



A comparison of five Korean snake species' reproductive organ sizes, *Oocatochus rufodorsatus* and *Rhabdophis tigrinus* in Colubridae and *Gloydus saxatilis*, *G. brevicaudus* and *G. ussuriensis* in Viperidae

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Abstract

Characteristics of snake reproductive organs that are relatively less affected by external environmental conditions can be used as an important means of classification; additionally, such characteristics can provide useful information on a species' reproductive system. In this study, we compared the testis weights, hemipenis lengths and retractor muscle lengths of male *Oocatochus rufodorsatus* and *Rhabdophis tigrinus* in Colubridae and *Gloydus saxatilis*, *G. brevicaudus* and *G. ussuriensis* in Viperidae. The snake snout-vent lengths (SVLs) were positively related to the three reproductive organ sizes, but the body weight only exhibited a positive relationship with the testis weight. The three organs did not significantly differ on the left and right sides. The relative testis weights and retractor muscle lengths (divided by the body weight and SVL, respectively) of the Colubridae snakes were greater than for the Viperidae snakes, but the relative hemipenis lengths (divided by SVL) did not differ between the two groups. The relative testis weight of *G. saxatilis* and the relative retractor muscle lengths of the Viperidae snakes were smaller compared with the Colubridae snakes. The relative hemipenis length of *O. rufodorsatus* was greater than for *R. tigrinus*, *G. saxatilis* and *G. brevicaudus*. Additional comparisons were not significant. Our results may facilitate further studies on hemipenial morphology and mating competition in Korean snakes. This is the first study on Korean snake reproductive organs.

Key words: Colubridae, mating system, reproductive organ, sexual selection, sperm competition, Viperidae

INTRODUCTION

The external morphological characteristics of a species may be largely shaped by natural selection in response to environmental factors. In contrast, internal organ morphology is affected by more specific factors, such as sexual selection or developmental constraints (Olsson and Madsen 1998, Hosken and Stockley 2004). Snake reproductive

organs are internal organs that do not typically exhibit intraspecific variation (Dowling and Savage 1960, Arnold 1986). Therefore, gonad size and morphological characteristics of the hemipenis have been used as important means of classification and to study reproductive biology in reptiles (Dowling and Savage 1960, Arnold 1986, Das

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and Purkayastha 2012).

Recently, several studies have shown that snake reproductive organ characteristics are closely related to their mating system and degree of mating competition (Olsson and Madsen 1998, Todd 2008, Friesen et al. 2013). A male snake's testis size directly affects the quantity of ejaculated sperm (Møller 1989), and hemipenis size is related to the quantity of sperm transported per unit of time (Eberhard et al. 2009, King et al. 2009). Transferring large quantities of sperm and related secretions, which might also be used as mating plug materials, into female cloaca should increase the successful insemination rate and may be beneficial for competing with other males' sperm in fertilizing eggs (Arnold and Duvall 1994, Olsson and Madsen 1998). Thus, understanding the mating system of a species through studying reproductive organs may provide basic information on the life history of a species and be useful for conserving a species in the field.

In Korea, 8 snake species in Colubridae, 3 in Viperidae and 3 in Elapidae have been identified (Kang and Yoon 1975). Recently, multiple studies have investigated Korean snakes, through verifying a specific species in the Korean peninsula (Lee et al. 2013); determining food, habitat use and home range (Kim et al. 2012b, Lee et al. 2012, Do and Yoo 2014, Kim and Oh 2014); and describing mating patterns, including courtship behaviors (Kim et al. 2012a). Despite such progress, the basic morphological and anatomical characteristics of Korean snakes remain poorly reported. Specifically, Korean snake reproductive organ sizes have not been compared.

In this study, we compared the testis weights, hemipenis lengths and retractor muscle lengths for five Korean snake species, *Oocatochus rufodorsatus* and *Rhabdophis tigrinus* in Colubridae and *Gloydus saxatilis*, *G. brevicaudus* and *G. ussuriensis* in Viperidae. Our results may facilitate further studies on the hemipenial morphology and mating systems of Korean snakes.

MATERIALS AND METHODS

Measuring physical characteristics and reproductive organs

The experimental procedure described in this report was conducted in accordance with the guidelines established by the Kangwon National University Institutional Animal Care and Use Committee (KW-150225-1). We used 30 adult male snakes, which consisted of 4 *O. rufodorsatus* and 6 *R. tigrinus* in Colubridae and 5 *G. saxatilis*, 8 *G.*

brevicaudus and 7 *G. ussuriensis* in Viperidae, after euthanizing the snakes through ether exposure. The snakes were illegally captured by poachers, confiscated by Han River Water Shed Environmental Management Office and provided for herpetological studies. To use only adult male snakes in the study, we selected male snakes with sizes greater than 80% of the known average adult size for each species (Kang and Yoon 1975).

For the physical characteristics, we measured the snout-vent length (SVL, the shortest distance from the tip of the snout to the middle of the cloaca) of each snake up to 0.1 cm using a tape ruler and the body weight up to 0.1 g using an electrical balance (TMB 120-1; Kern-Korea, Seoul, Korea). Based on previous studies (Todd 2008, Porto et al. 2013), we selected three parameters, testis weight, hemipenis and retractor muscle length, to compare reproductive organs of Korean male snakes. To measure the testis weight, we first ventrally dissected a snake, removed both the left and right testis and measured its weight up to 0.001 g using a digital balance (AR 2140; Ohaus Corp, Parsippany, NJ, USA). The longitudinal lengths of both the hemipenis and retractor muscle length from both the left and right sides were measured up to 0.01 mm using a digital Vernier caliper after the tail was dissected (CD-15CPX; Mitutoyo, Kawasaki, Japan).

Statistical analysis

For each snake, we calculated the average testis weight, hemipenis length and retractor muscle length using both the left and right side organ values. To statistically compare the Colubridae and Viperidae snakes and the five species following previous studies (Olsson and Madsen 1998, Todd 2008, Porto et al. 2013), the relative testis weight (calculated as testis weight / body weight \times 100), relative hemipenis length (calculated as hemipenis length / SVL \times 100) and relative retractor muscle length (calculated as retractor muscle length / SVL \times 100) for each snake were determined.

Because the SVL, body weight, hemipenis length, retractor muscle length and log-transformed testis weight showed a normal distribution (Kolmogorov-Smirnov, $P > 0.05$ for all cases), the relationships among the physical characteristics and reproductive organs were analyzed using the Pearson method, and the difference between the left and right side reproductive organs was analyzed using a paired t-test.

Additionally, the relative testis weight, relative hemipenis length and log-transformed relative retractor muscle length showed a normal distribution (Kolmogorov-

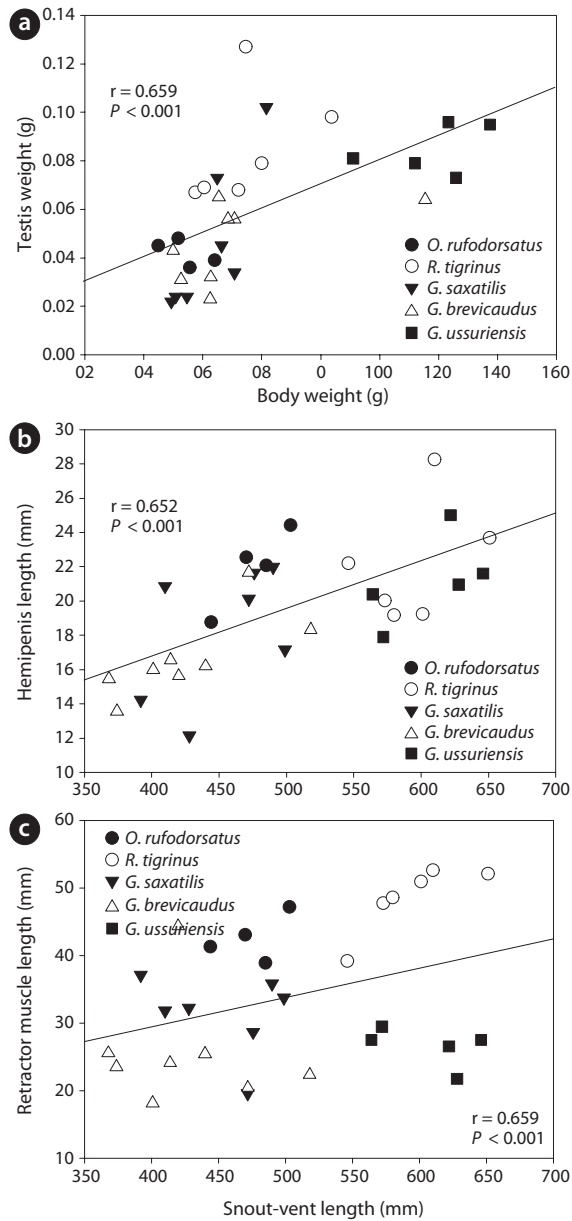


Fig. 1. The relationship between the physical characteristics and size of the five Korean snakes' reproductive organs ($N = 30$); (a) between the body weight and testis weight, (b) between the snout-vent length and hemipenis length, (c) between the snout-vent length and retractor muscle length.

Smirnov, $P > 0.05$ for all cases). The parameter differences between the Colubridae and Viperidae snakes and among five species were determined using a general linear model (GLM) in SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA), which was followed by a Tukey's *post-hoc* test if the results were significant. For a small sample size, the interactions between the groups and species were not considered. The data in the text are presented as the mean ± 1 standard error.

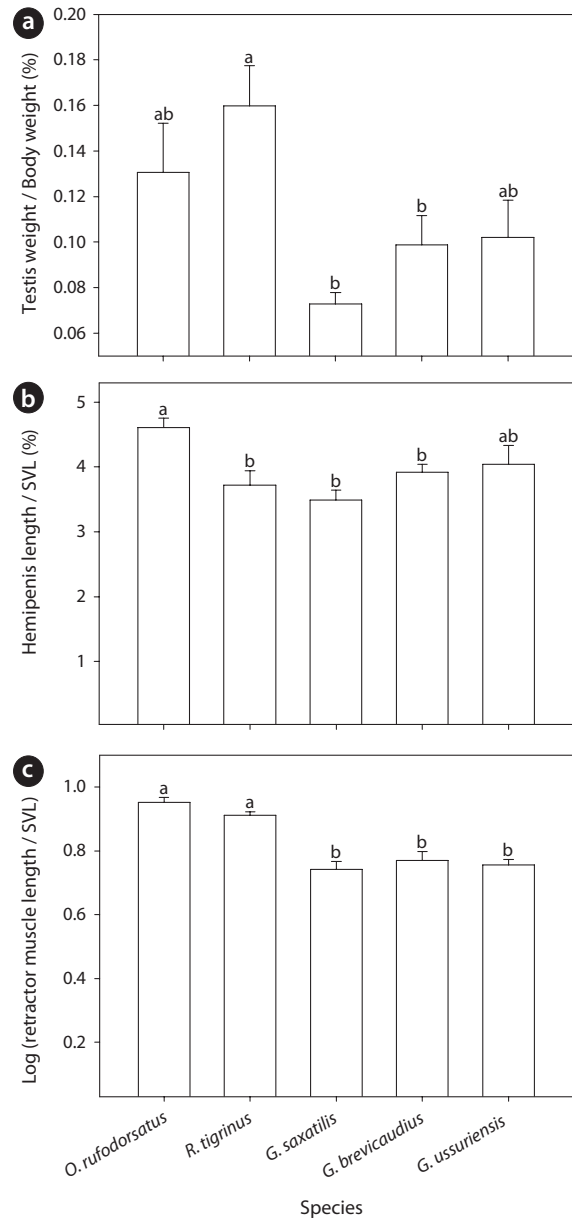


Fig. 2. Comparisons for the five Korean snakes' relative testis weights (a), relative hemipenis lengths (b) and relative retractor muscle lengths (c) ($N = 30$), *Oocatochus rufodorsatus* and *Rhabdophis tigrinus* in Colubridae and *Gloydius saxatilis*, *G. breviceaudus* and *G. ussuriensis* in Viperidae. The different characters on the bars represent a significant difference at $P < 0.05$.

RESULTS

A summary of the physical and reproductive organ characteristics is presented in Table 1, and the correlation test results are in Table 2. The body weight only positively correlated with testis weight ($P < 0.001$, Table 2 and Fig. 1a), while the SVL positively correlated with testis weight, hemipenis length and retractor muscle length ($P < 0.001$ for all cases, Table 2 and Fig. 1b and 1c). The three

reproductive organs exhibited significant positive correlations ($P < 0.036$, Table 2). We did not detect significant differences between the left and right testis weights ($P = 0.147$), hemipenis lengths ($P = 0.759$) and retractor muscle lengths ($P = 0.665$).

The relative testis weight of the Colubridae snakes (0.148 ± 0.014 , $N = 10$) was significantly greater than for the Viperidae snakes (0.094 ± 0.008 , $N = 20$, $F_{1,29} = 13.00$, $P = 0.001$). The difference in relative testis weights among the five species was also significant ($F_{4,29} = 4.34$, $P = 0.008$). Specifically, the relative testis weights of *O. rufodorsatus* and *R. tigrinus* were greater than for *G. saxatilis* and the three viper species, respectively ($P < 0.05$ for all cases, Fig. 2a). Other comparisons were not significant ($P > 0.05$ for all cases).

The relative hemipenis length of the Colubridae snakes (4.08 ± 0.20 , $N = 10$) did not differ from the Viperidae

snakes (3.85 ± 0.12 , $N = 20$), but the relative hemipenis length difference among the five species was significant ($F_{4,29} = 2.98$, $P = 0.038$). Specifically, the relative hemipenis length of *O. rufodorsatus* was greater than for *R. tigrinus*, *G. saxatilis* and *G. brevicaudus* ($P < 0.05$ for all cases, Fig. 2b). Other comparisons were not significant ($P > 0.05$ for all cases).

The relative retractor muscle lengths of the Colubridae snakes (0.148 ± 0.014 , $N = 10$) were significantly greater than for the Viperidae snakes (0.094 ± 0.008 , $N = 20$, $F_{1,29} = 13.00$, $P = 0.001$). The relative retractor muscle length difference among the five species was also significant ($F_{4,29} = 4.34$, $P = 0.008$). Specifically, the relative muscle lengths of *O. rufodorsatus* and *R. tigrinus* were greater than *G. saxatilis* and the three viper species, respectively ($P < 0.05$ for all cases, Fig. 2c). Other comparisons were not significant ($P > 0.05$ for all cases).

Table 1. Physical characteristics and size of the five Korean snakes' reproductive organs ($N = 30$), *Oocatochus rufodorsatus* and *Rhabdophis tigrinus* in Colubridae and *Gloydus saxatilis*, *G. brevicaudus* and *G. ussuriensis* in Viperidae

Species Parameters	<i>Oocatochus rufodorsatus</i> ($N = 4$)	<i>Rhabdophis tigrinus</i> ($N = 6$)	<i>Gloydus saxatilis</i> ($N = 5$)	<i>Gloydus brevicaudus</i> ($N = 8$)	<i>Gloydus ussuriensis</i> ($N = 7$)
Snout-vent length (SVL) (mm)	475.5 ± 12.5 444-503	593.5 ± 14.7 546-651	606.4 ± 16.2 564-646	425.9 ± 17.8 368-518	452.4 ± 15.9 392-499
Body weight (g)	34.0 ± 4.0 24.9-44	54.7 ± 6.8 37.4-83.7	117.9 ± 7.9 91.0-137.4	51.0 ± 9.6 29.9-115.4	42.6 ± 4.4 29.3-61.6
Testis (g)	0.042 ± 0.003 0.036-0.048	0.084 ± 0.010 0.067-0.127	0.085 ± 0.005 0.073-0.096	0.046 ± 0.006 0.023-0.065	0.046 ± 0.011 0.022-0.102
Testis/BW (%)	0.13 ± 0.02 0.09-0.18	0.16 ± 0.18 0.12-0.23	0.07 ± 0.01 0.06-0.09	0.10 ± 0.01 0.05-0.14	0.10 ± 0.02 0.07-0.17
Hemipenis (mm)	22.0 ± 1.2 18.8-24.4	22.1 ± 1.4 19.2-28.3	21.2 ± 1.1 17.9-25.0	16.7 ± 0.9 13.6-21.7	18.3 ± 1.5 12.1-22.0
Hemipenis /SVL (%)	4.61 ± 0.14 4.23-4.85	3.72 ± 0.22 3.20-4.63	3.49 ± 0.15 3.13-4.02	3.92 ± 0.12 3.54-4.59	4.04 ± 0.29 2.84-5.09
Retractor muscle (mm)	42.61 ± 1.74 38.91-47.18	48.53 ± 2.03 39.19-52.63	33.52 ± 1.47 28.88-37.08	25.92 ± 3.03 18.15-44.32	25.82 ± 1.07 21.74-29.49
Retractor muscle /SVL (%)	8.97 ± 0.32 8.02-9.38	8.17 ± 0.21 7.18-8.63	5.55 ± 0.32 4.61-6.48	5.98 ± 0.43 4.93-8.56	5.73 ± 0.23 4.70-6.71

The data are presented as the mean ± SE and the range.

Table 2. The relationships between the physical characteristics and size of the five Korean snakes' reproductive organs ($N = 30$), *Oocatochus rufodorsatus* and *Rhabdophis tigrinus* in Colubridae and *Gloydus saxatilis*, *G. brevicaudus* and *G. ussuriensis* in Viperidae

	Snout-vent length (mm)	Body weight (g)	Testis weight (g)	Hemipenis length (mm)
Testis weight (g)	$r = 0.779$ $P < 0.001$	$r = 0.659$ $P < 0.001$		
Hemipenis length (mm)	$r = 0.652$ $P < 0.001$	$r = 0.355$ $P = 0.054$	$r = 0.384$ $P = 0.036$	
Retractor muscle length (mm)	$r = 0.695$ $P < 0.001$	$r = 0.246$ $P = 0.189$	$r = 0.478$ $P = 0.008$	$R = 0.594$ $P = 0.001$

DISCUSSION

The snake reproductive organ sizes are closely related to the physical characteristics. We show that the testis weight was related to both the body weight and SVL, which suggests that condition factor (body weight/SVL) may affect testis weight. This result is consistent with previous findings that testis size depends on plasma testosterone levels and condition factor (Licht 1984). In contrast, the hemipenis and retractor muscle lengths were related to the SVL, but not the body weight, which suggests that the two parameters might directly depend on physical characteristics. Thus, these two parameters might be more useful for comparing differences between different species or genera.

In this study, the left and right sides of the reproductive organs did not differ significantly. Although several studies have shown that snake species also exhibit lateralization in reproductive organs and courtship behaviors (Wallach 1991, Shine et al. 2000), it is generally accepted that most snake species do not show such lateralization (Crews 1978, Tokarz and Slowinski 1990, Zweifel 1997). Our results show that the five Korean species used in this study do not exhibit lateralization in reproductive organs.

The Colubridae snake species may produce higher quantities of sperm than snakes in Viperidae. In snakes, the plasma testosterone level determines the testis weight (Licht 1984, Moore and Lindzey 1992), which directly affects the quantity of sperm produced (Licht 1984). Transferring high quantities of sperm should yield a long copulation period (Olsson and Madsen 1998, Eberhard et al. 2009, King et al. 2009). Subsequently, such activity may increase mating monopoly of a female, which may decrease additional mating with other males (Duvall et al. 1992, Arnold and Duvall 1994). In this study, the relative testis weight of the Colubridae snakes was greater than the Viperidae snakes, which indicates that the Colubridae snakes produce higher quantities of sperm. This might be caused by their large body size, greater activity and use of large habitats, which may facilitate a greater investment in reproduction. In addition, although the difference was not statistically significant, the testis weights differed among the three viper species. Specifically, *G. saxatilis* exhibited the lowest testis weight. Considering that the *G. saxatilis* population density was the lowest among the three viper species in the field (Lee et al. 2009, Kim et al. 2011), *G. saxatilis* may exhibit such a low testis weight because it might incur less mating competition. On the other hand, the viper reproductive system may be dissociative (mating occurs in autumn, but fertilization oc-

curs in spring), but the Colubridae snakes' reproductive system may be associative (both mating and fertilization occurs in spring) (Moore and Lindzey 1992). Thus, the different mating systems may involve the different testis weights among the Colubridae and Viperidae snakes observed in this study. Thus, the sex hormones and testis size should be measured throughout a year in both snake groups. Based on our current results, the snakes in Colubridae exhibit heavier testes and might produce large quantities of sperm, which likely reflects the associative mating system.

The relative hemipenis length differences between Colubridae and Viperidae snakes remain unclear. The Squamata genitalia, the hemipenis, consists of two cylindrical sacs, and during copulation, sperm is transported into the female cloaca by following the ducts on the hemipenis (Dowling and Savage 1960). The hemipenis lies in the tail when not in use, but during copulation, it turns inside out and inserts into female cloaca (Porto et al. 2013). In general, the hemipenis size is believed to reflect the sperm transportation ability per unit of time (Eberhard et al. 2009, King et al. 2009). Males garter snakes (*Thamnophis sirtalis*) prefer to use the larger hemipenis out of two hemipenises during copulation, which suggests that the different hemipenis sizes might be meaningful (Shine et al. 2000, Friesen et al. 2013). Our hemipenis length data are largely consistent with the testis weight data, which suggests that hemipenis size might adapt to sperm quantity for effective transport into the female cloaca. However, this explanation does not apply for *R. tigrinus* because it exhibited the highest relative testis weight but a relatively small hemipenis. Further study is necessary to resolve this inconsistency. Traditionally, the hemipenis morphology rather than size was studied to understand a mating system and discriminate different species or genera (Mao et al. 1984, King et al. 2009, Das and Purkayastha 2012, Friesen et al. 2013). Further studies on Korean snake hemipenial morphology and size as well as female cloacal structure may clarify the relationship between the hemipenis characteristics and mating systems. Currently, our results show that hemipenis length is consistent with testis weight; however, we observed species-specific aspects.

A difference in relative retractor muscle length was evident between the Colubridae and Viperidae snakes, but the lowest variation was observed within each family. Retractor muscle functions not only propel and retract the hemipenis but also control minor hemipenis movements during copulation (Porto et al. 2013). Thus, the muscle length might be closely related to hemipenis length, but this speculation was not clearly verified in this study. To

date, no study has reported a detailed structure of or on the function, origin and differences among species for the retractor muscle. Our results herein show large variations between groups but small variations within a group, which suggests that the relative retractor muscle length may be useful for discriminating different snake groups.

In conclusion, our results show differences in the Colubridae and Viperidae snakes' reproductive organs; however, certain aspects are species-specific. The results might be helpful to further study how differences in foraging mode and life history of the two representative snake families in Korea affect their reproductive behaviors and competitions. Additionally, we observed certain shift trends in the reproductive organs for the species in the following order: *G. saxatilis*, *G. brvicaudus* and *G. ussuriensis*. Our current results might facilitate further studies on hemipenial morphology and mating competition, which may then enhance our understanding of the Korean snake species' life history.

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