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SPERM PARAMETERS ASSOCIATED WITH REPRODUCTIVE ECOLOGY IN TWO SNAKE SPECIES

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ABSTRACT: The effect of temperature on sperm dynamic parameters in ectotherms in general, and reptiles in particular, remains poorly understood due to the lack of consistent evidence. As a group, snakes show considerable variability regarding mating systems, male reproductive behavior, thermoregulatory behavior, and preferred temperatures. Additionally, snakes present significant variability in sperm competition levels, which is determined by the species mating system. Because sperm longevity, motility, and velocity are positively related to reproductive success in both competitive and noncompetitive conditions, the sperm physiology of ectothermic organisms may functional optimally at ecologically relevant temperatures. The objective of this work was to analyze the effect of an ecologically plausible range of temperatures on sperm dynamic parameters of two species of snakes with contrasting mating systems and sperm competition levels: *Boa constrictor occidentalis* and *Waglerophis merremii*. To accomplish this, an in vitro incubation approach was used: sperm dynamic parameters (i.e., motility and velocity) were measured on sperm solution aliquots incubated at 25°C, 30°C, and 37°C for up to 10 h by means of a phase contrast video microscopy system. Results suggested that although an increase in temperature has a general negative impact on sperm motility and velocity, the two species studied present different degrees of sensitivity to high incubation temperatures. Moreover, these differences can be explained by the dissimilar thermal conditions that the sperm of the two species would experience during their reproductive seasons, which are a consequence of the differences in their reproductive behavior. In conclusion, sperm motility and swimming velocity respond mainly to environmental conditions imposed by mating systems rather than to selection by sperm competition.

Key words: Snakes; Sperm competition; Sperm dynamic parameters; Temperature effects

SNAKES are ectothermic organisms that occur in a great variety of climates and are exposed to a wide range of temperatures; even species with sympatric distribution show differences in thermoregulation and preferred temperatures (Llewelyn et al., 2005; Luiselli and Akani, 2002; Moore, 1978). To function over a wide range of temperatures, snakes display a diversity of physiological and behavioral adaptations that optimize numerous physiological processes and biochemical reactions at different temperatures (reviewed by Shine et al., 2003*b*).

In addition, snakes display considerable variability in reproductive traits among species (see Zug et al., 2001, for a partial analysis), exhibiting a wide range of mating systems and male reproductive behaviors (Duvall et al., 1992; Rivas and Burghardt, 2005; Shine, 2003). Those behaviors include extremes such as males that traverse long distances searching for spatially unpredictable females (Bertona and Chiaraviglio, 2003; Rivera et al., 2006) and males that participate in explosive mating aggregations with highly spatially predictable females (Shine et al., 2001, 2003*a*). Moreover, snakes present significant variability in sperm competition (competition between sperm of rival males to fertilize a given set of ova), which is associated with male–male and male–female encounter rates determined by the mating system (Tourmente et al., 2009).

Sperm competition has been reported to promote an increase in sperm length in several groups (reviewed in Gomendio and Roldan, 2008). It has been proposed (Gomendio and Roldan, 1991) that a longer flagellum would increase propelling force, thus resulting in faster sperm. Although snake sperm structure has been studied in several works (Al Dohki, 2004; Cunha et al., 2008; Oliver et al., 1996; Tavares-Bastos et al., 2007, 2008), most of them considered it only as a source of phylogenetic information. In contrast, Tourmente et al. (2006, 2008) described the spermatozoa of *Boa constrictor occidentalis*,

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Bothrops alternatus, and *B. diporus;* proposed several ultrastructural traits as adaptations to increase motility or longevity under sperm competition and storage conditions; and related them to male reproductive ecology in these species. More importantly, Tourmente et al. (2009) found that an increase in sperm competition (determined by mating system) was associated with an increase in total sperm length, which was accounted for mainly by an elongation of the midpiece.

Under sperm competition, a higher proportion of motile sperm (higher motility) would improve fertilization success and a higher swimming velocity would increase the probability of reaching the ovum and fertilizing it before rival sperm (Parker, 1998). In this regard, previous studies have found positive relationships between sperm competition and sperm motility (Firman and Simmons, 2010; Møller, 1988) and velocity (Fitzpatrick et al., 2009; Gomendio and Roldan, 2008).

Relatively few studies have focused on snake sperm motility (Fahrig et al., 2007; Mengden et al., 1980; Schulte-Hostedde and Montgomerie, 2006; Zacariotti et al., 2007), and all of them have estimated this parameter in a subjective manner. Only one study (Tourmente et al., 2007) has thoroughly described the basic sperm dynamic parameters (velocity and motility) of a snake species.

It is widely accepted that the regulation of sperm motility and fertilizing ability depends on the interaction of several factors, among which temperature is of vital importance (Ashizawa and Wishart, 1994). Still, the effect of temperature on sperm dynamic parameters in ectotherms in general (Costanzo et al., 1998; Crockett, 1998; Hellriegel and Blanckenhorn, 2002; Iguchi et al., 2007; Johnson and Yund, 2004), and reptiles in particular (Fahrig et al., 2007; Gist et al., 2000), remains poorly understood due to the lack of consistent evidence. To date, the only information available about the effects of temperature on snake sperm physiology comes from studies related to the effects of cold preservation on motility (Fahrig et al., 2007; Mengden et al., 1980), which have been performed at temperatures that are not expected to be experienced by the species in their natural environment.

Nevertheless, it is intuitive to think that, because sperm motility and velocity are the main determinants of male reproductive success both in competitive (Birkhead et al., 1999; Gage et al., 2004) and noncompetitive (Froman et al., 1999; Levitan, 2000; Malo et al., 2005) conditions, sperm physiology in ectothermic organisms may be adapted to function optimally at temperatures that are more frequently experienced in their reproductive seasons (i.e., ecologically relevant conditions) regardless of sperm competition pressures.

This study involved two snake species, *Boa constrictor occidentalis* (Boinae) and *Waglerophis merremii* (Xenodontinae), which share a large portion of their distribution in central Argentina. Although these two species are not close phylogenetically, they represent opposite extremes of male investment in sperm production and morphology, sperm competition levels, and mating systems (Tourmente et al., 2009).

Boa constrictor occidentalis (Philippi, 1873) is a viviparous, large-sized (females over 3 m length in some cases), nonvenomous snake (Bertona and Chiaraviglio, 2003; Cei, 1993). This species has relatively small testes in relation to body size (testes represent 0.47% of body weight) and short spermatozoa, which indicate low levels of sperm competition (Tourmente et al., 2009). The reproduction of *B. c. occidentalis* is marked by the formation of mating groups composed of one female and one to three males (Bertona and Chiaraviglio, 2003; Chiaraviglio et al., 2003). Although these groups appear during the dry season from midautumn to late winter (Bertona and Chiaraviglio, 2003; Chiaraviglio et al., 2003), copulations are presumed to take part in mid- to late winter (Bertona and Chiaraviglio, 2003) because they have only been observed in the lab during this season (M. Tourmente, personal observation). Spermatogenesis has been reported as prenuptial for this species, occurring through the autumn (Ibargüengoytía et al., 2006). Studies of the reproductive ecology of *B. c. occidentalis* have demonstrated that reproductive females alter their thermoregulatory behavior to maintain high and stable body temperatures (around 28°C with a maximum of 33°C; Bertona, 2003;

Chiaraviglio, 2006). Additionally, *B. c. occidentalis* has a prolonged-mate-search mating system in which males travel long distances in high temperatures to search for dispersed females (Bertona and Chiaraviglio, 2003; Cardozo and Chiaraviglio, 2008; Rivera et al., 2006); the males reach a mean active body temperature of 27°C and a maximum of 36°C (Bertona, 2003).

Waglerophis merremii (Wagler, 1824) is an oviparous, midsized (females reaching 1 m length in some cases), nonvenomous snake (Cei, 1993; Giraudo, 2001). Compared to *B. c. oddidentalis*, *W. merremii* has a roughly 10 times higher (4.09%) relative testes size and extremely long spermatozoa, which suggests a mating system with high levels of sperm competition (Tourmente et al., 2009). This species has an annual and associated reproductive cycle, with mating taking place in the spring coincident with peaks in gonadal activity and sexual hormones (Chiaraviglio, 1993). However, it is remarkable that the males of this species have a second peak of testicular and hormonal activity in the winter, during which they are not active (Chiaraviglio, 1993). This pattern suggests the occurrence of spermatogenesis in the winter, coupled with male sperm storage until the mating season. During the mating season, females are available only briefly (because hatchlings must emerge at the same time as juvenile anurans) and have high spatial predictability (in association with water bodies; Chiaraviglio, 1993; Leynaud et al., 2008). In this situation, which is similar to an explosive mating aggregation, the male–female encounter rate would be increased and males would not be forced to travel long distances to find females, and hence could avoid exposure to extreme temperatures. Moreover, there is no evidence indicating that females of this species possess thermoregulatory behaviors that generate high temperatures inside their bodies. Finally, thermoregulatory studies in other reptile species (Grigg et al., 1979; Manning and Grigg, 1997) have demonstrated that frequent immersion in water would produce rapid cooling in animals that are similar in size to *W. merremii*.

In this context, two working hypotheses could be formulated: (1) *B. c. occidentalis* spermatozoa are capable of maintaining their performance during prolonged periods of high temperatures (while males are searching for mates and inside females prior to fertilization), whereas *W. merremii* spermatozoa would instead show maximum performance at cooler temperatures and (2) at optimum temperatures, the species with higher levels of sperm competition would show higher sperm motility and velocity values. The objective of this work was to analyze the effect of an ecologically plausible range of temperature on sperm dynamic parameters of these two species of snakes with contrasting levels of sperm competition (inferred from relative testes mass, sperm length, and predicted mating system), using an in vitro incubation approach.

Materials and Methods

Specimens and Sperm Collection

Waglerophis merremii.—Thirteen sexually mature males were obtained from Córdoba Province (central Argentina) during the mating season of this species, from early November to late December (see Chiaraviglio [1993] for maturity criteria). The animals were euthanized by decapitation following anesthetic overdose with ketamine hydrochloride and xylazine. Semen samples were obtained from the caudal section of the vas deferens. Because snakes store mature sperm in the vas deferens and do not posses sexual accessory glands (Sever, 2004), the extracted semen would be equivalent to ejaculated semen.

Boa constrictor occidentalis.—Thirteen sexually mature males were captured in the district of Loreto (Santiago del Estero Province, Argentina) during the mating season, from late July to early October. The reproductive condition of these animals was determined in situ using ultrasound images (portable ecographer Sonosite 180 Plus, 7.5 Mhz transductor; see Chiaraviglio et al. [1998] for details on the procedure and Bertona and Chiaraviglio [2003] for maturity criteria), after which the animals were moved to the laboratory. Because *B. c. occidentalis* is a threatened species (CITES, 2008), semen samples were extracted from living animals. This has been achieved in other snake species by manual stimulation of the cloacal zone

(Mattson et al., 2007; Mengden et al., 1980; Schulte-Hostedde and Montgomerie, 2006) and electroejaculation (Quinn et al., 1989). However, the high muscular development of these snakes made these procedures unreliable. Consequently, we decided to explore the following surgical procedure: The animals were anesthetized with intramuscular injections of ketamine hydrochloride (40 mg/kg) and xylazine (0.6 mg/kg; Carpenter et al., 2001; Mader, 2005). Subsequently, males were immobilized with adherent hypoallergenic tape and a 5-cm incision was made approximately 10 cm anterior to the cloaca. The semen samples were extracted from the caudal section of one of the deferent tubes. To close the wound, a double suture was performed at muscular and dermal levels. The animals were maintained and observed for at least 15 d and then released into the wild. All animals survived the procedure and none of them exhibited any behavioral or physiological signs associated with pain or distress (IACUC, 2002) during the observation period. For each specimen, a semen sample was obtained that was both contamination-free and of sufficient volume for the experiment. This fact, together with the survival of all the individuals, indicates that this technique is acceptable for regular use in large snakes.

Capture, surgery, maintenance, and release procedures were carried out according to the American Society of Ichthyologists and Herpetologists' guidelines for use of live amphibians and reptiles in field and laboratory research (ASIH, 2004). Euthanasia and anesthesia were carried out according to the Institutional Animal Care and Use Committee (IACUC) guidelines. Specimens were collected with permission of the Agencia Córdoba Ambiente (Córdoba, Argentina), and the Government of the Santiago del Estero Province (Santiago del Estero, Argentina).

For both species, the semen samples were collected in 1.5-mL plastic tubes containing approximately 450 μL of phosphate-buffered saline ×1. Finally, snout–vent length (SVL; cm), body weight (g), testes length and width (mm), and testes weight (TW; g) were measured for each animal. Testes volume (mm^3) was calculated using the equation for the volume of an ellipsoid (Méndez and Villagrán, 1998). For *B. c. occidentalis*, testes weight was calculated using a linear regression of testes mass against testes volume (Tourmente et al., 2009).

Influence of Incubation Temperature on Sperm Dynamic Parameters

For both species, sperm concentration was estimated using a Neubauer chamber and the samples were diluted to a concentration of 1×10^6 cells/mL in Biggers, Whitten, and Wittingham culture medium (Biggers et al., 1971) supplemented with 4% bovine serum albumin. After dilution, 100-μL aliquots of these samples were fixed for photography with a solution of 2% formaldehyde in water. The samples were examined at ×400 magnification using phase-contrast microscopy. Microphotographs of the samples were taken using a Sony DSCW50 digital camera with a ×6 zoom. The lengths (μm) of sperm head, midpiece, and principal piece, and total sperm length were measured for a minimum of 20 spermatozoa per sample using software ImageJ v. 1.38 (U.S. National Institutes of Health). Mean trait values for each species were calculated from the means from each individual of that species.

In order to determine the effects of incubation temperature on sperm traits, aliquots (5 mL) of the diluted sperm samples were transferred to 15-mL plastic tubes and incubated at 25°C, 30°C, and 37°C in thermally stable water baths. The tubes were partially open to allow airflow. Total time elapsed between semen collection and beginning of incubation (0 h) was never greater than 10 min. All semen extraction and dilution procedures were performed at room temperature (25°C).

The selection of ecologically relevant incubation temperatures (25°C, 30°C, and 37°C) was based on two factors. (1) Previous studies on the thermal biology of *B. c. occidentalis* (Bertona, 2003; Chiaraviglio, 2006; Chiaraviglio and Bertona, 2007) indicated that males of this species have mean body temperatures near 27°C when they are moving, but are capable of reaching maximum body temperatures of 36°C in the mating season. (2)

Because data on thermal biology of *W. merremii* are not available, we performed a bibliographic search for field studies of thermoregulation in snakes of the families Viperidae, Colubridae, Pythonidae, and Elapidae (Beck, 1996; Blouin-Demers and Weatherhead, 2001; Brown and Weatherhead, 2000; Isaac and Gregory, 2004; Llewelyn et al., 2005; Luiselli and Akani, 2002; Moore, 1978; Peterson, 1987; Rosen, 1991; Shine et al., 2003*b*; Slip and Shine, 1988; Withaker and Shine, 2002). These studies included a wide range of body sizes, oviparous and viviparous species, and species that live in temperate and tropical habitats. We only excluded three species because they were reported to be mainly nocturnal (Llewelyn et al., 2005). The mean values of field body temperatures provided by these studies ranged from approximately 25°C in the diamond python (*Morelia spilota*; Slip and Shine, 1988) to 32.4°C in male *Natriciteres fuliginoides* (Luiselli and Akani, 2002). A review by Peterson et al. (1993) suggested that, in most cases, temperatures of active snakes have a modal value in the range of 28–32°C.

At 0, 2, 4, 6, 8, and 10 h of incubation, a 20-μL aliquot of sperm suspension was taken for each incubation temperature and placed in a plastic observation chamber and covered with a coverslip. Measurement of dynamic parameters was carried out at room temperature (25°C) using a video microscopy system composed of a phase contrast microscope (Zeiss) equipped with a video camera (Panasonic CCTV WVBL90) and a digital capture card (Pinnacle Studio 500 PCI). The software used to capture the digital videos was Virtualdub v.1.6.16 (Avery Lee). The samples were recorded at ×100 magnification for 5 min, with a random change of the microscope field every 5 s. Subsequently, individual sperm tracks were followed for 3 s in 50 cells/sample and transformed to a matrix of Cartesian coordinates using ImageJ v.1.38 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 Córdoba, Argentina): percentage of cells with progressive motility (MOT), linear velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), and linearity (LIN; LIN = VSL/VCL).

Data Analysis

The data were analyzed with a two-factor repeated-measures analysis of variance for each species, using incubation temperature and time as factors (with three and six levels, respectively). Differences between conditions were analyzed using a post-hoc multiple-comparisons test using the Bonferroni correction for α. To satisfy assumptions of normality, the variable MOT was transformed to the arcsine of the square root of the proportion of motile sperm. The statistical analyses were performed using SPSS Statistics (SPSS v.17.0.0; SPSS, IBM Corporation, Somers, New York, USA) with a significance level $\alpha = 0.05$.

Results

Body (SVL, body mass, gonadosomatic index, testes mass, and testes volume) and sperm dimensions (head length, midpiece length, principal piece length, and total sperm length) for both species are listed in Table 1. Micrographs of sperm from both species are shown in Fig. 1. Although sperm cells of both species were similar in morphology to the model described by Oliver et al. (1996), they show notable variation in component dimensions (Table 1).

Influence of Incubation Temperature on Sperm Dynamic Parameters

For both species, it was impossible to take representative velocity measurements at 10 h of incubation as a consequence of the low percentage of motile cells. Due to the same problem, no velocity measurements were taken from aliquots of *W. merremii* incubated at 37°C beyond 2 h.

Waglerophis merremii.—The MOT decreased significantly with increasing incubation time for the three temperatures (at 4 and 8 h for 25°C, at 2 and 6 h for 30°C, and at 2 and 8 h for 37°C). However, MOT showed an earlier and more pronounced decrease at higher incubation temperatures (Fig. 2a). At earlier incubation times (2 and 4 h), there were significant differences between MOT values at the three temperatures ($MOT_{25°C} >$

TABLE 1.—Body and sperm dimensions of *Boa constrictor occidentalis* and *Waglerophis merremii*. Data are expressed as ± SE. SVL: snout–vent length; GSI: gonadosomatic index = (testes mass/body mass) × 100. *Waglerophis merremii* n = 13 and *Boa constrictor occidentalis* n = 13.

	Variable	*B. c. occidentalis*	*W. merremii*
Corporal dimensions	Body mass (g)	4161.5 ± 939.9	84.7 ± 26.8
	SVL (cm)	179.8 ± 13.6	58.9 ± 6.1
	Testes volume (cm^3)	29.9 ± 15.4	4.1 ± 2.3
	Testes mass (g)	20.9 ± 10.8	2.6 ± 1.6
	GSI	0.5 ± 0.2	2.9 ± 1.5
Sperm dimensions	Head length (μm)	11.4 ± 0.3	11.3 ± 0.6
	Midpiece length (μm)	35.7 ± 0.7	111.1 ± 2.6
	Principal piece length (μm)	43.5 ± 1.2	35.7 ± 1.6
	Total sperm length (μm)	90.6 ± 1.1	159.3 ± 3

$MOT_{30°C} > MOT_{37°C}$). At later times (6, 8, and 10 h), the pattern was $MOT_{25°C} > MOT_{30°C} \approx MOT_{37°C}$ (Fig. 2a). VCL showed a significant increase at 25°C and 30°C (at 2 and 4 h, respectively) and a decrease at 37°C (at 2 h; Fig. 2b). VSL presented a similar pattern: it increased at 2 h for the aliquots incubated at 25°C and decreased at the same time for those at 37°C (Fig. 2c). The aliquots incubated at 25°C showed higher values of VCL and VSL than those incubated at 30°C and 37°C at all times (Fig. 2b,c), with the exception of VSL at 2 h (for which values were similar for the aliquots incubated at 25°C and 30°C). The interaction term between incubation temperature and time was significant in MOT, VCL, and VSL (Fig. 2). LIN did not show a significant effect of incubation temperature, only registering a slight increase at 8 h for the samples at 25°C and 30°C (Fig. 2d).

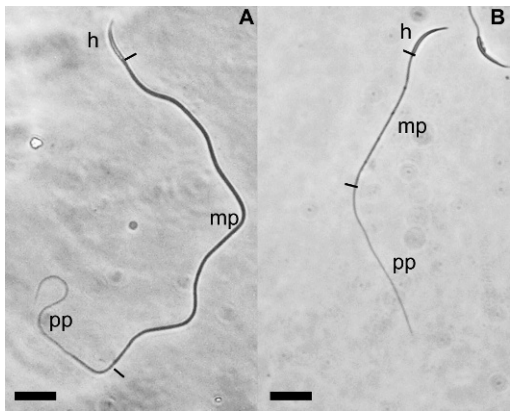

FIG. 1.—Phase contrast micrographs of the sperm of two snake species. (a) *Waglerophis merremii*. (b) *Boa constrictor occidentalis*. h: Head; mp: mipdiece; pp: principal piece. Lines indicate the beginning and end of the midpiece. Scale bar: 10 μm.

Boa constrictor occidentalis.—The MOT showed a low value (10.2%) at 0 h, increasing significantly at 2 h in all incubation temperatures. Subsequently to this initial increase, MOT decreased significantly at the three incubation temperatures (at 6 h for 25°C and 30°C, and at 4 and 6 h for 37°C; Fig. 3a). Additionally, the aliquot incubated at 37°C presented significantly lower MOT values than the ones incubated at 25°C and 30°C at all times after 2 h (Fig. 3a). VCL and VSL showed an early increase for the samples incubated at 25°C and 30°C (at 2 and 4 h, respectively). Later in time there was a decrease for the 30°C and 37°C aliquots (at 8 h for VCL and at 6 and 8 h, respectively, for VSL; Fig. 3b,c). In the case of VCL, the values presented by the aliquots incubated at 25°C and 30°C were similar and higher than those from the samples incubated at 37°C at 2, 4, and 6 h (Fig. 3b). From 4 to 8 h, VSL values for the samples incubated at 25°C and 30°C were also higher than those incubated at 37°C (Fig. 3c). The interaction term between incubation temperature and time was significant in MOT, VCL, and VSL (Fig. 3). LIN did not show a significant incubation temperature effect, registering only a significant decrease at 6 h for the 37°C samples (Fig 3d).

DISCUSSION

The results of this study suggest that, although snake spermatozoa are able to maintain their motility and velocity to a certain

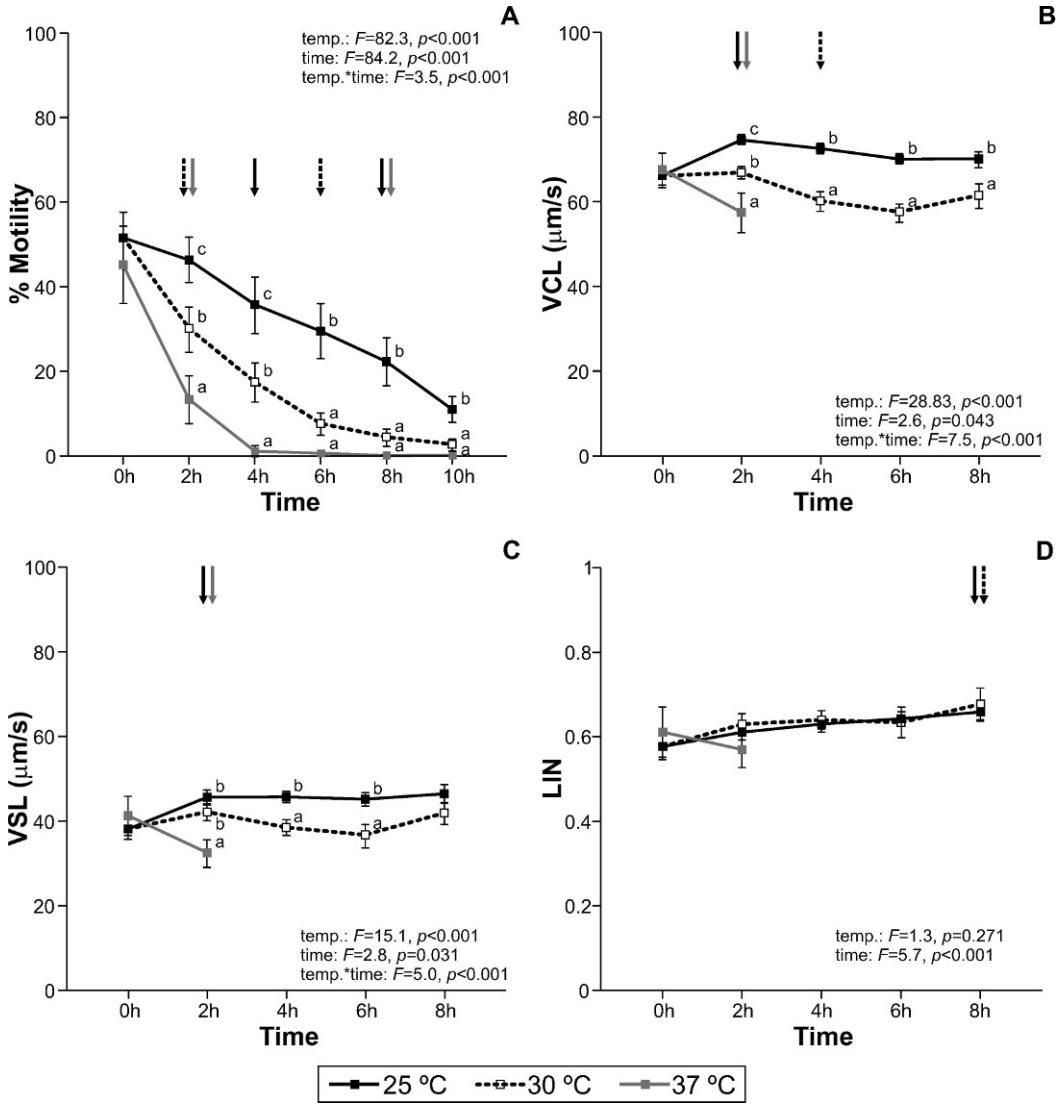

FIG. 2.—Effects of temperature and time of incubation on the sperm dynamic parameters of *Waglerophis merremii*. (a) Percentage of motile cells (% motility). (b) Curvilinear velocity (VCL, μm/s). (c) Linear velocity (VSL, μm/s). (d) Linearity (LIN = VSL/VCL). Values represented are ± SE. Different letters indicate significant differences among temperatures at the same time. Vertical arrows indicate significant differences relative to the previous time of incubation at the same temperature. Values of *F* and *P* were taken from a two-factor repeated-measures analysis of variance (α = 0.05). The significance of the differences was tested using a post-hoc multiple-comparisons test with Bonferroni correction. Full black line: 25°C; dashed black line: 30°C; full grey line: 37°C.

degree when exposed to a wide range of ecologically realistic temperatures, an increase in incubation temperature has a general negative impact on their values. Furthermore, because the effects of temperature depended on the length of incubation in both species, higher incubation temperatures not only affected the absolute values of sperm dynamic parameters, but would also change the temporal curve of these parameters, accelerating the time-related decrease in their values.

The deleterious effects of high incubation temperatures could be due to an increase in sperm metabolism and other cellular bio-

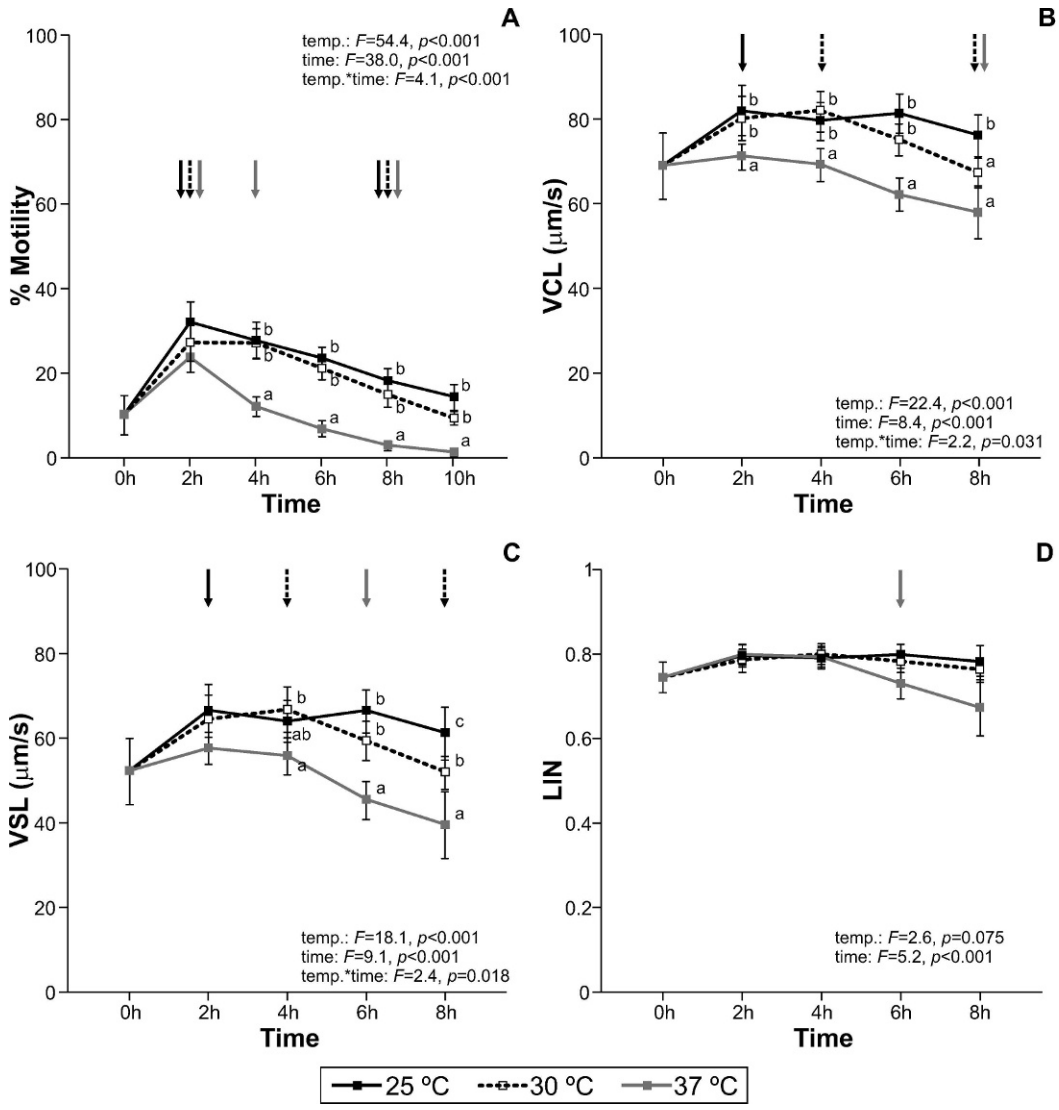

FIG. 3.—Effects of temperature and time of incubation on the sperm dynamic parameters of *Boa constrictor occidentalis*. (a) Percentage of motile cells (% motility). (b) Curvilinear velocity (VCL, µm/s). (c) Linear velocity (VSL, µm/s). (d) Linearity (LIN = VSL/VCL). Values represented are ± SE. Different letters indicate significant differences among temperatures at the same time. Vertical arrows indicate significant differences relative to the previous time of incubation at the same temperature. Values of *F* and *P* were taken from a two-factor repeated-measures analysis of variance ($\alpha = 0.05$). The significance of the differences was tested using a post-hoc multiple-comparisons test with Bonferroni correction. Full black line: 25°C; dashed black line: 30°C; full grey line: 37°C.

chemical processes associated with sperm motility, which would increase energy consumption and result in a general acceleration of sperm senescence (Auger et al., 1989; Kime et al., 1996; Makler et al., 1981).

In certain teleost species, higher temperatures have been shown to increase the beat frequency of sperm flagella (Billard and Cosson, 1992) but decrease the duration of sperm motility (Billard and Cosson, 1992; Mansour et al., 2002; Van Look, 2001; Williot et al., 2000) and viability (Mansour et al., 2002). In this regard, the results of the present study appear to be contradictory, because the

decrease in sperm motility at higher incubation temperatures was not associated with an increase in sperm velocity. In addition, studies in ascidian species have shown that an increase in sperm incubation temperature results in a decrease in sperm fertilizing ability by diminishing sperm longevity (Johnson and Yund, 2004).

Studies in turtles have suggested that sperm dynamic parameters (percentage of motile cells and velocity) increase when sperm are maintained at low temperature (Gist et al., 2000). This pattern contrasts with the idea of an acceleration of sperm metabolism, which has been suggested to promote an increase in sperm velocity, at least in endothermic organisms (Auger et al., 1989; Hammerstedt and Hay, 1980).

Additionally, high temperatures may cause negative effects on sperm motility due to enzyme denaturation (Mahi and Yanagimachi, 1973) or alteration of sperm membrane order, fluidity, permeability, and thickness (Crockett, 1998; Müller et al., 2008). However, sperm physiology in reptiles is insufficiently understood and, to our knowledge, there is not enough evidence in this field to draw a consistent conclusion about the effects of high temperatures on sperm function.

In the present study, we also show for the first time that the sperm dynamic parameters of the two studied snake species show different degrees of sensitivity to high incubation temperatures. *Waglerophis merremii* spermatozoa register higher motility and velocity values at 25°C than those registered at 30°C. Moreover, incubation at 37°C is highly deleterious to motility, precluding accurate velocity measurements. Conversely, the motility and velocity of *B. c. occidentalis* spermatozoa show a less pronounced effect of incubation temperature: the values of these parameters are similar at 25°C and 30°C, only decreasing at 37°C. Moreover, even at 37°C, the percentage of motile sperm is high enough to take enough velocity measures to assure a representative sample.

From an adaptive standpoint, any organism should ensure the maximum fertilization probabilities during its reproductive time window. Because environmental temperature has a great influence on body temperature in most ectotherms, it seems logical that differences in environmental temperature experienced during the mating season may result in animals having different body temperatures when mating. Thus, it would be intuitive to expect sperm dynamic parameters of different species to have favorable variations when subjected to thermal conditions that are frequently experienced by these snakes in their reproductive seasons. Coincidently, evidence in amphibians indicates that the spermatozoa of the species that undergo regular winter freezing are able to maintain higher fertilizing capabilities after cryopreservation periods (Costanzo et al., 1998). In addition, some turtle species that mate in winter show an increase in sperm speed and motility percentage when incubated at low temperatures (Gist et al., 2000).

If we take in account that *B. c. occidentalis* copulates during the winter (Bertona and Chiaraviglio, 2003), whereas *W. merremii* copulations occur during the spring (Chiaraviglio, 1993), the results appear to be contradictory: the species that reproduces when temperatures are colder shows a higher tolerance to high incubation temperatures than the species in which the mating season occurs at warmer temperatures. However, the results support our hypothesis that the differences between these two species in sperm sensitivity to high incubation temperature may be explained in terms of their differences in reproductive mode and mating systems features. These features could alter the environmental conditions experienced by the individuals of these species, consequently modifying environmental pressures on sperm dynamics.

As a product of their prolonged mate-search activities, males of *B. c. occidentalis* would be subjected to long periods of exposure to high temperatures, which would result in high body temperatures (Bertona, 2003; Rivera et al., 2006). Moreover, females of this species are able to maintain high and stable body temperatures during mating season (Bertona and Chiaraviglio, 2003; Chiaraviglio, 2006). Thus, because sperm inside these snakes would frequently be exposed to relatively high temperatures (both inside the males and the females), selective forces should favor the maintenance of sperm performance under such conditions.

There is no accurate information on the thermal biology of *W. merremii*. Nevertheless, due to the increased male–female encounter rates characteristic of the inferred mating system for this species (Chiaraviglio, 1993; Leynaud et al., 2008; Tourmente et al., 2009), male *W. merremi* would not be forced to engage in prolonged mate searching. Furthermore, the frequent exposure to water would lower the average body temperature (Grigg et al., 1979; Manning and Grigg, 1997).

According to theoretical predictions (Parker, 1998) and previous evidence from other taxonomic groups (Firman and Simmons, 2010; Møller, 1988), we should expect that species with higher sperm competition exhibit higher sperm motility. Nonetheless, our results in this regard are not conclusive: although at 25°C *W. merremii* (higher sperm competition) appears to have a higher percentage of motile sperm during shorter incubation periods, this pattern disappears with time. Moreover, the extremely deleterious effects of increasing incubation temperatures on *W. merremii* sperm motility render the comparison moot at 30°C and 37°C.

One last remarkable point of this comparison is the fact that *B. c. occidentalis*, the species with the shorter sperm and lower sperm competition level (according to relative testes size), also attained higher straight-line velocity by sperm. It has been proposed that sperm competition should select for longer sperm flagella, because they would yield higher propelling thrust and hence result in faster sperm (Gomendio and Roldan, 1991). This hypothesis has been supported by recent studies in mammals (Gomendio and Roldan, 2008), birds (Lüpold et al., 2009), and fish (Fitzpatrick et al., 2009). However, the results of the present work seem in conflict with this hypothesis. A possible explanation to the observed results is that larger sperm could maintain relatively stable speeds for a longer period of time than shorter ones, without necessarily achieving higher speeds. If we reduce the former analysis to the last 4 h of incubation (4, 6, and 8 h), then the effect of time on both velocity measures ceases to be significant for *W. merremii* but remains significant for *B. c. occidentalis*. In this regard, theoretical models (Parker, 1998) and empirical evidence (Levitan, 2000) for external fertilizers have pointed to the existence of an energetic conflict between speed and duration of sperm motility. Furthermore, Parker (1998) proposed that larger sperm could increase ejaculate competitiveness by increasing the duration of sperm motility when the risk of sperm competition increases as a function of the time between copulation and fertilization.

In conclusion, the results of the present study suggest that, in *W. merremii* and *B. c. occidentalis*, sperm motility and swimming velocity respond mainly to environmental conditions imposed by mating systems rather than to selection by sperm competition. However, it is important to note that in order to understand the different relationships among snake sperm dynamics, life history, and mating system features, it is necessary to increase the number of species studied, with focus on comparative studies that take in account the potential for sperm competition and the predominant conditions of species mating systems.

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