants of fertilization success. The size of different
71 sperm components has been considered important in ejaculate quality because each part of spermatozoa
72 contributes differentially with diverse sperm functions (Fitzpatrick et al., 2009; Lüpold et al., 2009;
73 Tourmente et al., 2011; Blengini et al., 2014). A positive contribution of principal piece length or flagellum
74 length to sperm velocity has been reported for several taxa (Fitzpatrick et al., 2009; Lüpold et al., 2009;
75 Tourmente et al., 2011), a trait that has been shown to be a major determinant of male fertility (Birkhead et
76 al., 1999; Gomendio and Roldan, 2008; Beausoleil et al., 2012). An increase of midpiece length may reflect
77 an increase in the energetic reserves for sperm (Ruiz-Pesini et al., 2007). Furthermore, an elongation of head
78 size may play an essential role during sperm storage, contributing to sperm-female interactions (Pitnick et al.,
79 2009), and reducing the drag experienced by the sperm cell (Malo et al., 2006). Studies concerning sperm
80 traits in lizards are scarce (Vieira et al., 2004; Blengini et al., 2014, 2016; Kahrl and Cox, 2015; McDiarmid et
81 al., 2017; Rheubert et al., 2017), and those completed showed among- and within- male variation in sperm
82 morphometric and dynamic traits in lizards in the context of sperm competition (Blengini et al. 2014;
83 Rheubert et al. 2017).

84 *Pristidactylus achalensis* belongs to the Leiosauridae family from austral South America (Etheridge and
85 Williams, 1985; Morando et al., 2015) and is endemic to the highest mountain areas in central Argentina
86 (Minoli and Avila, 2017). *Pristidactylus achalensis* is interesting because it is the only lizard species living in
87 the isolated highland ecosystem of Pampa de Achala (Torres et al., 2019). This granite plateau extending from
88 an altitude of 2000 m above sea level and to the highest peak of 3000 m, is an area with harsh climatic
89 conditions. At high altitudes, the daily and annual time for activities are constrained, affecting fundamental
90 life-history traits like growth and reproductive cycles (Boretto et al., 2018). We predict that *Pristidactylus*
91 *achalensis* needs to reproduce during a short period, which influences its reproductive physiology, measured
92 as sperm production and sperm quality. Surprisingly, little is known about the biology of this lizard.
93 *Pristidactylus achalensis* is sexually dimorphic in body size (Sinsch et al., 2002, Naretto and Chiaraviglio
94 2020), hibernates in rock crevices or belowground, and emerges during the warm spring, being active until
95 late summer (from October to March). Because of the lack of biological information, the small distribution
96 range and endemism, this species has been classified as vulnerable and baseline studies that impact
97 conservation actions are necessary (Abdala et al., 2012). In this study, we first characterized the period of
98 sperm availability in the ejaculate during the active season to determine the mating period. Secondly, we
99 characterized the spermatozoa of *P. achalensis* based on sperm morphometric and dynamic traits among
100 males and within an ejaculate. To our knowledge this information represents the first study on the
101 reproductive biology of this rare lizard species.

102

103 MATERIALS AND METHODS

104 **Species.**—Forty-four adult males of *Pristidactylus achalensis* were captured from wild populations in central
105 Argentina (31.50°W, 64.90S° to 31.63°W, 64.82°S; 2200 m above the sea level) throughout the active season
106 between October to March. Animals were captured by lassoing them around the neck (i.e., a pole with a loop
107 of string with a slipknot that tightens around the neck of the lizard). After capture, the lizards were
108 individually held in cloth bags and transported to the laboratory. For each individual, we recorded the
109 following data: date, location (GPS GARMIN etrex30), sex, and snout-vent length (SVL) with a digital
110 calliper to the nearest 0.01 mm. We identified individuals by the shape and size of a black spot at the neck
111 region to avoid sampling the same individual twice. We took a photo from the right lateral area trying to

112 extend the skin to standardize the photo. We focus on the shape, the position and the size of the spot. In case
113 of doubts about the individual identification we checked extra patterns around the main spot or particular
114 signals such as: spots in the labial scales, shape of the tail, lost fingers. Each lizard was released at the same
115 rock of its capture within five days of being removed.

116

117 **Semen collection.**— Males were acclimated to lab conditions for 3 or 4 days before semen collection. In the
118 laboratory, lizards were kept at individually plastic containers with rocks as refuges. Light and temperature
119 were provided with fixed light (0900 to 1800 h, Zoomed UVB 5.0 UV tubes). We controlled the temperature
120 between 25 °C and 28 °C during the day and between 18 and 20 °C at night; larvae of *Tenebrio molitor* and
121 water *ad libitum* were provided. We follow the semen collection protocol proposed by López Juri et al.
122 (2018), a detailed protocol developed for reptiles, particularly for medium-sized lizards. Briefly, the
123 specimens were restrained by manual immobilization of the head and cloacal region to avoid any aggressive
124 reaction to manipulation. The cloaca was cleaned with sterile saline solution (0.9% NaCl) to eliminate feces.
125 A metal probe (length 14 mm, diameter 3.5 mm) was inserted into the cloaca. Lizards were electro-stimulated
126 by performing up to three series, each one consisting of five stimuli and each stimulus lasting 5 s. A period of
127 a minimum of 5 s was allowed between each stimulus to enable muscles to relax and sperm to flow. The
128 ejaculate was collected in 30 μ l of saline solution in Eppendorf tubes using a micropipette. All procedures
129 were done at room temperature of 25°C to avoid variability of body temperature among males. Each semen
130 sample was divided in two aliquots, one for sperm morphometry assessment, and one for sperm velocity.

131

132 **Sperm Morphometry.**— Aliquots of sperm samples were fixed for photography in 2% formaldehyde and
133 stained with Coomassie Brilliant Blue (Blengini et al., 2014, 2016). The samples were examined at 1000x
134 magnification under a phase-contrast Nikon Eclipse Ti microscope (Nikon Instruments Inc, Tokyo, Japan).
135 Microphotographs were taken using Nikon DS-Qi1Mc digital camera with a controller DS-U2 (Nikon
136 Instruments Inc, Tokyo, Japan). Total sperm length, length of the sperm head, midpiece, and principal piece
137 were determined in 40 spermatozoa per individual. The curvature of the spermatozoa was considered when
138 measuring total and partial lengths using the software Image J version 1.43u (Schneider et al., 2012).

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140 **Sperm Velocity.**—Sperm aliquots (5 μ L) were incubated in 45 μ l of Ham-F10 medium supplemented with
141 1% albumin at 25°C in a thermally stable water bath for 10 min (Blengini et al., 2014). Sperm suspension (5
142 μ L) was placed in a plastic observation chamber and covered with a coverslip. Dynamic parameters were
143 measured at room temperature of 25°C using a video microscopy system composed of a phase-contrast
144 microscope (Eclipse 50i, Nikon Instruments Inc, Tokyo, Japan). Videos were captured using Nikon DS-
145 Qi1Mc digital camera with a DS-U2 controller (Nikon Instruments Inc, Tokyo, Japan); spermatozoa were
146 recorded at 40x for 3 min, in different fields that were randomly changed every 5 s. Subsequently, 20 sperm
147 tracks from each male were followed for 3s and transformed to a matrix of Cartesian coordinates using
148 ImageJ version 1.43u (Schneider et al., 2012) and its plug-in MtrackJ v. 1.1.0 (Meijering et al., 2012).
149 Straight-line velocity (VSL; μ m/s) which is the linear distance travelled by the spermatozoon divided by the
150 time and curvilinear velocity (VCL) which is equivalent to the curvilinear distance that is travelled by the
151 spermatozoon divided by the time were calculated using AG paNoel 1.0.0.ijm macro for ImageJ (Universidad
152 Nacional de Cordoba, Argentina <http://www.iibyt.conicet.unc.edu.ar/software/>).

153

154 **Statistical Analyses.**—Statistical differences in the mean of sperm traits and differences in within- ejaculate
155 variation among males were determined by a non-parametric Kruskal-Wallis test and Brown-Forsythe test,
156 respectively. The outliers were removed from each data set following ROUT method before performing the
157 analyses. Statistic were conducted using R version 3.5.2 (R Core Team 2018) and GraphPad Prism version
158 6.00, GraphPad Software (La Jolla California USA).

159

160 **RESULTS**

161 We examined 44 males by electrostimulation, and we only obtained ejaculate with spermatozoa in 13 males.
162 The smallest male with sperm was 92.55 mm SVL. There was no difference in the body size of males with
163 and without sperm (mean SVL males with sperm = 104.84 ± 6.43 mm; mean SVL males without sperm =
164 104.84 ± 5.55 mm; $F_{1,42} = 8.39$, $P = 0.99$). We observed a seasonal pattern in the sperm availability, with
165 concentrated at the beginning of the active season from October to December (Fig.1). In October, 100% of the
166 males possessed spermatozoa in their ejaculates, while this percentage decreased during the spring until
167 December when only 20% of males had sperm in their ejaculates.

168 We analyzed 11 sperm samples from individual males. However, in only 9 samples sperm velocity
169 was assessed due logistical problems with the microscope when we were recording videos from two samples.
170 Sperm of *Pristidactylus achalensis* are filiform; the head has a narrow elongated shape, and its rostral portion
171 (which contains the acrosomal vesicle) was slender and ended in a curve shape (Fig. 2). Sperm morphometric
172 and dynamic traits have been presented in Table 1. Sperm velocity had the highest variation, based on the
173 coefficient of variation (CV; Table 1). There were differences among males in all sperm traits we measured
174 (head length: $H_{(10,418)} = 250.9$; $P < 0.0001$; midpiece length: $H_{(10,422)} = 108.4$; $P < 0.0001$; principal piece
175 length: $H_{(10,420)} = 215.8$; $P < 0.0001$; total sperm length: $H_{(10,420)} = 202.4$; $P < 0.0001$; VSL: $H_{(8,170)} = 71.95$; P
176 < 0.0001 , VCL $H_{(8,170)} = 101.1$; $P < 0.0001$) (Fig. 3). The within ejaculate variation of sperm morphometric
177 traits was different among males (head length: $F_{10,418} = 3.283$; $P = 0.0004$; midpiece length: $F_{10,422} = 5.749$; P
178 < 0.0001 ; principal piece length: $F_{10,420} = 3.970$; $P < 0.0001$; total sperm length: $F_{10,420} = 2.275$; $P = 0.0031$).
179 However, within ejaculate variation in sperm velocity was not found ($F_{8,170} = 1.755$; $P = 0.0892$) (Fig. 3).

180

181 **DISCUSSION**

182 Our study constitutes an essential step in expanding the knowledge of reproductive biology in *Pristidactylus*
183 *achalensis* by providing and analyzing sperm trait data on this endemic lizard species. The sperm traits such
184 as size and speed, influence fertilization efficiency and thus are the focus of selective pressures (Birkhead et
185 al., 1999; Snook, 2005). Males have sperm in their ejaculates at the beginning of the active season suggesting
186 that reproductive activity (or mating) is restricted to spring (October, November and December). Seasonal
187 information about sperm availability and seminal analysis are basic information that should be taken into
188 account for conservation programs (Swanson, 2006; Kouba et al., 2009; Browne et al., 2019). The genus
189 *Pristidactylus* is an attractive system due to the climatic restrictions of the environment in which it occurs
190 (Minoli and Avila, 2017), and because it was the earliest divergent taxon of its clade, with the northernmost
191 distribution (Morando et al., 2015). Future comparison within the genus could help to understand the
192 evolutionary relationships within this genus and how reproductive traits have evolved (Tourmente et al.,
193 2008).

194 Spermatozoa of *Pristidactylus achalensis* share a similar morphology with the general model
195 described for lizards (Blengini et al., 2014, 2016; Kahrl and Cox, 2015). Spermatozoa are filiform, possess a

196 narrow head with its anterior portion covered by the acrosome, a short midpiece, and a principal piece almost
197 ten times longer than midpiece length. *Pristidactylus achalensis* shows intra-specific variation in sperm traits
198 not only among males but also within-ejaculate. These results are similar to sperm variation found in other
199 species of lizards (Blengini et al., 2014). However, within-ejaculate variation was only observed in sperm
200 morphometric traits. At the intra-specific level, post-copulatory sexual selection may regulate the variability
201 of sperm size among and within male (Calhim et al., 2007; Kleven et al., 2007; Immler et al., 2008;
202 Helfenstein et al., 2010). To understand the importance of the variability in sperm size within an ejaculate and
203 the consequences in sperm physiology and sperm velocity, further studies are needed where sperm size and
204 velocity are assessed in the same spermatozoa, allowing to establish robust correlation between both
205 parameters.

206 *Pristidactylus achalensis* is a sexually dimorphic and sexual dichromatic species (Naretto and
207 Chiaraviglio, 2020) and we have observed intrasexual agonistic encounters suggesting a high level of
208 competition pre copula. Moreover, the relatively short period of sperm presence could be another potential
209 factor that can increase reproductive competition. Several hypotheses have been proposed to explain the role
210 of sperm competition in sperm trait variation, and it is necessary to raise questions about post-copulatory
211 strategies of *Pristidactylus* males (Blengini et al., 2014). An increase in sperm competition pressure could
212 reduce sperm size variation or produce a diversity of sperm to improve fitness (Calhim et al., 2007; Kleven et
213 al., 2007; Immler et al., 2008; Helfenstein et al., 2010; Immler et al., 2010; Lifjeld et al., 2013; Bakker et al.,
214 2014; Van der Horst and Maree, 2014). Phenotypic variation within individual ejaculates may be the result of
215 sexual selection pressures; males may produce different specialized sperm, each one trending toward a
216 different optimum (Pizzari and Parker, 2009; Blengini et al., 2014). Further studies are needed to explain the
217 intra-specific variation found in *P. achalensis*, taking into account characters of the mating system such as the
218 rate of multiple paternity, the variability of secondary sexual traits, coloration, male competition for females
219 and duration of copula in order to integrate pre and post-copulatory selected traits (Friesen et al., 2014, 2017;
220 McDiarmid et al., 2017). This information is key to improve our knowledge about the reproductive strategies
221 (sperm competition, mating systems, sperm storage and fertilization) of this endemic species and lizards in
222 general. The results of this work will help in the establishment of parameters which would be useful to

223 evaluate the reproductive potential of the populations of *P. achalensis* and provide invaluable information for
224 the study of sperm physiology and sperm evolution in lizards.

225

226 **DATA ACCESSIBILITY**

227 The datasets generated during and/or analyzed during the current study are available from the corresponding
228 author on reasonable request.

229

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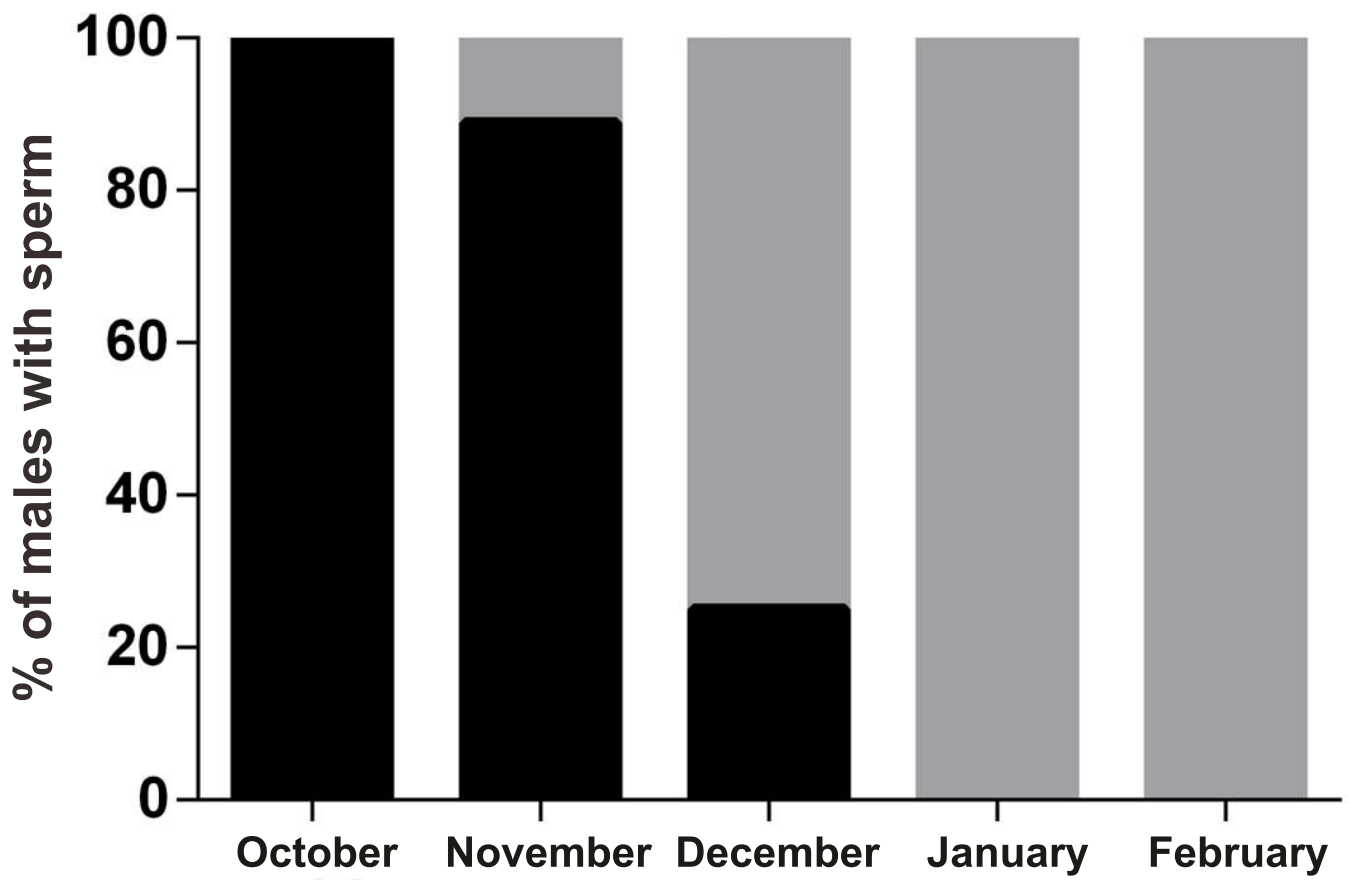
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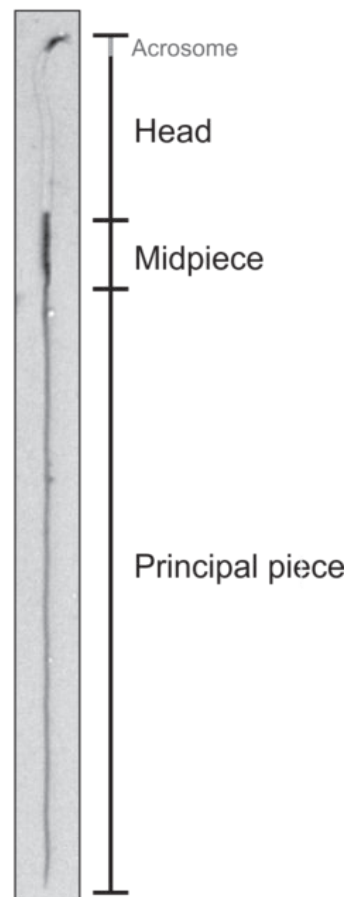
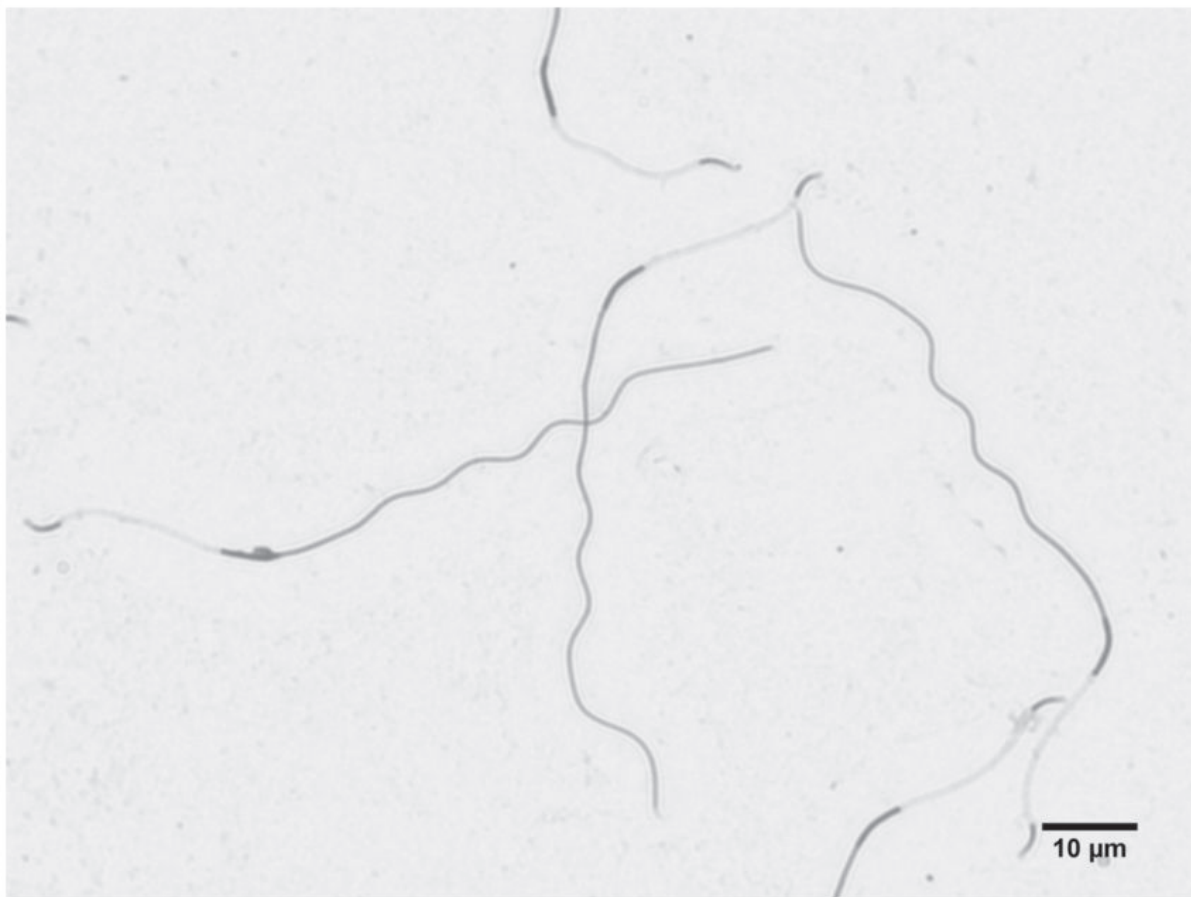
443 **FIGURE CAPTIONS**

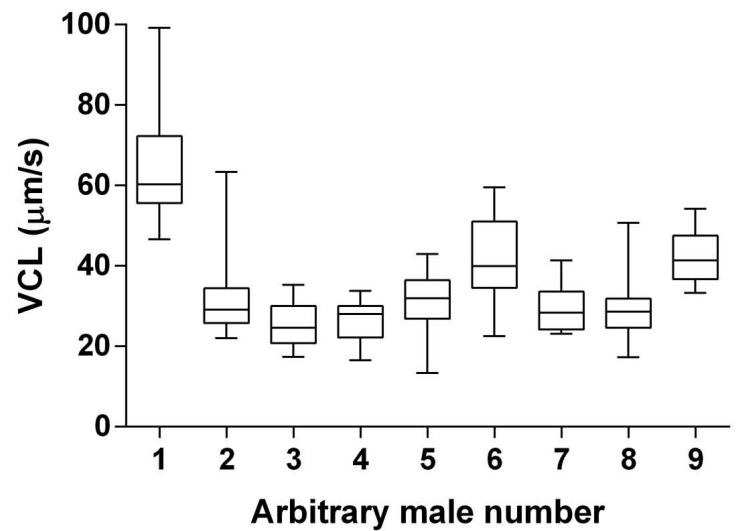
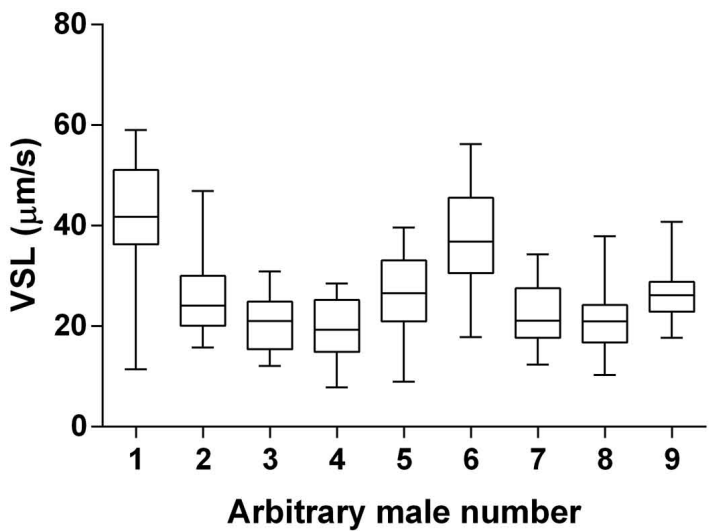
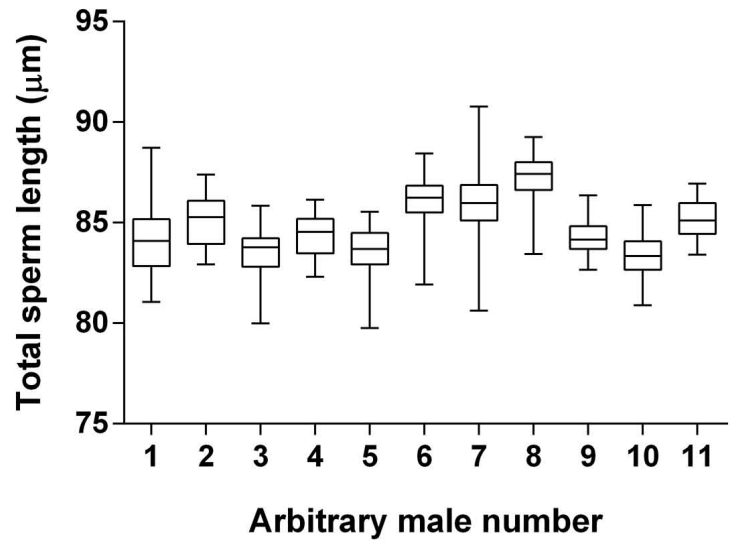
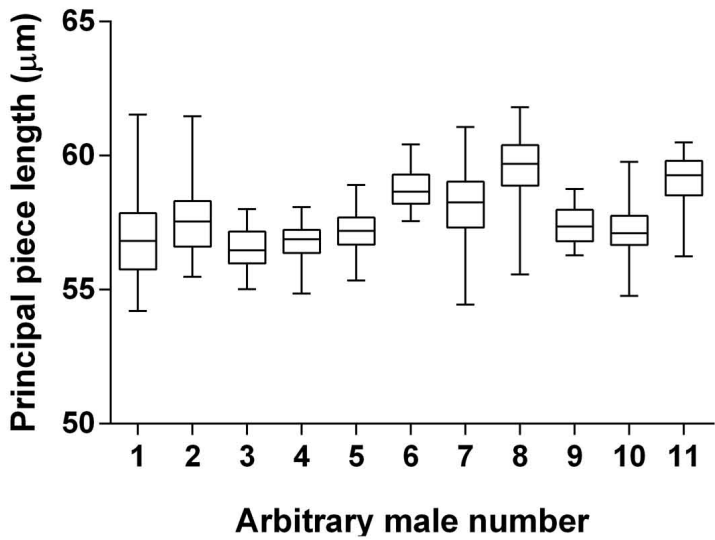
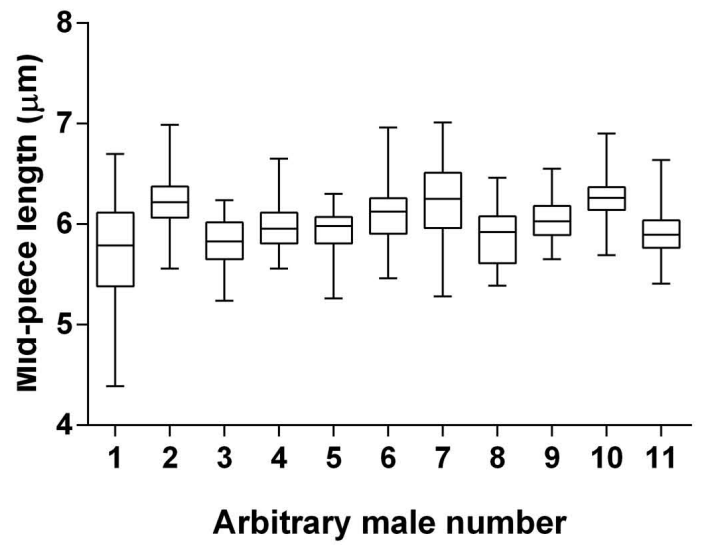
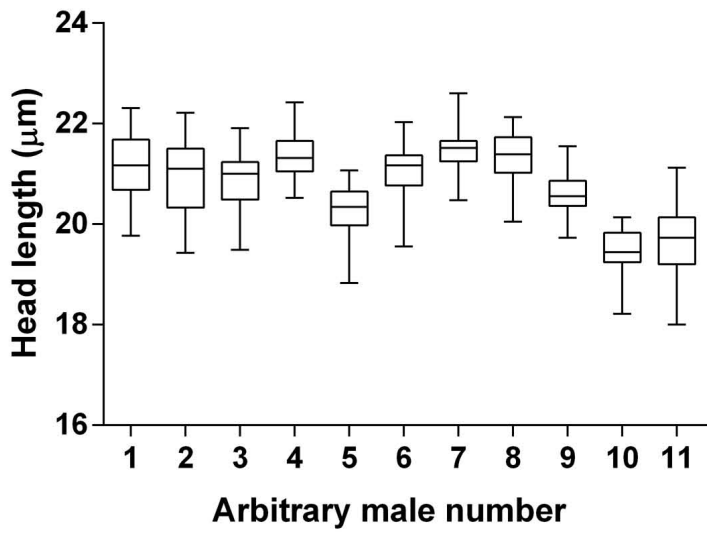
444 **Fig. 1** Seasonal pattern of sperm presence in *Pristidactylus achalensis*. Black: males with sperm, grey: males
445 without sperm. (October n= 3, November n= 9, December n= 8, January n= 15, February n= 9).

446 **Fig. 2** Light microscopy image of the spermatozoa of *Pristidactylus achalensis*. Bar scale: 10 μ m.

447 **Fig. 3** Within- and among-male variation in sperm morphometric and dynamical traits in *Pristidactylus*
448 *achalensis* lizards.







1 **Table 1.** Sperm traits of *Pristidactylus achalensis*. n (number of males), SD (standard deviation), CV
 2 (coefficient of variation), Min (Minimum value), Max (Maximum value), VSL (Straight-line velocity), VCL
 3 (Curvilinear velocity).

Sperm traits	<i>n</i>	Mean	SD	CV	Min	Max
Head length (μm)	11	20.68	0.98	4.75	15.62	26.10
Midpiece length (μm)	11	5.99	0.50	8.34	2.04	8.54
Principal piece length (μm)	11	57.68	1.64	2.85	45.05	63.81
Total sperm length (μm)	11	84.71	2.01	2.37	73.15	94.44
VSL ($\mu\text{m/s}$)	9	27.10	10.76	39.71	7.85	59.01
VCL ($\mu\text{m/s}$)	9	35.84	12.43	34.69	25.30	64.92

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